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β-defensins and LL-37 in bronchoalveolar lavage fluid of patients with cystic fibrosis☆

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Abstract

Background: The antimicrobial peptides human β-defensin 1 and 2 (hBD-1 and 2) and the cathelicidin LL-37/hCAP-18 are key factors in innate immune responses of the respiratory tract. The aim of this study was to determine the concentrations of these peptides in airway surface fluid of CF patients with mild lung disease. *Methods*: We measured the concentrations of hBD-1, hBD-2, and LL-37 in bronchoalveolar lavage fluid of 20 patients (5–34 years) participating in the prospective BEAT-study (bronchoalveolar lavage for the evaluation of anti-inflammatory treatment) using an immuno-dot blot-assay. *Results*: All three peptides could be detected in lavage fluid of the study population. Increased levels of inflammatory markers in bronchoalveolar lavage fluid were associated with elevated concentrations of LL-37/hCAP-18 (total cell count, $P=0.006$; relative neutrophil count, $P=0.002$). Deterioration of lung function, measured by MEF_{25} (maximal flow rate at 25% of residual forced vital capacity), correlated with decreased hBD-2 ($P=0.026$), but increased LL-37/hCAP-18 concentrations ($P=0.016$). *Conclusions*: The data suggest that concentrations of antimicrobial peptides are correlated with severity of CF lung disease: Levels of LL-37/hCAP-18 are associated with bronchial inflammation and, therefore disease severity, whereas decreased levels of β-defensins in advanced lung disease likely contribute to a secondary defect of the local host defense.

Keywords: Antimicrobial peptides; Cystic fibrosis; Bronchoalveolar lavage fluid; Innate immunity

1. Introduction

Cystic fibrosis (CF) is the most common autosomal recessive disorder in the Caucasian population and is caused by mutations in the gene coding for the CF transmembrane conductance regulator (CFTR) [1]. There is evidence of bronchial inflammation and bacterial colonization even before the onset of clinically overt lung disease [2], pointing to a primarily inherited modulation of immune response rather than a defect, which occurs secondarily with progression of the disease.

β-defensins and cathelicidins are the two principal families of antimicrobial peptides expressed in the epithelium of the human lung and include the human β-defensins 1 to 4 (hBD-1 to hBD-4) [3–10] and the cathelicidin LL-37/hCAP-18 [11–13]. LL-37/hCAP-18 is expressed as pre-pro-peptide. The propeptide is called hCAP-18 and stored in cells. After secretion, the propiece is cleaved off and the C-terminal peptide is called LL-37. LL-37 is the active peptide with antimicrobial and multiple other activities [14]. Antimicrobial peptides contribute to innate immunity by direct antimicrobial activity [15]. Additionally, defensins and LL-37/hCAP-18 have multiple other functions, such as the activation

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Table 1
Patients' characteristics

	BAL 1 ($n=20$) Median (95% CI)	BAL 2 ($n=20$) Median (95% CI)	BAL 3 ($n=14$) Median (95% CI)
Age (years)	11 (9–14)	13 (10–16)	14 (11–15)
FEV_1 (% pred.)	97 (93–101)	88 (83–103)	91 (78–101.5)
MEF_{25} (% pred.)	58 (45–72)	42 (28–72)	55 (27–67)
Cellular viability pooled fraction (%)	88 (80–92)	82 (76–86)	83 (78–86)
Cell count pooled fraction ($\times 10^4$/ml)	20 (14–51)	24 (16–46)	42 (19–66)
Neutrophils pooled fraction (%)	22 (0.8–89)	32 (0.20–92)	56 (0.70–94)
Positive for *Staphylococcus aureus* (BAL)	13 patients	9 patients	7 patients
Positive for *Pseudomonas aeruginosa* (BAL)	7 patients	6 patients	5 patients
Positive for *Staphylococcus aureus* and *Pseudomonas aeruginosa* (BAL)	2 patients	2 patients	2 patients

FEV_1 = forced expiratory volume in one second, MEF_{25} = maximal flow rate at 25% of remaining forced vital capacity. Some patients were not colonized of colonized with more than one species.

