Aspergillus fumigatus germ tube growth and not conidia ingestion induces expression of inflammatory mediator genes in the human lung epithelial cell line A549

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Received 28 July 2008
Accepted 20 October 2008

INTRODUCTION

Invasive pulmonary aspergillosis (IPA) is an opportunistic infection whose incidence is increasing at the same rate as the number of severely immunocompromised patients (Lin et al., 2001). Neutropenia and high-dose steroid treatments are the main risk factors for IPA, and these underline the importance of an appropriate inflammatory response if fungal invasion is to be avoided. Most authors stress the important roles that alveolar macrophages and neutrophils play in controlling infection (Romani, 2004; Walsh et al., 2005). The release of innate immune-related molecules from professional phagocytic cells in the course of Aspergillus fumigatus infection has been widely studied (Meier et al., 2003; Pylkkanen et al., 2004; Cortez et al., 2006). However, the respiratory epithelium is the first tissue that inhaled conidia encounter, and it probably participates in the efficient coordinated response against IPA. In animal models of IPA, most inhaled conidia are trapped in the upper respiratory tract, and the respiratory epithelium coordinates with alveolar macrophages (Stephens-Romero et al., 2005).

A few studies have investigated the production of inflammatory mediators by studying the human lung epithelial cell line A549 after stimulation with moulds or mould extracts (Borger et al., 1999; Kauffman et al., 2000; Huttunen et al., 2003; Zhang et al., 2005; Tai et al., 2006). These studies have focused on fungi as the source of allergens, and on species other than A. fumigatus, which are...
often more allergenic (Kaufman et al., 2000). To our knowledge, there are no reports on the response of A549 cells to germinating A. fumigatus or on comparisons with other mould species. Spores of other mould species usually outnumber A. fumigatus conidia in inhaled air, although A. fumigatus is the main aetiological agent of IPA. Therefore, differences observed between A. fumigatus conidia and the spores of other species after the challenge of respiratory epithelium could help explain why A. fumigatus is a frequently encountered aetiological agent of invasive infection.

The aim of our study was to investigate the early inflammatory signals in airway epithelial cells after exposure to A. fumigatus conidia using real-time quantitative PCR for reliable quantification of expression of selected inflammatory mediator genes (Stordeur et al., 2002; Asselah et al., 2005). We used in our study Penicillium chrysogenum as a control for conidia ingestion (Botterel et al., 2008). P. chrysogenum is a mould that is highly predominant in the environment, with a conidial size (3–4 μm diameter) similar to that of A. fumigatus. Although P. chrysogenum can germinate at 37 °C (de Hoog et al., 2000), this fungus is rarely responsible for invasive disease. We also aimed to explore the effect of steroids on the development of the inflammatory response of the A549 cell line after challenge with live A. fumigatus conidia.

METHODS

Fungal strains. A. fumigatus strain IP 2279.94 and P. chrysogenum strain IP 1652.86 (both from the Pasteur Institute) were grown in 2% malt agar tubes (Bio-Rad) for 7 days at their optimal growth temperature: 37 and 27 °C for A. fumigatus and P. chrysogenum, respectively. The conidia were harvested with PBS/0.01% Tween 20 (Sigma-Aldrich) and 1 ml of conidia suspension (10^6 ml^-1) was used to seed the wells containing A549 cells. For some experiments, conidia were inactivated either chemically with 10% (v/v) formaldehyde or by heating at 100 °C in water for 1 h, and were resuspended in culture medium at 10^6 conidia ml^-1.

Cell lines. The alveolar epithelial cell line A549 was purchased from the German Collection of Micro-organisms and Cell Cultures (DSMZ) and cultured in Dulbecco’s modified Eagle’s medium (DMEM; Invitrogen), with 10% heat-inactivated fetal bovine serum (Biowest) and 25 μg gentamicin ml^-1 (Sigma-Aldrich). The medium was changed every 2 days and cells were confluent after 5 days. Cell cultures were checked for the presence of Mycoplasma spp. every month using the Venor GeM Mycoplasma Detection PCR kit (BioValley). A549 cells were cultivated in 24-well microtitre plates (VWR). Confluent A549 cells (~5×10^4 cells per well) were seeded with conidial suspensions. Plates were centrifuged for 5 min at 1500 r.p.m. to optimize and standardize contact between the cells and conidia. The supernatants were removed and the conidia were counted. Wells were refilled with fresh, pre-warmed culture medium. Cell exposure was terminated at 2, 4, 8 and 24 h. These experiments were performed at least three times under all conditions.

