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Molecular mechanisms of epithelial host defense in the airways

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CHAPTER 7

SUMMARY AND GENERAL DISCUSSION

INTRODUCTION

In this thesis studies are described in which the molecular mechanisms of host defense in bronchial epithelial cells were investigated. The central question underlying these studies was:"What are the mechanisms employed by bronchial epithelial cells to cope with respiratory infections?". The aims were *(i)* to delineate the early host defense response in bronchial epithelial cells and *(ii)* to characterize the expression of (novel) epithelial-derived host defense effector molecules. As described in **Chapter 1**, airway epithelial cells are central to host defense by *(i)* forming the primary physical barrier [1] and *(ii)* by producing host defense effector molecules [2]. Effector molecules including antimicrobial peptides, proteinase inhibitors, cytokines and chemokines have been described as key players in host defense. Nevertheless, the repertoire of gene products involved in the early epithelial host defense response had not been systematically investigated.

Large scale gene expression analysis has become feasible through the introduction of genomics technology. Recent studies in host-pathogen research that were aimed at gene expression profiling using state-of-the-art genomics techniques revealed novel insights in the cross-talk between the host and the pathogen [3,4]. A review of the recent literature on this topic is provided in **Chapter 2**. In the study described in **Chapter 3** the SAGE technology was applied to assess the transcriptional changes that occur in primary bronchial epithelial cells upon exposure to *P. aeruginosa* and the pro-inflammatory cytokines IL-1β and TNFα. **Chapter 4** describes the transcription kinetics of the S100 calcium-binding proteins S100A8 and S100A9 in cultured bronchial epithelial cells. These S100 calcium-binding proteins were found to be among the most abundantly transcribed genes that were differentially expressed after microbial exposure genes as determined by SAGE. S100A8 and S100A9 display interesting features related to host defense [5]. We assessed the expression kinetics of S100A8 and S100A9 and investigated whether bronchial epithelial cells were able to synthesize and release the S100A8/A9 protein complex upon microbial exposure. In **Chapter 5** we assessed the protein levels of selected antimicrobial peptides and proteinase inhibitors in a range of mucosal secretions. Each mucosal secretion is characterized by a unique pattern of antimicrobial peptides and proteinase inhibitors. Many of the assessed molecules were present in multiple mucosal secretions, indicating their potentially unified function in host defense at different epithelial sites of the body. To further delineate commonalities in epithelial host defense we performed a comparative genomics approach on our generated SAGE data. **Chapter 6** describes a comprehensive *in silico* analysis of SAGE gene expression profiles obtained from from two established culture models of epithelial inflammation. In these inflammation models, the host defense response of bronchial epithelial cells and keratinocytes was assessed upon exposure to pro-inflammatory cytokines. This comparative genomics analysis revealed a unique genetic signature that is associated

with epithelial host defense. Expression of this genetic signature appears to be restricted to epithelial tissues of the airways and skin.

APPLICATION OF GENOMICS TECHNOLOGY IN HOST-PATHOGEN RESEARCH

Application of SAGE has been successful in identifying (novel) host defense effector molecules and delineating the epithelial host defense response [6]. The advantages of the SAGE technology are its *(i)* efficient way of generating gene expression profiles of both known and unknown genes; *(ii)* its ability of studying (novel/unknown) genes in a biological context; and *(iii)* its ability to elucidate genetic networks associated with epithelial host defense. These advantages have been utilized in the investigations described in **Chapter 3** and **Chapter 6**.