of inflammatory cells, the regulation of adaptive immunity [16,17], and the induction of angiogenesis [18]. The role of antimicrobial peptides in the pathogenesis of cystic fibrosis lung disease is unclear. In the high salt hypothesis of Welsh and colleagues [19], the activity of antimicrobial peptides is inhibited by the high salt concentration in CF airway surface fluid. Other reports indicate that the salt-independent antimicrobial activity of CF airway surface fluid is decreased [20]. Defensins and the cathelicidin also may exert proinflammatory or cytotoxic effects on airway epithelial cells as has been proposed for α-defensins found in lavage fluid of CF patients [21]. Polymorphisms of the genes coding for defensins are associated with susceptibility to Candida carriage in type I diabetes and with the clinical course of chronic obstructive pulmonary disease [22]. These association studies support the role of defensins in host defense and inflammation. Based on their host defense and proinflammatory functions, antimicrobial peptides likely have a central role in the pathogenesis of CF lung disease.

It was the aim of this study to measure concentrations of hBD-1, hBD-2, and LL-37/hCAP-18 in bronchoalveolar lavage fluid of CF patients with early mild lung disease. We hypothesized that peptide concentrations correlate with colonization status, pulmonary functions parameters, or inflammatory markers in bronchioalveolar lavage fluid (BALF).

2. Material and methods

2.1. Patients

Flexible fiber optic bronchoscopy and bronchoalveolar lavage (BAL) were performed three times within three years (time interval 18 months) in CF patients (5–34 years) during the course of the BEAT-study (bronchoalveolar lavage for the evaluation of anti-inflammatory treatment) [23]. Twenty patients took part at the first BAL, 20 at the second BAL (18 months), and 14 at the third BAL (36 months) (Table 1). All patients were free of infectious exacerbations within 6 weeks before BAL. Informed consent had been given by each patient/the parents before the procedure, which was approved by the local Ethics Committee. Measurements of pulmonary function tests were performed according to current guidelines [24]. Bacterial cultures were obtained in 2 ml of the pooled BALF sample prior to processing.

2.2. Flexible fiber optic bronchoscopy and BAL

BAL was performed at 08.00 h. No rhDNAse was inhaled before the procedure, but β2-agonists were permitted. No patient received inhaled steroids or amino glycosides. Flexible fiber optic bronchoscopy (Olympus, external diameter 3.5 or 4.9 mm, Hamburg, Germany) and BAL were performed in deep sedation according to standard methods [23]. The first and pooled (second and following) fractions were analyzed separately, only the pooled fraction was used in this investigation. Total cell count of the BAL was measured by two experienced laboratory assistants, the cell differentials by an experienced cytopathologist, who counted at least 500 cells in each preparation.

2.3. Measurement of concentrations of antimicrobial peptides

Concentrations of hBD-1, hBD-2, and LL-37/hCAP-18 in BALF were measured as described previously [20,25,26]. In brief, after centrifugation of BALF at

1000 g for 20 min, 2 μl of each sample were dotted onto a nitrocellulose membrane, immunolabeled with polyclonal rabbit antibodies to hBD-1, hBD-2 and LL-37/hCAP-18 (dilution 1:1000), followed by a peroxidase-conjugated anti-rabbit antibody from goat (Sigma Chemical Co., 1:5000). Antibodies against the antimicrobial peptides were generated by performing a standard immunization protocol in rabbits [9,11], and tested for specificity to the corresponding antimicrobial peptide. Comparisons of the immune- with the pre-immune-sera in blotting experiments showed specific reactivity of the polyclonal antibodies to their corresponding peptides (data not shown). Additionally, the assays were tested by addition of a known amount of peptides to BALF and measuring the increase of peptide concentrations. The measurements showed the expected change in concentration. After the washes, bound antibodies were visualized by using chemiluminescent substrate (ECL – enhanced chemiluminescence-Western blotting detection kit; Amersham, Arlington Heights, IL) and exposure to X-ray films. Signal intensity was analyzed using a GelDoc 2000 system (Biorad, Munich, Germany). To characterize the molecular forms of the peptides in airway secretion, we prepared Western blots of lavage fluid from selected patients as described before using chemically synthesized peptides as standards (defensins from IPF Pharmaceuticals, Hanover; LL-37 from Dr Henklein, Charité, Berlin, Germany).