Quantification of conidia ingestion. The internalized conidia were quantified using the fluorescence brightener Blankophor-P-fluessig [4,4’-bis(4-anilino-6-substituted-1,3,5-trazine-2-yl) amino stilbene-2,2’-disulfonic acid], kindly provided by Bayer (Botterel et al., 2008). The brightener stained only the external conidia. Ten microlitres of the dye was diluted in 1 ml culture medium and 300 μl of this dilution was added to the wells and incubated for 10 min at 37 °C to stain non-internalized conidia. The wells were washed twice to collect any remaining unattached conidia and to remove the dye. The A549 cells were then lysed with 300 μl distilled water to allow counting of all conidia associated with or ingested by the cells. An Axioskop 40 microscope (Zeiss) with a filter combination including a barrier filter at 420 nm was used to classify the conidia. When UV light was stimulated below 400 nm, the brightener emitted a very intense bluish, yellowish or white light (Ruchel & Magraff, 1993). The blue conidia were classified as non-internalized and the non-fluorescent conidia as internalized.

Dexamethasone experiments. A549 cells were also challenged with A. fumigatus conidia in the presence of 10^{-6} and 10^{-7} M dexamethasone (Sigma). Dexamethasone was dissolved in 90% ethanol, diluted in culture medium and added to the A549 cells just after centrifugation of the plates and removal of the supernatant. The growth of A. fumigatus in DMEM at 37 °C in the presence of 10^{-6}–10^{-7} M dexamethasone was also monitored for effects on fungal growth at 37 °C. Fungal germination was analysed at 4, 8 and 24 h.

mRNA quantification. RNA was extracted using an RNAble kit (Eurobio). Determination of the concentration of nucleic acids was performed using a spectrophotometer (Nanodrop ND 1000) and the concentrations were adjusted to 250 ng μl^-1. Reverse transcription was carried out in a final volume of 20 μl as follows: 1 μg (4 μl) RNA extract was added to 2 μl 0.1 M dithiothreitol (Invitrogen), 0.5 μl Superscript II reverse transcriptase (200 U μl^-1; Invitrogen), 4 μl 5× first-strand buffer [250 mM Tris/HCl (pH 8.3), 375 mM KCl, 15 mM MgCl2; Invitrogen], 2 μl 5 mM dNTP mix (Amersham Pharmacia), 6 μl pdN1 random hexamers (0.5 μg μl^-1; Amersham Pharmacia) and 0.5 μl RNasin (40 μU μl^-1; Promega). Sterile water was used to dilute the cDNAs (1:20) before storing at −80 °C until amplification. For each reaction, negative controls without RNA and without reverse transcriptase were carried out.

The primers used are listed in Table 1. Real-time PCR was carried out in a LightCycler 1.5 (Roche) in a 20 μl final volume containing 2 μl 10× Fast Start SYBR Green Buffer (Fast Start SYBR Green kit; Roche Diagnostics), 3 mM MgCl2 for granulocyte–monocyte colony-stimulating factor (GM-CSF; CSE2 gene) and interleukin (IL)-8 (IL-8 gene) and 5 mM MgCl2 for tumour necrosis factor (TNF)-α (TNF gene), 50 μM sense and antisense primers, 8 μl cDNA (diluted 1:20) and sterile water. The thermal cycling conditions were: initial denaturation at 95 °C for 10 min, followed by 50 cycles of 95 °C for 15 s and 65 °C for 1 min. Quantitative values were obtained from the cycle threshold (Ct) number. Samples from three independent experiments were analysed in duplicate. The melting curves of each PCR were checked. The presence of contaminating DNA was checked by amplifying an intron of the human albumin gene. Each sample was normalized on the basis of its content compared with two reference mRNAs that were not co-regulated (Bieche et al., 2005). The first gene, P0, also known as 36B4 (GenBank accession no. NM_001002), encodes acid ribosomal RNA. DNA was checked by amplifying an intron of the human albumin gene. Each sample was normalized on the basis of its content compared with two reference mRNAs that were not co-regulated (Bieche et al., 2005). The first gene, P0, also known as 36B4 (GenBank accession no. NM_001002), encodes acid ribosomal phosphoproteins. The second, the TBP gene (GenBank accession no. NM_003194), encodes a component of the DNA-binding protein complex TFIID. The results, expressed as the fold change in target gene expression relative to the P0 or TBP gene (termed N_{target}), were determined by the formula N_{target} = 2^{(ΔC_{t} target)}. The ΔC_{t} sample was calculated by subtracting the mean C{t} value of the target gene from the mean C{t} value of the TBP or the P0 gene. The N_{Target} values of the samples were subsequently normalized so that the median N_{Target} value of the non-fungus control samples was 1.