In **Chapter 3** the generation of three SAGE libraries derived from bronchial epithelial cells is described. Cells were exposed to medium alone, to the pro-inflammatory cytokines IL-1β/TNFα and to *P. aeruginosa*. Since SAGE is based on random sampling, the accuracy of expression profiles depends on the number of sequenced tags. To profile the complete mammalian transcriptome, it has been estimated that at least 1.2 million SAGE tags should be sequenced [7]. In contrast, ~20,000-50,000 tags are generally sequenced per single SAGE library. These numbers of tags appear to be sufficient for detecting genes with medium to high abundant levels of expression [8]. To ensure the reliability of our SAGE libraries, the expression profiles were verified by quantitative real-time polymerase chain reaction (qPCR). SAGE and qPCR perform equally in estimating the expression levels of high abundant genes. For genes expressed at low levels, both SAGE and qPCR correctly estimate the directional change in expression. However, discrepancies in the magnitude of differential gene expression were observed between SAGE and qPCR. These discrepancies are due to the difference in sensitivity of the technologies: qPCR is by far more sensitive than SAGE. The investigation in **Chapter 3** revealed four classes of genes that are implicated in the early epithelial host defense response: keratins, proteinase inhibitors, S100 calcium-binding proteins and IL-1 family members [6].

S100A8 AND A9: MULTIFUNCTIONAL EFFECTOR MOLECULES OF EPITHELIAL HOST DEFENSE

S100 calcium-binding proteins were found to be abundantly expressed in bronchial epithelial cells. The family members S100A8 and S100A9 have been described as novel effector molecules of innate immunity [5]. In **Chapter 4** we investigated the expression of these molecules in bronchial epithelial cells in more detail. We demonstrated that

mRNA expression of S100A9 and S100A8 is transiently increased upon microbial exposure. Furthermore, we demonstrated that bronchial epithelial cells are able to synthesize and release the S100A8/A9 protein complex. Secretion of this protein complex was also transiently increased upon microbial exposure. These observations indicate that the S100A8/A9 complex is particularly involved in the early epithelial host defense response. We also observed that oxidative stress resulting from cigarette smoke exposure increased S100A8/A9 gene expression. These observations are in line with others that demonstrated the increased expression of these genes induced by cellular stress [9]. This finding may be highly relevant for understanding the function of the S100A8/A9 complex in smoking-induced lung disease.

COMPARATIVE ANALYSIS OF HOST DEFENSE EFFECTOR MOLECULES IN MUCOSAL SECRETIONS

A second feature of epithelial host defense is the production of effector molecules with antimicrobial activity. We analyzed the composition of different mucosal lining fluids for similarities and differences. The aim of the study described in **Chapter 5** was to assess the levels of epithelial-derived antimicrobial peptides and proteinase inhibitors in different mucosal fluids [10]. Each mucosal fluid displayed an unique protein expression pattern of antimicrobial peptides and proteinase inhibitors. Differences in effector molecule profile of mucosal fluids most likely reflect tissue-specific requirements for optimal antimicrobial action at that particular site of the body. It should be noted that gene expression levels do not necessarily reflect protein expression levels. Nevertheless, we did observe that components such as the proteinase inhibitors SLPI, elafin and cystatin M/E were present in nasal secretions and were also detected by SAGE analysis in bronchial epithelial cells. In nasal secretions, the presence of the inducible antimicrobial peptides hCAP18/LL-37 and β-defensin-2 is relatively low in healthy individuals. Our findings in **Chapter 3** as well as other studies indicate that the levels of host defense effector molecules increase during infection.

APPLICATION OF COMPARATIVE GENOMICS TO STUDY EPITHELIAL HOST DEFENSE

Bioinformatics and experimental genomics approaches are indispensable in elucidating genetic networks. To identify gene expression patterns associated with epithelial host defense, we conducted a comparative analysis of our previously generated SAGE expression profiles [6,11]. Whereas the contribution of bronchial epithelial cells and kerati-

nocytes to epithelial host defense is widely recognized, few common protective mechanisms have been described for these cell types. The aim of **Chapter 6** was to investigate whether bronchial epithelial cells and keratinocytes employ similar mechanisms of host defense [12]. We addressed this question by utilizing a combined approach of bioinformatics and genomics. To identify a unique pattern of gene expression in these types of epithelial cells the "Tissue Preferential Expression (TPE)" algorithm [13] was applied. The advantage of this *in silico* method is that it allows clustering of SAGE data without the necessity of having full knowledge of the repertoire of genes expressed. Experimental verification is essential to ensure the reliability of the TPE predictions for subsequent data interpretation. Collectively, **Chapter 3** and **Chapter 6** illustrate the power of an unbiased research approach using open gene expression profiling and bioinformatics to delineate novel mechanisms in epithelial function. The investigation in **Chapter 6** revealed a genetic signature that characterizes the response of bronchial epithelial cells and keratinocytes to an inflammatory stimulus.