2.4. Statistical analysis

Data of the three BAL's were pooled. Group median of numerical data were tested for significant differences by the Mann–Whitney U-Wilcoxon rank sum W test, correlations by the Spearman correlation test with two-tailed level of significance. All levels of significance were set at $P \leq 0.05$.

3. Results

3.1. Mucosal antimicrobial peptides are present in airway secretion of CF patients with mild lung disease

To determine whether antimicrobial peptides are present in the airways of CF patients with mild lung disease, we analyzed bronchioalveolar lavage fluid by antigen capture assay and Western blot. The antigen capture assay was validated earlier to measure the amounts of peptides in human body fluids and reached a sensitivity of 5 ng/ml [9,11,20]. We first addressed the question, whether mucosal antimicrobial peptides are present in airways of patients with CF, and identified detectable amounts of hBD-1, hBD-2, and LL-37/hCAP-18 in BALF from patients of different ages and degrees of lung disease. The concentrations of the peptides are in the range from undetectable to 15 μg/ml. To characterize the molecular structures of the peptides in airway secretion, we performed Western blot analysis and immunopositive bands representing mature peptides were identified (Fig. 1).

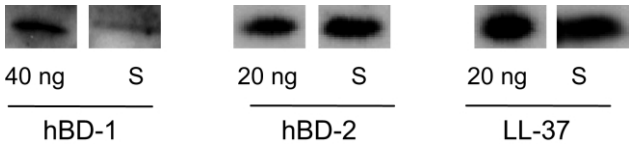

Fig. 1. Western blots of airway surface fluid showing bands representing mature peptides for hBD-1, hBD-2, and LL-37. Lung lavage fluid of arbitrarily selected patients was extracted in acetonitrile (final concentration 60%) and trifluoroacetic acid (TFA, final concentration 1%) overnight at room temperature. After centrifugation and lyophilization, the material was applied to Western blotting. 20 or 40 ng of synthetic peptide, S = sample.

3.2. Levels of antimicrobial peptides correlate with disease severity

We next asked whether levels of antimicrobial peptides correlate with markers of inflammation in the BALF, microbiological data, or pulmonary function tests. Data were correlated with levels of antimicrobial peptides in a cross sectional analysis.

With increasing levels of inflammatory markers (total cell count and relative neutrophil count) the concentration of LL-37/hCAP-18 increased (Table 2 and Fig. 2a,b). hBD-1 and hBD-2 correlated positively with each other, while they did not show a correlation with LL-37 (Table 2).

Deterioration of lung function as measured by MEF_{25} was associated with increased LL-37/hCAP-18 and lower levels of hBD-2 (Table 2 and Fig. 3a,b). Presence of bacteria was not associated with significantly increased levels of antimicrobial peptides in the study population. There were no significant changes of the concentrations of the antimicrobial peptides during the study in a longitudinal analysis. The data represent a cross-sectional analysis.

4. Discussion

The results of our study demonstrate that the antimicrobial peptides hBD-1, hBD-2 and LL-37/hCAP-18 are present in BALF of patients with CF. The main finding is that levels of these peptides are associated with pulmonary inflammation and disease severity. The concentration of the cathelicidin LL-37/hCAP-18 increases with lung inflammation, as judged by neutrophil airway inflammation. In contrast, the levels of hBD-1 and hBD-2 are not changed by endobronchial inflammation. Disease severity as estimated by reduced lung function is associated with increased levels of LL-37, whereas concentrations of hBD-2 are decreased. The

Table 2
Correlation of the antimicrobial peptides with inflammatory parameters in BALF (with correlation coefficient '*r*' and, in parenthesis point of significance '*P*')

	FEV_1	MEF_{25}	Total cell count	Relative neutrophil count	hBD-1	hBD-2
hBD-1	0.200	0.266	−0.051	−0.125	–	–
	(0.148)	(0.052)	(0.714)	(0.374)		
hBD-2	0.144	**0.3020**	−0.024	−0.180	**0.417**	–
	(0.299)	(**0.026**)	(0.862)	(0.896)	**0.002**	
LL-37	−0.261	**−0.326**	**0.370**	**0.414**	−0.224	−0.037
	(0.057)	(**0.016**)	(**0.006**)	(**0.002**)	0.103	0.791

Bold printed are the significant correlations with $P \leq 0.05$.