Aspergillus fumigatus and cytokine production

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Table 1. Primers sequences used in the study

<table>
<thead>
<tr>
<th>Gene target</th>
<th>Sense primer (5’→3’)</th>
<th>Antisense primer (5’→3’)</th>
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<tbody>
<tr>
<td>IL-8 (IL-8 gene)</td>
<td>CACCGGAAGAACCATCTCTACTGT</td>
<td>TCCCTGGCAAAAACCTGCACCTTCA</td>
</tr>
<tr>
<td>TNF-α (TNF gene)</td>
<td>GCCCAAGAAGTCAGATCATCTT</td>
<td>CCTCAGCTTGAGGTTGCTACA</td>
</tr>
<tr>
<td>c-Fos (FOS)</td>
<td>ACCACTAACCAGGAGACTCCT</td>
<td>CCAGGTCCTGCAAGATCTCT</td>
</tr>
<tr>
<td>GM-CSF (CSF2)</td>
<td>GAGACACTGGCTGCTGAGATGAGT</td>
<td>CAGGTGGCTCTCTGAGGT</td>
</tr>
<tr>
<td>P0</td>
<td>GGCAGACTGGAAGTCCAACT</td>
<td>CCATCAGCAACACAGCCTTC</td>
</tr>
<tr>
<td>TBP</td>
<td>TGACAGGGACCAAGAGTGA</td>
<td>CACATCAGACTCCCAACCAACCA</td>
</tr>
<tr>
<td>Albumin (intron)</td>
<td>GCTGTATCTCTTGTGAGCCTGT</td>
<td>ACTCATGGGAGCTGCTTGTC</td>
</tr>
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</table>

Statistical analysis. Data are presented as means ± SEM of three independent experiments. Statistical analysis was performed using XLSTAT 2007. Differences were considered statistically significant for P < 0.05. A one-way analysis of variance (ANOVA) test was used to detect significant variations in mRNA levels for each target gene during exposure. A t-test was used to detect significant differential mRNA levels with or without dexamethasone.

RESULTS AND DISCUSSION

Using quantitative PCR, we observed a significant increase in mRNA levels in A549 epithelial cells for all three selected inflammatory mediators (IL-8, TNF-α and GM-CSF) when they were exposed to live A. fumigatus conidia. This increase was observed at 8 h and continued up to 24 h. This overexpression of mRNA was not observed when the cells were exposed to inactivated A. fumigatus conidia. This overexpression of the mRNA levels with or without dexamethasone.

Standardization of techniques

After centrifugation of cell cultures with conidial suspensions, approximately 10% did not adhere to the A549 cells and were removed. There was no statistical difference between the ingestion rates of conidia for A. fumigatus (35.3 ± 3.3 %) and P. chrysogenum (37.7 ± 2.0 %) at 4 h post-exposure.

After RNA extraction, we first checked for contaminating genomic DNA by amplifying an intron of the human albumin gene. The Ct values were always >37 and were similar for all of the cDNAs, confirming the absence of DNA contamination. To normalize mRNA expression from different wells, a relative quantification of the target genes and of the endogenous reference P0 and TBP genes using ΔΔCt was performed. Because similar results were obtained for both reference genes, only the results standardized with the P0 gene are reported here.

mRNA quantification

We focused our study on three mediators whose role has been well established in the inflammatory response. The absence of this response is one of the well-known risk factors for IPA, i.e. prolonged neutropenia and steroid therapy. IL-8 is a chemokine involved in both the recruitment of neutrophils and the neutrophil phagocytosis of A. fumigatus conidia (Richardson & Patel, 1995), TNF-α is a cytokine that plays a role in chemokine production and neutrophil activity (Mehrad et al., 1999; Phadke & Mehrad, 2005) and GM-CSF is a growth factor known to be crucial in the maturation process of granulocytes and macrophages (Walsh et al., 2005). These three mediators are released in response to activation of the NF-κB transcriptional complex.