The majority of preferentially expressed genes that make up this signature has been previously described to be involved in the formation of the cornified envelope in keratinocytes [14]. This rigid protein structure is known to form the primary physical barrier in skin [15]. Much less is known about similar structures in bronchial epithelial cells. Therefore, our finding that bronchial epithelial cells express multiple structural elements and enzymes that form a cross-linked protein envelope provides novel insight into the cell biology of the bronchial epithelial cell. Despite these similarities, there are obvious differences. One such difference that we noted was the expression of loricrin and involucrin by bronchial epithelial cells and keratinocytes. Whereas in keratinocytes, these molecules form more than 70% of the protein mass of the cornified envelope, no expression was observed by SAGE in bronchial epithelial cells. However, this does not implicate that other components of the cornified envelope do not contribute to barrier function in bronchial epithelial cells. It has been demonstrated that absence of loricrin and involucrin expression does not affect skin epithelial barrier function [16,17]. In knock-out mice, the absence of these molecules was compensated by other components of the protein envelope. These include members of the small proline rich proteins and members of S100 calcium-binding protein family [16]. These genes were abundantly expressed in bronchial epithelial cells. These findings demonstrate that the relative abundance of these components may vary widely in the protein envelope [14]. Consequently, the proposed composition of the protein envelopes in bronchial epithelial cells and keratinocytes may differ substantially [18]. It is tempting to speculate that dynamic regulation of a protein structure may contribute to an effective barrier against respiratory pathogens in the airway.

NOVEL INSIGHTS INTO EPITHELIAL HOST DEFENSE AND FUTURE RESEARCH PERSPECTIVES

Our studies provide novel insight into the mechanisms employed by bronchial epithelial cells in host defense against infection. Important functions are suggested for structural barrier proteins, S100 proteins, proteinase inhibitors and IL-1 family members in epithelial host defense ***(Table 1)***. Particularly little is known about the contribution of barrier proteins and IL-1 homologues in epithelial host defense in the airways. Our studies demonstrated that epithelial cells of the the airways and the skin utilize similar mechanisms in host defense against infection, despite a markedly different structure of these epithelia. How do these findings increase our understanding of host defense in the lung and what are their implications for inflammatory and infectious lung disorders?

Our systematic comparison of bronchial epithelial cells and skin keratinocytes in the response to pro-inflammatory cytokines revealed remarkable similarities. These observations are in line with the findings that genetic susceptibility to asthma and atopic dermatitis partly involves polymorphisms in genes specifically expressed in the epithelium of the skin and airways [19]. The association of epithelial cell function and susceptibility to asthma and atopic dermatitis is further underlined by a recent study demonstrating that loss-of-function variants of the epidermal barrier protein filaggrin are risk factors for the development of atopic dermatitis with or without asthma [20]. Investigating filaggrin in bronchial epithelial cells could provide novel insights into the role of this barrier protein in asthma.

Also other studies in atopic dermatitis may have important implications for understanding lung disease. It has been demonstrated that the expression of antimicrobial peptides in lesional skin of patients with atopic dermatitis is lower than that in psoriasis or normal skin [21,22]. This finding is relevant because patients with atopic dermatitis frequently suffer from skin infections, which - as suggested by these recent findings - may in part be due to a local deficiency in antimicrobial peptides. Further studies showed that Th2 cytokines such as IL-4, IL-13 and IL-10 [23,24] decrease the expression of antimicro-

Table 1. Summary of SAGE the data. Expression of genes in bronchial epithelial cells upon exposure to *P. aeruginosa*.