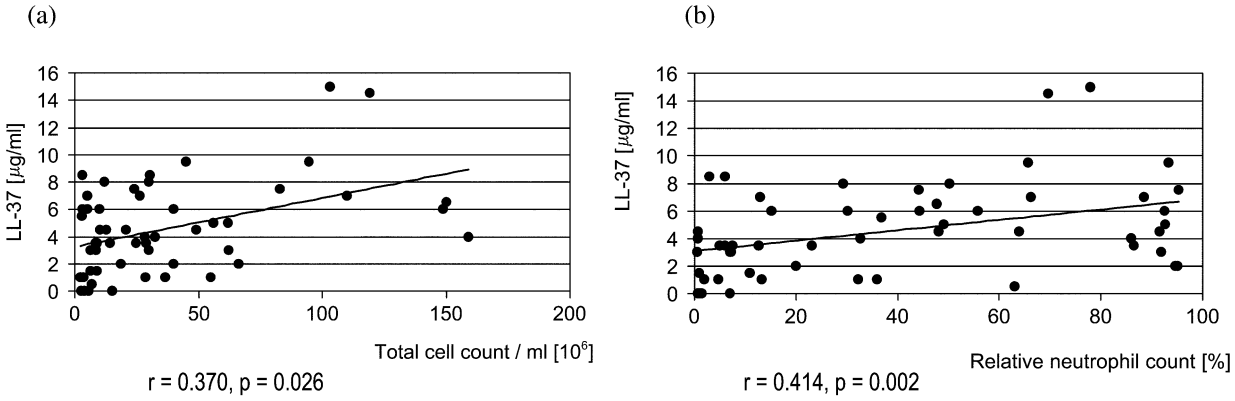

Fig. 2. Concentrations of LL-37 are correlated with pulmonary inflammation. Levels of LL-37 increase with elevated numbers of total cells/ml (a) or relative neutrophil count (b).

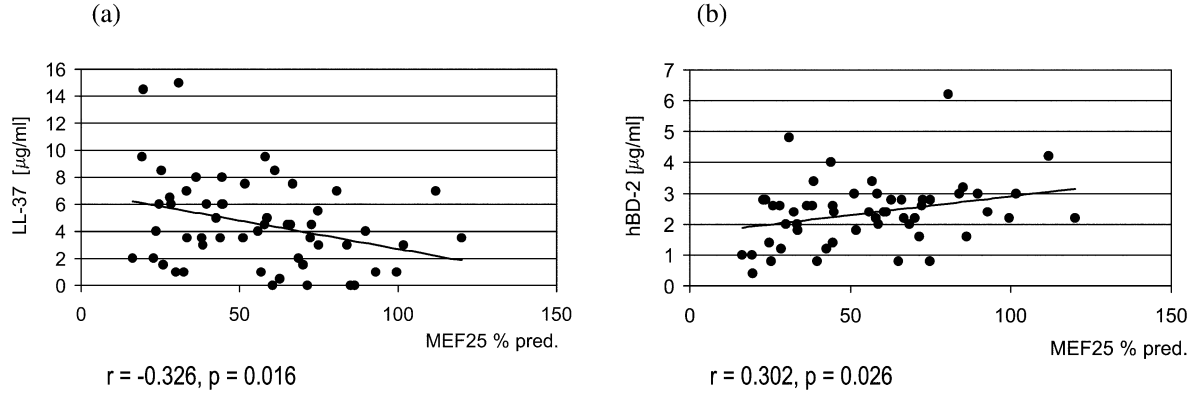

Fig. 3. Concentrations of LL-37 and hBD-2 are correlated with pulmonary function. With decreased MEF_{25} the levels of LL-37 in BALF increase (a), whereas concentrations of hBD-2 are decreased (b).

