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Therapeutic effects of recombinant forms of full-length and truncated human surfactant protein D in a murine model of invasive pulmonary aspergillosis

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ABSTRACT

Aspergillus fumigatus (*Afu*) is an opportunistic fungal pathogen that can cause fatal invasive pulmonary aspergillosis (IPA) in immunocompromised individuals. Previously, surfactant protein D (SP-D), a surfactant-associated innate immune molecule, has been shown to enhance phagocytosis and killing of *Afu* conidia by phagocytic cells *in vitro*. An intranasal treatment of SP-D significantly increased survival in a murine model of IPA. Here we have examined mechanisms via which recombinant forms of full-length (hSP-D) or truncated human SP-D (rhSP-D) offer protection in a murine model of IPA that were immunosuppressed with hydrocortisone and challenged intranasally with *Afu* conidia prior to the treatment. SP-D or rhSP-D treatment increased the survival rate to 70% and 80%, respectively (100% mortality on day 7 in IPA mice), with concomitant reduction in the growth of fungal hyphae in the lungs, and increased levels of TNF-α and IFN-γ in the lung suspension supernatants, as compared to untreated IPA mice. The level of macrophage inflammatory protein-1α (MIP-1α) in the lung cell suspension was also raised considerably following treatment with SP-D or rhSP-D. Our results appear to reaffirm the notion that under immunocompromised conditions, human SP-D or its truncated form can offer therapeutic protection against fatal challenge with *Afu* conidia challenge. Taken together, the SP-D-mediated protective mechanisms include enhanced phagocytosis by recruited macrophages and neutrophils and fungistatic properties, suppression of the levels of pathogenic Th2 cytokines (IL-4 and IL-5), enhanced local production of protective Th1 cytokines, TNF-α and IFN-γ, and that of protective C–C chemokine, MIP-1α.

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1. Introduction

Aspergillus species are increasingly recognized as major fungal pathogens in immunocompromised (especially neutropenic) patients. *Aspergillus fumigatus* (*Afu*) is responsible for nearly 90% of cases of invasive pulmonary aspergillosis (IPA), which is a prominent cause of infectious morbidity and mortality in patients with hematologic malignancies and in recipients of stem cell, and organ transplants (Labbé et al., 2007; Magill et al., 2008). As the use of long courses of corticosteroids as well as aggressive antineoplastic chemotherapeutic regimens is becoming frequent, the number of patients susceptible to *Aspergillus* infection is rising (Staber et al., 2007). IPA is characterized by hyphal invasion and destruction of pulmonary tissue and dissemination to other organs

Abbreviations: *Afu*, *Aspergillus fumigatus*; IPA, invasive pulmonary aspergillosis; SP-D, surfactant protein D; rhSP-D, a recombinant fragment of human surfactant protein D composed of homotrimeric neck and CRD regions; MIP-1α, macrophage inflammatory protein-1α; AmB, amphotericin B; PMN, polymorphonuclear cells; CRD, carbohydrate recognition domain; ABPA, allergic bronchopulmonary aspergillosis; SDA, sabouraud dextrose agar; CFU, colony forming unit; H & E, hematoxylin and eosin; PTX3, long prototypic pentraxin 3; DC, dendritic cell; Th, helper T cell; SP-D$^{-/-}$, mice genetically deficient in SP-D gene.

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occurs in approximately 20% cases (Bodey and Vartivarian, 1989). In spite of correct diagnosis and best available treatment, IPA results in mortality of >80% of the patients (up to 95% mortality rate among patients of bone marrow transplantation) (Sternberg, 1994; Denning, 1996; Walsh et al., 1998). The currently used anti-fungal drugs have some limitations due to their side effects and therapeutic inadequacy (Stevens et al., 2000; Patterson, 2002).

The respiratory tract appears to be portal of entry in the majority of cases of IPA (Bardana, 1981). Host defence against *Aspergillus* infections is mediated by phagocytic cells including macrophages and neutrophils (Chilvers et al., 1989; Levitz et al., 1986; Roilides et al., 1993a,b; Schaffner et al., 1982; Waldorf et al., 1984). Macrophages can bind and phagocytose *Afu* conidia and kill conidia as well as hyphae (Schaffner et al., 1983; Kurup, 1984; Levitz et al., 1986; Roilides et al., 1994). It is becoming increasingly evident that innate immune molecules, such as collectins, toll-like receptors and pentraxin 3, are crucially involved in lung resistance to *Afu* infection (Kishore et al., 2002; Madan et al., 2005; Garlanda et al., 2002). Since IPA is rare in immunocompetent individuals, therapy aimed at strengthening the host's immune response to the organisms, offers a promising therapeutic or prophylactic approach.