Gene family	Members most abundantly expressed	Function
Keratins	KRT5, KRT6B, KRT14, KRT17, KRT19	Structural/barrier proteins
Small proline-rich proteins	SPRR1A, SPRR1B, SPRR2A, SPRR3	Structural/barrier proteins
Proteinase inhibitors	SLPI, elafin, CSTA, CSTB, SERPINB5	Protease inhibition, antimicrobial, cell proliferation and anti-inflammatory activity
S100 calcium-binding proteins	S100A2, A6, A8, A9, A10, A11, A14, A16	Barrier proteins, antimicrobial, signal transducer activity, calcium ion binding
IL-1 members	IL-1β, IL-1RN, IL-1F9	Cell signaling, IL-1 receptor activity

bial peptides in cultured keratinocytes. These findings link Th2 type inflammation to a local deficiency in epithelial host defense. Since also patients with atopic airway disease (rhinitis, sinusitis and asthma) suffer from airway infections, the question arises whether this deficiency is also present in airway epithelial cells. Especially IL-13 has been found to have marked effects on airway epithelial function and differentiation and has been identified as an inducer of goblet cell differentiation [25]. Recent studies show that IL-13 may also markedly increase expression of small proline-rich proteins (SPRR) 2A and 2B in mouse airway epithelium during allergic inflammation [26]. Our observation that SPRRs are part of the repertoire of genes specifically expressed by both bronchial epithelial cells and keratinocytes in response to pro-inflammatory cytokines (**Chapter 6**) emphasizes the suggested importance of these proteins in host defense. A third recent illustration of the importance of comparing epithelial tissue biology at different sites in the human body, is the observation that patients with ileal Crohn's disease frequently display a relative deficiency in expression of the human α-defensin HD5 [27]. This deficiency is at least in part related to polymorphisms in the pattern recognition receptor NOD2. Also in view of the observation that patients with Crohn's disease may display (subclinical) pulmonary complications, such mechanisms could play a role in lung disease [28,29]. Collectively, these studies underline the clinical relevance of studying shared defense mechanisms in inflammatory disorders affecting epithelial surfaces. Therefore, future studies into potential alterations in epithelial host defense function in inflammatory and infectious lung disorders are needed.

We have identified regulation of expression of epidermal barrier proteins in bronchial epithelial cells in response to microbial exposure. Whereas these components have been associated with the extracellular epidermal barrier in skin, relatively little is known about their function in the airways. Studies on *e.g.* SPRRs in bronchial epithelial cells have largely focused on squamous differentiation [30,31]. As a follow-up to our findings, one of the first questions that needs to be addressed is whether these structural proteins truly form a functional barrier in bronchial epithelial cells. This can be addressed partly by using confocal scanning microscopy to determine whether these proteins are indeed organized into a structure reminiscent of a barrier. Their role in host defense against infection can be addressed by studying polymorphisms in selected patient populations, since single nucleotide polymorphisms (SNPs) in these proteins have been associated with atopic disease, especially atopic dermatitis. One approach could be to study whether SPRRs act as modifier genes in cystic fibrosis, based on the observation that deficiencies in the innate immunity molecule mannose binding lectin (MBL) are associated with increased bacterial colonization and decreased lung function in cystic fibrosis [32].

Our studies also led to the unexpected finding that the IL-1 family is the most abundantly expressed cytokine family in bronchial epithelial cells exposed to *P. aeruginosa* (**Chapter 3**). The IL-1 cytokine family might be of special interest for epithelial host

Table 2. Expression of IL-1 family members by bronchial epithelial cells and keratinocytes. IL-1 family members detected in the SAGE libraries of bronchial epithelial cells and keratinocytes. Listed are the SAGE tag sequence, IL-1 family member, the Tissue Preferential Expression (TPE) value for the particular tag in the PBEC and KC SAGE libraries and the preferential expression in epithelial cells. TPE values were calculated in the SAGE libraries generated from PBEC and KC that were exposed to pro-inflammatory cytokines. TPE values of ≥9 are indicative for preferential expression of the gene for the particular cell type. Only those tags with TPE ≥9 in both libraries are considered as preferentially expressed by epithelial cells.

