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Proteomic demonstration of the recurrent presence of inter-alpha-inhibitor H4 heavy-chain during aspergillosis induced in an animal model

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ABSTRACT

Invasive pulmonary aspergillosis remains a matter of great concern in oncology/haematology, intensive care units and organ transplantation departments. Despite the availability of various diagnostic tools with attractive features, new markers of infection are required for better medical care. We therefore looked for potential pulmonary biomarkers of aspergillosis, by carrying out two-dimensional (2D) gel electrophoresis comparing the proteomes of bronchial-alveolar lavage fluids (BALF) from infected rats and from control rats presenting non-specific inflammation, both immunocompromised. A bioinformatic analysis of the 2D-maps revealed significant differences in the abundance of 20 protein spots (ANOVA P-value < 0.01; q-value < 0.03; power > 0.8). One of these proteins, identified by mass spectrometry, was considered of potential interest: inter-alpha-inhibitor H4 heavy-chain (ITIH4), characterised for the first time in this infectious context. Western blotting confirmed its overabundance in all infected BALF, particularly at early stages of murine aspergillosis. Further investigations were carried on rat serum, and confirmed that ITIH4 levels increased during experimental aspergillosis. Preliminary results in human samples strengthened this trend. To our knowledge, this is the first description of the involvement of ITIH4 in aspergillosis.

Introduction

Invasive pulmonary aspergillosis (IPA), which affects immunocompromised patients (Nivoix et al., 2008), is increasingly common in various weakened medical populations, such as patients undergoing solid-organ or bone marrow transplantation, patients with haematological malignancies or treated by chemotherapy for cancers (Barnes and Marr, 2007; Salman et al., 2011), and individuals in intensive care units (ICU). The incidence of IPA in patients undergoing allogeneic stem-cell transplantation may reach 13% (Garcia-Vidal et al., 2008). Despite major progress in our understanding of the disease (Chai and Hsu, 2011) and the development of new antifungal drugs (Herbrecht et al., 2002), IPA-related mortality remains high, at over 30% (Kontoyiannis et al., 2010; Kousha et al., 2011; Segal, 2009). These high mortality rates may be accounted for, in part, by difficulties establishing a rapid diagnosis, although various exploratory approaches are now available according to expert opinions from the European Organisation for Research and Treatment of Cancer/*Mycoses Study Group* (EORTC/MSG) (De Pauw et al., 2008; Marchetti et al., 2011). For example, the halo sign is highly suggestive of IPA on chest CT-scans, but it may actually reflect bacterial pneumonia, or infection with a fungus

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other than *Aspergillus* spp. (Caillot et al., 2010). Detection of the *Aspergillus* galactomannan (GM) antigen in serum has made a major contribution to diagnosis in the last ten years, although a few false-positives have been recognised, particularly in rare cases of concomitant treatment with semisynthetic β-lactams or polyvalent immunoglobulins (Sulahian et al., 2003; Wheat and Walsh, 2008). Furthermore, GM tests may be negative in cases of IPA occurring in patients from clinical departments other than haematology (Pfeiffer et al., 2006). (1–3)-β-D-Glucans, which have recently been incorporated into the EORTC/MSG criteria for IPA diagnostic, are serum pan-fungal biomarkers with a high negative predictive value (NPV), but no specificity for *Aspergillus* (De Pauw et al., 2008; Obayashi et al., 1995). Real-time PCR on serum appears a promising approach, but the lack of standardisation must be rectified before more widespread clinical use and its incorporation into the EORTC-MSG criteria for IPA (Mengoli et al., 2009). Several international molecular works currently underway should publish their results soon (White et al., 2010). *In vitro* mycological cultures of respiratory samples often fail to distinguish between real infections and simple colonisation of the airways (Marr and Leisenring, 2005; Maschmeyer et al., 2007; Ostrosky-Zeichner, 2012), other than for samples that should normally be sterile, such as pleural fluid aspiration samples (De Pauw et al., 2008). Furthermore, mycological culture from bronchial-alveolar lavage fluid (BALF) does not appear to be sensitive enough in neutropenic patients (Bergeron et al., 2012), and endoscopy is not always possible in such weakened subjects. Finally, although lung biopsy can provide a firm diagnosis of invasive fungal infection (De Pauw et al., 2008), the features of the hyphae observed on histological preparations are often insufficient on their own for formal identification of the fungal genus/species (Guarner and Brandt, 2011). Furthermore, this invasive surgical intervention, associated with a potential risk of infection, is not always feasible, in patients with coagulopathy for example (Yeo and Wong, 2002).

