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Expression of β-Defensin 1 and 2 in Nasal Epithelial Cells and Alveolar Macrophages from HIV-infected Patients

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Abstract:
Background: The incidence of respiratory infection is high in HIV-infected patients. β-defensins are anti-microbial peptides derived from epithelia on the mucosal surfaces of the respiratory, gastrointestinal and urinary tract. Nothing is known about the rate of expression of β-defensin 1 and 2 mRNAs in nasal epithelial cells and alveolar macrophages in HIV-infected patients.
Methods: Semiquantitative rt-PCR measurement of β-defensins 1 and 2 and β-actin were carried out on nasal epithelial cells of 109 patients (76 HIV-infected) and alveolar macrophages from 56 patients (18 HIV-infected).
Results: The levels of β-defensin 1 and 2 mRNAs in nasal epithelial cells did not differ significantly between HIV-infected and non-infected patients. In the nasal epithelial cells of HIV-negative patients who suffered from respiratory infections β-defensin levels were decreased. β-defensin 1 mRNA expression was significantly reduced in alveolar macrophages from HIV infected patients. β-defensin 2 mRNA expression in alveolar macrophages was very low. β-defensins 1 and 2 mRNA expression did not correlate with CD 4 cell numbers in the blood of HIV-infected patients.
Conclusion: HIV infection and CD 4 cell numbers in the blood do not influence β-defensin 1 and 2 expressions in nasal epithelial cells. In alveolar macrophages, β-defensin 1 expression is decreased in HIV-infected patients.

Introduction

The lungs are protected against infections by different systems, primarily the mucociliary escalator and the cough reflex. Malfunctions of these systems lead to pulmonary infections, for instance in patients suffering from ciliary dysfunction or from cystic fibrosis [1]. The next protection systems are the cellular and non-cellular aspects of innate immunity: alveolar macrophages, neutrophils and antimicrobial peptides such as defensins [2, 3]. Finally, there are the components of the adaptive immune system: lymphocytes and immunglobulins. As we know from studies of different patients, including HIV-infected patients, disturbances in the adaptive immune system allow uncontrolled proliferation of many microbes [4].

Disturbances of the innate immune system seem not to cause serious problems because of the redundancy within this system. β-defensin 1 deficient mice do not die earlier than wild type mice, though they have a delayed clearance of Haemophilus influenzae and Streptococcus pneumoniae from the lungs [5]. In immuncompromised patients, malfunctions in the innate immunity might be more serious.

Both β-defensin 1 and β-defensin 2 have been detected in the respiratory tract [6, 7]. β-defensin 1 seems to be constitutively expressed by different epithelial cells as well as alveolar macrophages. It cannot be up-regulated by LPS or chemokines. β-defensin 2 is expressed by epithelial cells after inflammatory stimulation. After stimulation of Toll-like receptor 2 in a human tracheobronchial epithelial cell model, β-defensin 2 expression was stimulated and resulted in a small reduction of bacterial numbers on the cell surface [8]. β-defensin 2 is expressed at a higher level in nasal epithelial cells from patients suffering from exacerbated COPD than in the same cells from healthy COPD patients [9].

Nothing is known about the expression of β-defensins 1 and 2 in respiratory epithelial cells or alveolar macrophages in HIV-infected patients. Recently, it has been shown that β-defensins 2 and 3 are induced in a human oral mucosal cell line infected with HIV 1. These defensins were able to block HIV 1 replication, whereas β-defensin 1 could not [10]. Beside their potential role in preventing oral mucosal transmission of HIV, the β-defensins might have an additional important function in the protection of mucosal surfaces, especially in patients with an impaired cellular immune response. The function of the soluble (non-cellular) components of the innate immune system has not yet been examined in immunocompromised patients.

Methods

Subjects

We investigated nasal epithelial cells from 76 HIV-infected patients and from 33 patients not infected with HIV. Informed consent was obtained from all patients. The protocol was approved by the Ethics Committee of the Ruhr-University.

Within the HIV infected group, 21 patients suffered from upper respiratory tract infection and 17 from

lower respiratory tract infection, while 38 had no respiratory infections. The lower respiratory tract infection group included all patients whose chest X-rays showed signs of bacterial infection and an infiltrate. The upper respiratory tract infection group included patients with tracheobronchitis, sinusitis and common cold. Within the non-HIV group, 20 patients were without respiratory infection while the other 13 suffered from sinusitis, common cold or pneumonias.

