Aspergillus fumigatus and the human lung

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CHAPTER 5.2

Fungi are a heterogenous group of micro-organisms. From a clinical perspective *Aspergillus* species are of special interest and have been recognized as causative organisms in human disease since long. *Aspergillus* is able to cause infection of virtually every organ in the human body although aspergillosis mostly presents as a pulmonary infection (**chapter 1.1** and **1.2**). The different manifestations of pulmonary aspergillosis range from innocuous non-pathogenic saprophytic colonization in healthy individuals to acute and fulminant invasive processes in patients vulnerable to infection. *Aspergillus* may also act as a powerful allergen which may result in the development of an allergic state in individuals with a predilection to develop allergic sensitization (atopy) (**chapter 1.2**). The most pathogenic members of this genus include *A. fumigatus*, *A. flavus*, *A. niger* and *A. terreus*. **Chapter 1.3** gives an overview of the pathology caused by these and other *Aspergillus* species. **Chapter 1.4** and **chapter 1.5** describe the cellular and humoral mechanisms that protect against *Aspergillus* and the fungal related factors that facilitate *A. fumigatus* infection.

In recent years many researchers have focussed on the putative role of fungal proteases in the pathogenicity of *A. fumigatus*. Within the *Aspergillus* genus, circumstantial evidence links the production of elastinolytic proteases with the ability to cause disease. Yet, none of these reports have conclusively demonstrated that extracellular proteases contribute significantly to the virulence of *Aspergillus* in man. **Chapter 2.2** reviews most of the *in vitro* and *in vivo* studies on the contribution of proteases to virulence of *A. fumigatus*. This chapter also presents two models for the role of proteases in human disease. Basically, the first model states that proteases secreted by *Aspergillus* induce a self perpetuating cycle of inflammation at the airway epithelium by causing epithelial cell desquamation and inducing release of pro-inflammatory cytokines from the epithelial cells. These pro-inflammatory cytokines may subsequently call in inflammatory cells followed by processes that cause further damage of the epithelium, thereby creating a site for fungal attachment and infiltration. Damage of the epithelium causes further development of inflammation. An alternative hypothesis states that proteases play a role in early events related to infection. The airway epithelium constitutes a tight barrier between the internal and external environment, therefore, one of the prime targets for the fungus may be the epithelial cell barrier. Here, fungal proteases may become important in their ability to induce epithelial cell desquamation.

In this thesis the contribution of proteases to the pathogenicity was studied in different ways. To ascertain the release of proteases in patients with aspergillosis *in vivo*, we studied the IgG antibody production specific for fungal proteases in sera from patients with ABPA and PA (**chapter 2.3**). Routine fungal culture media do not favor protease production therefore *A. fumigatus* was cultured on collagen medium, resulting in the excretion of high levels of fungal

protease activity. Protein profiles of collagen medium filtrate show several proteins not found in conventional culture filtrates, including a prominent 32 kD glycoprotein, which coisolates with elastase activity, as well as 67 kD and 94 kD proteins. These constituents elicit IgG production in patients with ABPA and PA, suggesting production of these proteins during disease *in vivo*.

As was discussed before, the epithelium is essential in the protection against pathogens by its function as a physical barrier. In recent years evidence has been presented indicating a role of airway epithelial cells in the orchestration of local inflammatory responses. To investigate whether fungal proteases could induce an inflammatory response or cause loss of the barrier function of the epithelium, the production of pro-inflammatory cytokines and epithelial cell desquamation in respons to protease-containing *A.fumigatus* culture filtrates was studied *in vitro* (**chapter 2.4**). It was found that fungal serine protease activity induced the production of IL-8, IL-6 and MCP-1 and caused cell desquamation in a dose-dependent fashion. Both phenomena were inhibited by serine protease inhibitors including ALP. By causing cell desquamation, fungal proteases may decrease the physical barrier function of the epithelium; however, by eliciting a cytokine response, the epithelium may signal the mucosal inflammatory response against *A. fumigatus*.

Chapter 2.5 presents further studies on the induction of a pro-inflammatory cytokine response and cell desquamation in airway epithelial cells with mite (*Lepidoglyphus destructor* and, *Dermatophagoides pteronyssinus*), Timothy grass pollen and birch pollen extracts. Production of IL-8, IL-6, MCP-1 and GM-CSF and cell detachment were monitored in pulmonary epithelial cell lines and primary epithelial cell cultures from the nose. With the mite extracts, cytokine production and cell detachment was largely dependent on protease activity. With the pollen extracts cytokine production without cell detachment seemed to be protease-independent. Results from this study are of special interest since the factors responsible for inducing or maintaining allergic airway inflammation in patients with asthma and rhinitis are poorly understood. The observation of local airway inflammation in non-allergic patients with asthma or rhinitis including those with nasal polyposis suggests that 'non-IgE-related' mechanisms exist that may lead to airway inflammation. The findings presented in chapter 2.5 support the view that epithelial cells contribute to the pathogenesis of airway disease by their interaction with inhalant allergen extracts. Furthermore, allergen extracts may enhance airway inflammation by means other than their IgE-binding activity via protease-dependent and -independent mechanisms.