We have previously shown that two hydrophilic lung surfactant proteins, SP-A and SP-D, can selectively enhance the contribution of the innate immune mechanisms against *A. fumigatus*. SP-A and SP-D, which belong to a family of proteins, called collectins, are composed of subunits, each of which contains a collagen-like triple-helical region, followed by an α-helical, trimerizing neck region and three CRDs at its C-terminal end (Holmskov et al., 2003; Kishore et al., 2005, 2006). SP-A and SP-D recognize carbohydrate structures present on the surfaces of a wide range of pathogens, such as viruses, bacteria and fungi, via their carbohydrate recognition domains (CRDs), and enhance phagocytosis and killing by neutrophils and alveolar macrophages (Crouch and Wright, 2001; Wright, 2005). SP-A and SP-D can bind and agglutinate *Afu* conidia *in vitro* in a sugar and calcium-dependent manner, and enhance killing of conidia by circulating neutrophils and alveolar macrophages via phagocytosis and superoxidative burst (Madan et al., 1997; Allen et al., 1999). The β-glucan on the *Afu* surface has been found to be one of the ligands that interact with SP-D (Allen et al., 2001). Intranasal administration of SP-D or a recombinant fragment of SP-D composed of trimeric neck and CRD regions (rhSP-D) in a murine model of IPA, where mice were immunosuppressed with hydrocortisone and challenged intranasally with *Afu* conidia, has been shown to have a protective effect. Untreated IPA mice showed 100% mortality at 7 days, whereas SP-D or rhSP-D treatment rescued 60% and 80% of the IPA mice respectively. Interestingly, SP-A did not have a significant effect on survival, suggesting an important role for SP-D in the ability of host to resist *Afu* challenge and subsequent infection (Madan et al., 2001a,b).

In the present study, we have intranasally administered various doses of recombinant forms of full-length (hSP-D) and truncated human SP-D (rhSP-D) in a murine model of IPA and examined their effects on animal survival, pulmonary fungal load, and cytokine and chemokine profiles in the lungs. hSP-D or rhSP-D treatment dramatically increased the survival rate to 80% (100% mortality on day 7 in IPA mice), with concomitant reduction in the growth of fungal hyphae in the lungs, lowered IL-4 and IL-5 and increased TNF-α and IFN-γ levels of in the lung cell suspension, as compared to untreated IPA mice. The level of macrophage inflammatory protein-1α (MIP-1α) in the lung cell suspension was also raised considerably following treatment with hSP-D or rhSP-D. Our results appear to suggest that under immunosuppressed conditions, full-length human SP-D or its truncated form can offer therapeutic protection against fatal challenge with *Afu* conidia challenge.

2. Materials and methods

2.1. Afu conidia

Afu strain 285, isolated and cultured from the sputum of an allergic bronchopulmonary aspergillosis (ABPA) patient (Madan et al., 2001a), was transferred to fresh sabouraud dextrose agar (SDA) slants and incubated for 3 days at 37 °C. Conidia were harvested and suspended in sterile PBS with 0.025% (v/v) Tween 20. The resulting suspension of conidia was filtered through sterile gauze to remove clumps and hyphal debris, and then washed once and re-suspended in sterile PBS. The number of spores present in the suspension was determined using a haemocytometer and then diluted with PBS to make challenge concentrations of 10^8 conidia per 50 μl. The spore viability of challenge inoculum was assessed by plating 10^6 and 10^7 dilutions on the SDA plates. The number of conidia determined by the haemocytometer was consistent with the number of viable colony forming unit (CFU) counts found by serial dilution and plating of the suspension.

2.2. Preparation of full-length human SP-D

A recombinant form of full-length human SP-D (hSP-D) was stably expressed in Chinese Hamster Ovary-K1 cells, as previously described (Hartshorn et al., 1996). Briefly, the secreted protein was isolated from conditioned medium by maltosyl-agarose affinity chromatography and the dodecameric forms of hSP-D were fractionated via gel filtration chromatography. SDS-PAGE and silver staining confirmed the purity of SP-D. The levels of endotoxin were monitored using a chromogenic assay (Cambrex). The assay was linear over a range of 0.1–1.0 EU/ml (10 EU = 1 ng of endotoxin) and found to be <2 pg/mg of hSP-D.

2.3. Expression and purification of rhSP-D

A recombinant fragment of human SP-D (rhSP-D), which included a short stretch of eight Gly-X-Y triplets, the α-helical coiled-coil neck region (residues 203–235) and the globular CRD region (residues 236–355), was expressed and purified as described recently (Singh et al., 2003). The rhSP-D was found to bind appropriate phospholipids and maltosyl-BSA (Kishore et al., 1996). The crystallographic structure of the rhSP-D, complexed with maltose in the carbohydrate binding pockets, has also been solved (Shrive et al., 2003). The endotoxin level in the protein preparation was assessed by QCL-1000 Limulus amoebocyte lysate system (Bio-Whittaker, Walkersville, MD). The assay was linear over a range of 0.1–1.0 EU/ml (10 EU = 1 ng of endotoxin) and found to be 4 pg/μg of rhSP-D.

2.4. Mice

Male BALB/c mice (National Institute of Nutrition, Hyderabad, India), weighing 20–22 g each, were housed in polycarbonate shoebox cages bedded with dried corncob. Mice took a standard laboratory rodent diet and had water *ad libitum*. Mice were immunosuppressed by three intradermal injections of 2.5 mg/(mouse day) (125 mg/kg body weight) of hydrocortisone acetate (Wycort) 1 day before, on the day, and the day after conidia challenge, as described previously (Madan et al., 2001a).

2.5. Study design summary

Various groups of 40–45 mice each were selected and randomised. On the day 0, mice were lightly anaesthetised with ether and 10^8 conidia of *Afu* in 50 μl sterile PBS were administered intranasally in the IPA group of mice and 50 μl of PBS alone in

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