Bronchoscopy, including bronchoalveolar lavage and culture of alveolar macrophages, was performed on 18 HIV-infected patients and 38 patients not infected with HIV.

In all patients, CRP (C-reactive protein) was measured in the blood as a marker of infection. In HIV-infected patients, CD 4 cell numbers in the blood were also determined.

Bronchoalveolar Lavage

Human alveolar macrophages were isolated from the bronchoalveolar lavage (BAL) fluid from patients undergoing investigative bronchoscopy for diagnostic reasons. Topical 2% lidocaine anesthesia was applied by inhalation and a fiber optic bronchoscope was passed into the airways and wedged in the right middle lobe, or in the most disease-affected area. BAL was performed by instillation of ten units (each 20 ml) of PBS followed by gentle suction after each unit. Recovery was 65-80 % (120 – 150 ml). The BAL fluid was filtered through gauze and centrifuged at 250 X g for 10 min at 4° C. The supernatant was discarded and normal saline was added to the cells (1% of the recovery volume). Cell numbers were determined using a hemocytometer and differential cell analysis was performed after cytospin preparation and HAEMA staining.

Culture of Alveolar Macrophages

A cell suspension containing 2 x 10^6 alveolar macrophages in 1500 µl RPMI 1640 was placed in each well of a six well plate. After 60 min, the supernatants were discarded and the wells were washed twice with 2000 µl normal saline. The adherent cells were confirmed as alveolar macrophages by esterase staining (92 –98 % AM) and viability was tested with trypan blue (> 90 %).

Lysis buffer (350 µl) containing 10 % mercaptoethanol was added to the remaining alveolar macrophages before RNA extraction. Total RNA was separated on a RNeasy Mini Spin Column (RNeasy®Mini Kit, Qiagen).

RNA from Nasal Epithelial Cells

Nasal epithelial cells were collected from the inside of each nostril using a short brush similar to those used in bronchoscopy. The cells were suspended in 350 µl DMEM. After quick but gentle centrifugation, the supernatant was discarded and the remaining cells were lysed in 350 µl of lysis buffer containing 10 % mercaptoethanol before RNA extraction. Total RNA was separated on a RNeasy Mini Spin Column (RNeasy®Mini Kit, Qiagen).

cDNA- Transscription

RNA solution (10 µl) from nasal epithelial cells or alveolar macrophages was mixed, after the RNeasy Mini Spin Column preparation, with 1.5 µl 10x PCR-buffer (500 mM KCl, 100 mM Tris-HCl pH 8.3, 0.01% (w/v) gelatine in 1.5 ml); 3.3 µl $MgCl_2$ (25 mM); 6.0 µl dNTPs (2.5 mM each); 1.5 µl random hexamer (50 µM); 0.5 µl RNase inhibitor (20 U/µl); and 0.8 µl MultiScribe Reverse Transcriptase (50 U/µl) (TagMan" Reverse transcription reagents, Applied Biosystems). Water was added to a final volume of 30 µl. The solution was incubated for 10 min at 25 °C, 45 min at 48 °C and 5 min at 95 °C to transcribe the RNA into cDNA.

HBD 1 and HBD 2 mRNA Quantification

After total RNA had been extracted from the alveolar macrophages or nasal epithelial cells, PCR was performed with specific primers for the β-defensins and for β-actin, constructed with the help of a software program (P.E. Applied Biosystems). β-actin RNA was used to normalize the RNA content. This PCR measurement is semiquantitative because the housekeeping gene ß-actin is used for comparison. An expression level of 0 means the same amounts of ß-actin and the defensin. A negative expression level means more defensin than ß-actin, because the product becomes detectable at an earlier cycle in the PCR.

The intron-spanning primers and PCR conditions were as follows:

HBD 1 Forward Primer:
AGATGGCCTCAGGTGGTAACTTT;
HBD 1 Reverse Primer:
GGGCAGGCAGAATAGAGACATT;
HBD 2 Forward Primer:
TGATGCCTCTTCCAGGTGTTT;
HBD 2 Reverse Primer:
GGATGACATATGGCTCCACTCTT;
β-actin Forward Primer:
TCACCCACACTGTGCCCATCTACGA;
β-actin Reverse Primer:
CAGCGGAACCGCTCATTGCCAATGG.

Each PCR reaction was performed with 12.5 µl SYBR Green PCR Mix, 5 µl cDNA solution and 5 µM of each primer. Detection was performed using a GeneAmp Sequence Detection System (P.E. Applied Biosystems). Constitutively expressed β-actin RNA was used to normalize the defensin RNA content.