Opposing the fungus, the human defense system tries to deal with potentially life-threatening micro-organisms. Both the adaptive and the native immune systems have been studied extensively for the past decades. Recent findings have suggested a role for ALP in the

mucosal defense against bacteria. ALP is a major protease inhibitor of mucous secretions and, in the human lung, is produced locally by various cell types including those from the lining epithelium. Based on previous findings on the antibacterial activity of ALP we studied its antifungal activity towards *A. fumigatus* and *C. albicans* (**chapter 3.2**). rALP had pronounced fungicidal activity toward metabolically active *A. fumigatus* conidia and *C. albicans* yeast cells; however, metabolically quiescent *A. fumigatus* conidia were totally resistant. In contrast with the protease inhibitory activity of rALP, the fungicidal activity was found to be localized primarily in the NH_2-terminal domain. On a molar base, the fungicidal activity of rALP was comparable with that of human defensins and lysozyme and is probably related to the cationic nature of the protein. In addition, rALP caused inhibition of *C. albicans* yeast cell growth. By exhibiting antifungal activity ALP may play an important role in the innate mucosal defense against human pathogenic fungi. **Chapter 3.3** discusses the potential of ALP as a therapeutic option in the treatment of infectious diseases in view of the unpropitious development of drug resistance of infectious micro-organisms in the human population.

Many studies have focussed on IgG antibody responses in different diseases, however, the factors that lead to increased production of specific IgG subclasses in aspergillosis are still largely unknown. Of particular interest is the role of IgG4 in immune responses. It has been suggested that IgG4 is a marker of protection against allergy. Others suggest that IgG4 is a marker of chronic antigen exposure, and it may play a role in protecting the host from protracted inflammatory reactions. **Chapter 3.4** presents results from studies on IgG subclass production in patients with ABPA and PA as models of transient and chronic exposure to *Aspergillus* antigens, respectively. Increased IgG4 responses were found under conditions of chronic exposure to *A. fumigatus* antigen. *A. fumigatus*-specific-IgG1 was increased in patients with PA compared with those in patients with ABPA. Patients with PA having IgE responses to *A. fumigatus* and/or other inhalant allergens showed significantly higher *A. fumigatus*-specific-IgG4 responses than patients with PA having negative IgE responses or patients with ABPA. Surveillance studies over time in individual patients showed that both the *A. fumigatus*-specific-IgG1 and IgG4 levels followed the course of disease progression and treatment. Immunoblotting revealed correlations between *A. fumigatus*-specific-IgG1 and IgG4 binding to most, but not all, antigenic *A. fumigatus* components. This study documents for the first time increased IgG4 levels under conditions of chronic exposure to fungal antigen in PA and a significantly higher IgG4 response in those patients with PA who produce IgE. The transient exposure to *A. fumigatus* antigen during exacerbation of ABPA gives rise to transient elevations in IgG4 levels.

In addition to studies on the more fundamental processes that underlie pathogenicity of *Aspergillus* and the human host defense, diagnosis of fungal infection in patients with aspergillosis is of major importance from a clinical point of view. Aspergillosis may present as a highly destructive disease with grave prognosis especially in patients who are immunocompromised. Diagnosis may be difficult to reach in some patients underlining the need for diagnostic tools. Early diagnosis of fungal infections is of crucial importance to limit fungal tissue invasion and irreversible tissue destruction. In **chapter 4** two studies are presented on the evaluation of the IgG-antibody responses in the serodiagnosis of *Aspergillus* infection in immunocompetent and immunocompromised patients. **Chapter 4.2** describes the monitoring of disease and treatment in an immunocompetent patient with exacerbations of PA during a 9-year follow-up. A rise in IgG response as identified by ELISA coincided with a recrudescence of clinical symptoms whereas a decrease in IgG response after treatment paralleled clinical improvement. IgG-binding to the 32 kD serine protease, and to 60 and 94 kD proteins produced with collagen-containing culture medium closely corresponded with IgG-ELISA titers. IgG-binding to a 40 kD metalloprotease remained at very low levels until symptoms and fungal growth became well advanced, when a sharp rise was seen. Responses to all antigens rapidly diminished after the start of successful antifungal treatment with itraconazole. Serology may be a useful adjunct in the monitoring of disease progression and the efficacy of antifungal treatment in patients with PA. Immunologic responses to fungal proteases raised with collagen-containing culture media may reflect fungal proteolytic involvement during disease progression and treatment more closely than responses to proteins raised with conventional media.

Finally, **chapter 4.3** presents data from a retrospective study on the serodiagnosis of aspergillosis in immunocompromised patients after lung transplantation. Four patients from the center for lung transplantation from the University Hospital Groningen were included in the study. All patients had proven *A. fumigatus* infections after lung transplantation and fatal outcome. The IgG antibody response specific for *A. fumigatus* antigens raised with conventional media was measured by means of ELISA and compared with radiographic features, cytologic findings, microbiological cultures, and clinical diagnosis. Increasing IgG antibody responses closely paralleled cytologic or microbiological identification of *A. fumigatus* from bronchoalveolar lavage fluid and decrease of lung function. Increasing specific IgG antibody responses were found to precede radiographic identification of lung cavitation by 1 to 2 wk, to precede the diagnosis of aspergillosis by 2 to 20 wk, and to detect fungal reinfection. In most cases, successful antifungal treatment decreased specific IgG antibody response. A decrease in specific IgG antibody response correlated with the inability to culture or identify *A. fumigatus*

from bronchoalveolar lavage fluid and with radiographic and clinical improvement. IgG antibody responses in serum may, therefore, provide important information that is helpful in the diagnosis and early treatment of pulmonary fungal infections and in monitoring antifungal treatment in patients who have had lung transplantation.