Tag	IL-1 member	TPE in PBEC	TPE in KC	Preferential expression
ACTAGCACAG	IL-1F9	9.55	9.98	Yes
CCCTCAATCC	IL-18 (IL1-F4)	7.39	9.35	No
CTATGAGCAG	IL-1F5	8.05	N/A	No
GATTTCAGCT	IL-1F10	<4	N/A	No
CAATTTGTGT	IL-1β	6.07	N/A	No
ACTCGTATAT	IL-1RN	7.83	N/A	No

defense. Abundantly expressed members were the classical members IL-1β, IL-1 receptor antagonist (IL-1RN) and IL-18, but also the novel member IL-1F9. Recently, scanning the human genome for homologs of IL-1α/β revealed six novel members of the IL-1 family, named IL-1F5 to IL-1F10 [33]. Interestingly, the investigations described in **Chapter 3** and **6** demonstrated that IL-1F9 expression is a shared element in the response of bronchial epithelial cells and keratinocytes to inflammatory stimuli and indicates a potentially important role of these novel IL-1 homologues in epithelial function. Equally interesting is the fact that expression of IL-1F9 appears to be characteristic for epithelial cells ***(Table 2)***. Our studies demonstrated preferential expression in bronchial epithelial cells and keratinocytes. Evaluation of publicly available SAGE and EST databases revealed that also colon epithelial cells are capable of expressing IL-1F9. It has been suggested that IL-1F9 exerts modulatory functions in IL-1 signalling [34]. Based on our data, we hypothesize that IL-1F9 is particularly involved in both the amplification and fine tuning of the epithelial innate immune response. Classically, abundant IL-1 production has been largely ascribed to macrophages. Based on our findings and data available in the literature, the effects of macrophage-derived IL-1 on target cells are presumably actively controlled by IL-1F9 and potentially other novel IL-1 family members. These findings shed new light on our understanding of the pathophysiology of epithelial inflammatory diseases.

In our laboratory, the contribution of IL-1F9 to the epithelial innate immune response is a topic of current research. Recently, we have shown that, *P. aeruginosa* not only induces the expression of IL-1F9, but also increases the expression of the related IL-1 homologues IL-1F6 and F8. Importantly, we have demonstrated that bronchial epithelial cells express IL-1Rrp2, the receptor for these novel IL-1 homologues. Exposure of bronchial epithelial cells to these novel IL-1 family members results in a functional response in these cells [35]. Due to the highly preferential expression pattern, IL-1F9 is potentially

an attractive target for the development of intervention therapies for the treatment of inflammatory diseases of epithelial tissues. With respect to atopic disease, the IL-1 family member IL-18 (also known as IL-1F4) may also represent an interesting intervention target. Recently, IL-18 has been associated with atopic diseases such as atopic dermatitis due to its enhancing effects on the expression of the Th2 cytokines IL-4 and IL-13 [36]. Overexpression of IL-18 in mouse skin has been shown to initiate atopic dermatitis-like inflammation, a process that is further accelerated by IL-1α/β [37]. These studies suggest an important role for IL-18 and IL-1α/β. Since IL-1F6, F8 and F9 may display activities that partly overlap with those of IL-1α/β and IL-18, studies on the role of these novel IL-1 homologues in atopic diseases are warranted. Functional studies in epithelial cells and investigating novel IL-1 homologues in disease are necessary in order to address whether these IL-1 family members indeed form candidate targets for future therapeutic interventions.

In summary, this thesis provides a strong basis for further investigations into epithelial function in host defense. Several novel targets were identified that may help to direct future research into the role of the airway epithelium in inflammatory and infectious lung disease. Especially members of the small proline-rich proteins and the novel IL-1 homologues (and their receptor) are of interest in view of their highly preferential expression in epithelial cells. The ultimate aim of such studies would be to develop novel and better treatment strategies aimed at controlling epithelial cell function in patients with inflammatory and infectious lung disease.

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