Agarose-gel Electrophoresis

PCR products were compared with a DNA length standard (Boehringer Mannheim, Germany) on a 4% agarose gel at 175 V and 0.08A.

StatisticaL Analysis

Statistical analysis was performed using SPSS Windows V11.5. Data are expressed as mean ± SEM. Patient groups were compared using a Mann-Whitney-U-

Test. Correlations between CD 4 cells and defensins were measured by the Pearson formula.

Results

Patients

Nasal epithelial cells: Clinical and laboratory data from the 109 patients whose nasal epithelial cells were investigated are summarized in Table 1.

Alveolar macrophages: We investigated alveolar macrophages from 18 HIV-infected patients and from 38 patients not infected with HIV.

CD 4 Cell Numbers

Within the HIV-infected patient group, CD 4 cell numbers differed between those with no respiratory infection or upper respiratory tract infections (478/µl ± 321 and 396/µl ± 329, resp.) and those with lower respiratory tract infections (106/µl ± 120) (Table 1).

CRP

CRP was normal in patients without respiratory infection, whether they were HIV-infected or HIV-negative (4 and 2 mg/l, respectively). It was slightly increased in patients with upper respiratory tract infections (18 mg/l) and high in patients with lower respiratory tract infections (126 and 60 mg/l) (Table 1).

HBD 1 and HBD 2 mRNA Quantification

HBD 1 and HBD 2 were measured semiquantitatively by rt-PCR by comparison with the expression of β-actin. Higher mRNA expression numbers represent a lower expression rate.

HBD 1 mRNA Expression in Alveolar Macrophages

HBD 1 mRNA expression in alveolar macrophages was significantly lower in HIV-infected patients (5.5 ± 0.73) than in the control group (3.76 ± 0.38); p = 0.04 (Fig. 1)

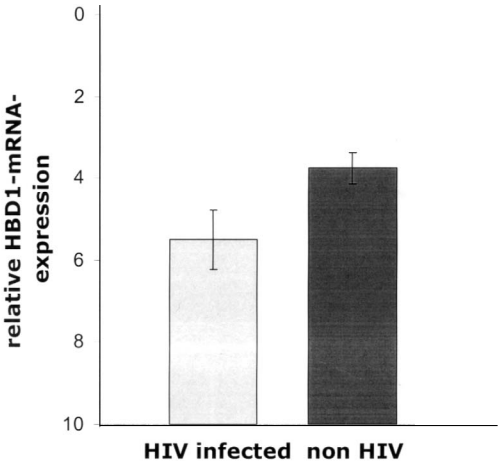

Fig. 1. β-defensin 1 mRNA expression in alveolar macrophages from HIV infected and HIV noninfected patients. HBD 1 mRNA expression in alveolar macrophages of HIV infected patients are significantly lower. (p = 0.04)
(The higher the number of the mRNA expression the lower the expression rate – so, numbers on y-axis were reversed)

HBD 2 mRNA Expression in Alveolar Macrophages

HBD 2 mRNA expression was lower in HIV-infected patients (14.7) than in the control group (14.0). HBD

Table 1. Study population. Laboratory data of HIV infected and HIV noninfected patients whos` nasal epithelial cells were investigated.

Patients	**HIV-infected**			**non HIV**	
	Without respiratory infection	**upper respiratory infection**	**Lower respiratory infection**	**without respiratory infection**	**respiratory infection**
Numbers	38	21	17	20	13
mean age	40.4	41.6	39,1	35.7	42.1
HIV-diagnosis	1998 ± 4.12	1994 ± 5.96	1996 ± 6.32		
CD4 /ul	478 ± 321 (80-1584)	396 ± 329 (1-931)	106 ± 120 (1-372)		
HIV-PCR [copies/ml]	41626 (40-516000)	71740 (40-844000)	44134 (40-125000)		
CRP [mg/l]	4 ± 4.98	18 ± 24.62	126 ± 119.37	2 ± 1.2	60.3 ± 10.3
PCP-prophylaxis	7	3	11		
HAART	30	19	17		