Exposure to live A. fumigatus conidia induced a progressive increase in IL-8, TNF-α and GM-CSF mRNA levels (Fig. 1). A significant overexpression of the mRNA of all three was observed as early as 8 h post-exposure (P ≤ 0.005, P ≤ 0.002 and P ≤ 0.003, respectively). This increase was not observed when the cells were exposed to inactivated A. fumigatus conidia. No significant variation for any of the three inflammatory mediators was observed when the epithelial cells were exposed to live or inactivated P. chrysogenum conidia. Germinating A. fumigatus conidia were observed in A549 cells as early as 8 h post-exposure. In contrast, with live P. chrysogenum conidia and with the inactivated conidia of both species, no germination was observed during the entire 24 h of the experiment.

We hypothesized that ingestion of conidia by A549 cells could be the triggering factor for the inflammatory response. Overexpression of chemokines and of chemokine receptor genes (IL-8 gene, CCL4, CXCL20 and CXCL2) has been described as early as 2 h in human monocyte models using cDNA microarray analysis (Cortez et al., 2006). In our study, we were unable to detect any significant increase before 8 h. If conidia ingestion were the triggering event, an inflammatory response would have occurred for both fungal species because the rate of internalized conidia was similar for both species: around 30–40 %, as reported previously (Wasylnka & Moore, 2002).

However, in our study, a sharp increase appeared at the same time as the hyphal growth of A. fumigatus, which started after 6–8 h of culture in liquid medium (Manavathu et al., 1999). In addition, exposure to inactivated A. fumigatus conidia did not induce any significant modifications of the mRNA levels of the factors studied. Similar results for cytokine production using ELISA were obtained using non-viable fragments of mycelium and inactivated A. fumigatus conidia (Zhang et al., 2005). Recently, infection with hyphae, but not with
conidia, was shown to stimulate endothelial cells to synthesize IL-8 and TNF-α in vitro using ELISA (Chiang et al., 2008). We cannot rule out the fact that heating or chemical inactivation may have destroyed epitope structures and heat-labile molecules such as proteases, which are potentially involved in inflammatory signalling. However, our results are in agreement with a recent report on the role of germinating conidia in the innate immune system in vitro and in animal models (Hohl et al., 2005). Conidial swelling is accompanied by an enrichment of β-glucans on the membrane surface, which triggers inflammatory responses via Dectin-1 recognition (Hohl et al., 2005).

**mRNA quantification in the presence of dexamethasone**

Exposure of A549 cells to live conidia of *A. fumigatus* in the presence of $10^{-6}$ M (not shown) and $10^{-7}$ M dexametha-
sone resulted in the absence of overexpression at 8 h for IL-8 (P < 0.005), TNF-α (P < 0.002) and GM-CSF (P < 0.003) and at 24 h (P < 0.01 for each) (Fig. 2). In addition, dexamethasone at concentrations of $10^{-3}$–$10^{-7}$ M did not modify the growth of A. fumigatus conidia (data not shown).

Given the significant role that germinating conidia play in IPA, the disappearance of overexpression when the conidia-exposed cells were treated with $10^{-7}$ M dexamethasone supports the role of steroids as a risk factor for IPA, not only at the macrophage level, but also at the respiratory epithelium level (Nissen & Yamamoto, 2000). Although the role that steroids play in the NF-κB pathway of the cells is a probable explanation, we cannot completely rule out the fact that steroids could inhibit the synthesis of a specific fungal compound that could be responsible for the immune stimulation of A549 cells.

These initial results show that germinating A. fumigatus conidia induce overexpression of inflammatory mediators by epithelial cells in the first 24 h after exposure. Further studies are needed to define in more detail the chemokines, adhesion molecules and other possible pathways involved. However, these results suggest that the respiratory epithelium plays a role in initiating innate immunity to prevent the occurrence of IPA. Our results also confirm that steroids significantly modify the normal inflammatory response of airway cells exposed to A. fumigatus, and this could explain at least in part why steroids are a risk factor for IPA.

**ACKNOWLEDGEMENTS**

This work was supported by grants from L’Agence Nationale de la Recherche (no. 0014705), ADEME (no. 0575C0030) and AFSSSET (no. ES-2005-012).

**REFERENCES**