In this context, the search for new biomarkers of IPA that are easily detectable (*e.g.* in serum) remains a matter of high priority (De Pauw and Viscoli, 2011; Thornton, 2010). Several animal models have been developed for studies of experimental IPA, reliably mimicking the characteristics of IPA in humans (Clemons and Stevens, 2005). Using an original model of rats immunocompromised by cyclophosphamide treatment (Chandenier et al., 2009; Desoubeaux and Chandenier, 2012), we compared the BALF of rats infected with *Aspergillus fumigatus* with that of control rats sensitised by exposure to a bacterial endotoxin (Signor et al., 2004). Differential analysis, by two-dimensional (2D) electrophoresis, identified 20 protein spots overrepresented in the BALF from infected animals. Their characterisation by nano-liquid chromatography coupled with tandem–mass spectrometry (LC–MS/MS) showed the corresponding proteins to be of host rather than fungal origin. One protein appeared of particular interest: inter-alpha-inhibitor H4 heavy-chain (ITIH4), which has never before been described in this situation, to our knowledge. Its overabundance in all infected BALF was attested by western blotting (WB). Further WB explorations of rat serum samples confirmed this trend. Although preliminary, first explorations in human samples highlighted a global trend to the increase of ITIH4 in BALF.

Materials and methods

Rodent model of aspergillosis

Experimental infection

This protocol was based on the animal model described by Chandenier et al. (2009) and Desoubeaux and Chandenier (2012) and approved by the ethics committee for animal experimentation of Val-de-Loire (France). Briefly, 17 six- to eight-week-old male Sprague-Dawley rats (Janvier®) were immunocompromised by repeated intraperitoneal injections of cyclophosphamide (Endoxan® Baxter™). The doses used were 75 mg/kg on D-5 and 60 mg/kg on D-1, D3 and D7 (*i.e.* D0 being the day of experimental infection). The rats were housed in cages placed in controlled-environment racks. They were fed with a low-protein diet (Safe Diets™), and had free access to sterile water supplemented with antibiotics (tetracycline, Sigma–Aldrich™) to prevent possible opportunistic bacterial infections, and with paracetamol (Efferalgan 150®, Bristol-Myers-Squibb™) as a painkiller.

On D0, each animal was anaesthetised with 5% isoflurane (Aeranne®, Baxter™). The rats had initially been randomised to three different groups, each one receiving, on D0, a tracheal administration of 100 μL of one of three aerosolised solutions/suspensions *via* a MicroSprayer® Aerosolizer – Model IA-1B (PennCentury™): nebulised saline (0.9% sodium chloride solution) for group A ($n = 2$), 1 mg/kg bacterial lipopolysaccharide (*Salmonella enterica* Serovar Typhimurium 2 mg/mL LPS, Sigma–Aldrich™) for group B ($n = 5$), and 10^6 *Aspergillus* conidia for group C ($n = 10$). The *Aspergillus* suspension had been prepared from a culture of *Aspergillus fumigatus* in Sabouraud-dextrose agar containing chloramphenicol and gentamicin (BBL Becton-Dickinson™). The strain used was initially isolated from the BALF of a patient with proven IPA, hospitalised in the Oncology/Haematology Department of Tours University Hospital. The ITS and β-tubulin genes were sequenced, and the strain was formally deposited as No. BRFM 1827 in the registered collection of the WFCC-MIRCEN *World Data Centre for Microorganisms* (CIRM-champignons filamenteux collection, UMR 1163 INRA, in Marseille, FRANCE).

Collection of biological samples

At baseline, 1 mL of blood was systematically sampled by puncture of the lingual vein in all included rats, just before experimental nebulisation (D-1) (Angelov et al., 1984; Directive, 2010/63/EU, 2010).

The healthy control animals of group A were systematically sacrificed 24 h after the intratracheal nebulisation of saline solution (D1). The rats of group B were killed between 24 and 48 h after LPS instillation (D1 or D2), when the inflammatory reaction was maximal (Signor et al., 2004). Once an experimentally infected rat (group C) began to present deleterious clinical signs consistent with the development of IPA, decisions concerning euthanasia were taken on the basis of the clinical score obtained with a validated grid (Morton and Griffiths, 1985). If no clinical sign of IPA was observed, the animal was sacrificed at the end of the protocol, on D10. All rats were anaesthetised, as described above, and then killed by the intraperitoneal injection of 0.5 mL of 2.5% thiopental (Pentothal®, Abbott™). A laparotomy was performed, and blood was collected from the abdominal aorta. Immediately after death, bronchial-alveolar lavage was carried out with 8 mL of sterile saline, which was distributed throughout the lungs and then reaspired in two batches, with a three-way stop-cock with a closing cap (Axel Med™). The fluid collected was rapidly centrifuged ($1000 \times g$ for 10 min at 4 °C), dispensed into aliquots and stored at −80 °C until use. We used 100 μL of the remaining pellet to inoculate Sabouraud-dextrose agar supplemented with chloramphenicol and gentamicin (BBL Becton-Dickinson™). Petri dishes were placed at 37 °C, and colony-forming units (CFU) were counted three days later.

GM antigen levels were determined in 300 μL of each rat serum sample (collected at baseline and at the time of death), in accordance with the kit manufacturer's instructions (EIA Platelia *Aspergillus*®, Bio-Rad™) (Marr and Leisenring, 2005). A pathological microscopic examination of the lungs was also carried out