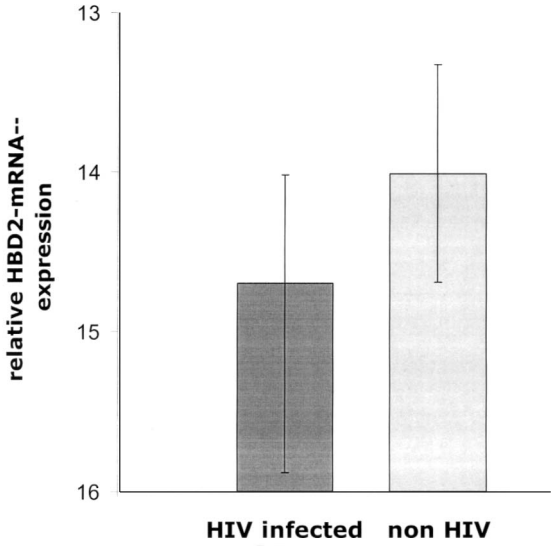

Fig. 2. β-defensin 2 mRNA expression in alveolar macrophages from HIV infected and HIV noninfected patients. HBD 2 mRNA expressions are low in both groups and differences are not statistically different.
(The higher the number of the mRNA expression the lower the expression rate – so, numbers of y-axis were reversed)

2 expression rates were very low and the difference was not statistically significant (p = 0.68) (Fig. 2)

HBD 1 mRNA Expression in Nasal Epithelial Cells

HBD 1 mRNA expression in nasal epithelial cells was higher in HIV-infected patients without respiratory tract infections (0.84) than in the healthy control group (2.39). The difference was not statistically significant (Mann-Whitney-U-test: p = 0.299). Differences in HBD 1 mRNA expression between HIV-infected patients with (0.78) and without (0.84) respiratory infections were not statistically significant (p = 0.55). (Fig. 3)

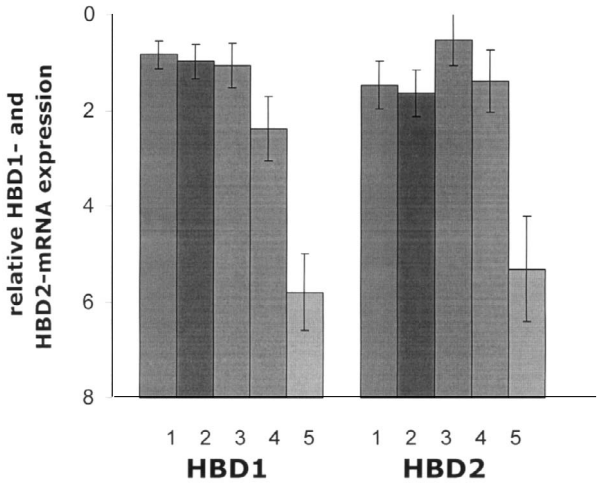

Fig. 3. Semiquantitative β-defensin 1 mRNA expression and β-defensin 2 mRNA expression in nasal epithelial cells from HIV infected and HIV noninfected patients. Group 1: HIV positiv without respiratory infection; group 2: HIV positiv with upper respiratory infection; group 3: HIV positiv with lower respiratory infection (pneumonia); group 4: HIV negativ without respiratory infection; group 5: HIV negativ with respiratory infection.
(The higher the number of the mRNA expression the lower the expression rate – so, numbers of y-axis were reversed)

HBD 2 mRNA Expression in Nasal Epithelial Cells

HBD 2 mRNA expression in nasal epithelial cells in HIV-infected patients without respiratory infection (1.47 ± 0.49) was almost as high as in the healthy control group (1.39 ± 0.64). There was no statistically significant difference (Mann-Whitney-U-test: p = 0.55). There were no significant differences in HBD 2 mRNA expression between HIV-infected patients with (1.64 ± 0.49) and without (1.47 ± 0.49) respiratory infections (p = 0.95). HBD 2 mRNA expression was higher in HIV-infected patients with lower respiratory tract infection (0.54 ± 0.53) than in those without (1.47 ± 0.49), but again the difference was not statistically significant (p = 0.44) (Fig. 3)

HBD 1- and HBD 2- mRNA Expression in Nasal Epithelial Cells in Patients not Infected with HIV

HBD 1- and HBD 2- mRNA expression in nasal epithelial cells from patients who were not infected with HIV and had no respiratory infections were significantly higher than in nasal epithelial cells from HIV-negative patients with respiratory tract infection. HBD 1 expression in the patients without respiratory infections was 2.39 ± 0.67, and in those with respiratory infection it was 5.8 ± 0.8. This difference was statistically significant (Mann-Whitney-U-test: p = 0.02). HBD 2 expression in patients without respiratory infections was 1.39 ± 0.64 and in those with respiratory tract infection it was 5.31 ± 1.1. The difference was again statistically significant (Mann-Whitney-U-test: p = 0.049). (Fig. 3)