concentrations of antimicrobial peptides in the present study are equivalent to the levels described in other pathologies [26–28]. We were not able to detect a longitudinal change of the peptide concentrations during the course of the study in individual patients. This is likely caused by the limited number of patients and the relatively short duration of the study. A small number of patients did not undergo the third BAL. These patients likely had more severe lung disease. This could possibly result in underestimation of the effects demonstrated in the study.

An early step in the development of CF lung disease is the incapability of the local host defense system to clear the bacterial colonization and inflammation, which might be linked to the absence or inhibition of antimicrobial substances [29]. Antimicrobial peptides like hBD-1, hBD-2, and LL-37/hCAP-18 play an important role in innate and adaptive immunity. The antimicrobial

activity of airway epithelial cells from CF patients is decreased as compared to non-CF cells [7,19,20]. The nature of this defect remained largely speculative. One hypothesis stated that the salt concentration of CF airway secretions is increased and that this results in inactivation of antimicrobial substances [19]. Another hypothesis described defects in secretion of antimicrobial factors [20]. Lacking appropriate methods to sample airway surface liquid from patients it has not been possible to verify or falsify these hypotheses. The concentrations of these peptides in mild CF lung disease and their correlation with disease severity have been unknown. The present study shows that β-defensins and LL-37/hCAP-18 are present in this patient group, and alterations of their levels are associated with disease severity. The cathelicidin LL-37/hCAP-18 is associated with airway inflammation and disease severity as determined from pulmonary function parameters. Its production by epithelial cells, neutrophils, and macrophages [11–13,30] contributes to the amount of peptide detected in BALF. Beside its direct antimicrobial function, LL-37 binds to formyl peptide receptor like 1 (FPRL1), and chemoattracts human peripheral blood neutrophils, monocytes, and T cells [31]. Increased concentrations of LL-37/hCAP-18 in the airway lumen likely result in the recruitment of further inflammatory cells.

In contrast to LL-37/hCAP-18, concentrations of β-defensins were found decreased in patients with more impaired lung function. Several explanations may account for this finding: (1) Chronic airway inflammation results in damage and loss of epithelial cells that secrete most of the β-defensins; (2) Chronic inflammation differs significantly from acute responses and results in down regulation of several molecules involved in inflammation and host defense, possibly also of β-defensins [32]. Furthermore, it is possible that microorganisms actively down regulate the synthesis, secretion and/or expression of certain antimicrobial peptides [33]; and (3) the increased load of luminal proteases could result in fastened cleavage and inactivation of defensins. As an example, the metalloproteinase matrilysin (MMP-7) has been described to be increased in CF airways [34], and is able to cleave defensins [35]. The relatively lower concentrations of β-defensins during advanced stages of CF lung disease likely contribute to a secondary defect of the host defense altering the natural history of the disease. It would be interesting to correlate the altered concentrations of the peptides with functional assays. Due to the small amount of material and the dilution of the BAL samples it was not possible to perform functional assays such as antimicrobial or cell physiological tests.

In conclusion, mucosal antimicrobial peptides are present in mild CF lung disease. Alterations of their concentrations are associated with disease severity indicating a link to the mechanisms of lung destruction. LL-37/hCAP-18 is a marker for the degree of bronchoalveolar inflammation, whereas concentrations of β-defensins are lower in more advanced lung disease. Since antimicrobial peptides act in a concentration-dependent manner, therapeutic modifications (inhibition or augmentation) could be regarded as a feasible alternative approach to treatment of CF.

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Appendix A:

The BEAT-study group consists of M. Ballmann, H. von der Hardt, Hannover, M. Griese, D. Reinhardt, München, F. Ratjen, Essen, E. Rietschel, Köln, U. Wahn, Berlin, and K. Paul, Berlin, the director of this study.

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