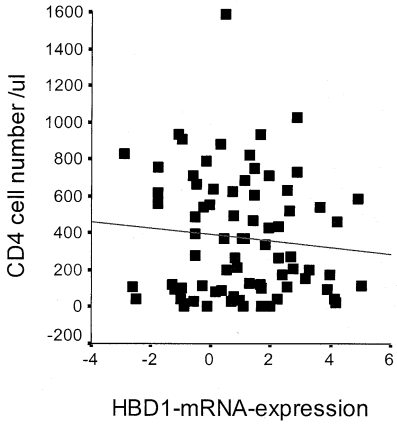

Fig. 4. Correlation of β-defensin 1 mRNA in nasal epithelial cells and CD 4 cell numbers in blood in HIV infected patients. There is no correlation.

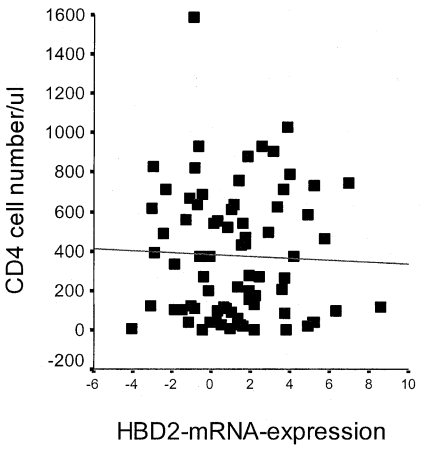

Fig. 5. Correlation of β-defensin 2 mRNA in nasal epithelial cells and CD 4 cell numbers in blood in HIV infected patients. There is no correlation.

Discussion

We have demonstrated a new component of impaired bacterial killing in HIV-infected patients: β-defensin 1 expression in alveolar macrophages is significantly lower in HIV-infected patients than in HIV-negative patients. The low level of β-defensin 1 might reduce not only the direct antimicrobial function of the alveolar macrophages, but also the capacity of these macrophages to activate dendritic cells, an important link between the innate and adaptive immune systems [11].

Our results pertaining to β-defensin 1 expression in alveolar macrophages from HIV-negative patients confirm the data of Druits et al., who demonstrated high levels of constitutive β-defensin 1 expression that were not up-regulated by external stimuli [12]. In our group of HIV-infected patients, β-defensin 1 expression was significantly lower than in controls. These data seem inconsistent with the constitutive expression of the peptide. A disturbance of alveolar macrophage maturation could explain the apparent contradiction, since Druits et al [12] showed that β-defensin 1 expression increased during this maturation process, but no such disturbance has yet been demonstrated.

The results pertaining to defensins in alveolar macrophages are different from those pertaining to defensins in nasal epithelial cells. In nasal epithelial cells, β-defensin 1 expression is even higher than normal in HIV-infected patients. However, there is an obvious explanation for this difference: we showed that nasal epithelial cells produce β-defensin 2, and β-defensins 2 and 3 protect epithelial cells against HIV infection [10]. Therefore, nasal epithelial cells are protected against HIV infection, whereas alveolar macrophages produce almost no β-defensins 2 and 3. The normal level of expression of these defensins in nasal epithelial cells from HIV-infected patients might also explain why bacterial colonization of the airways is no higher in healthy HIV-infected subjects than in healthy HIV-negative people [13].

We found a further interesting result in our HIV-negative patients: the expression of both β-defensin 1 and 2 mRNAs decreased during respiratory tract infections. In vitro studies on cell lines suggest that the levels of β-defensin 1 remain unchanged, while those of β-defensin 2 increase, as a result of LPS or interleukin activation. Others have shown that the β-defensin 1 and 2 peptide concentrations are reduced in BAL from patients suffering from cystic fibrosis, who have more impaired lung function [14]. Chen suggested that chronic airway inflammation might result in damage and loss of the epithelial cells that secrete most of the β-defensins. Here, we have shown similar results in patients with acute respiratory infections. We conclude that during chronic as well as acute respiratory infections, β-defensins are decreased. Perhaps β-defensin function is no longer necessary because the first line of defense has already been overturned.

Moreover, we could not demonstrate any correlation between CD 4 cell numbers in the blood as an indicator of disease progress and β-defensin expression in epithelial cells.

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