Increased serum concentration of G-CSF in cystic fibrosis patients with chronic *Pseudomonas aeruginosa* pneumonia

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Abstract

**Background:** Chronic *Pseudomonas aeruginosa* lung infection is the major reason for premature death in patients with cystic fibrosis (CF). Infected patients experience a progressive deterioration of the lung tissue caused by a persistent accumulation of PMNs. We investigated if the pulmonary accumulation of PMNs is reflected as a migration of PMNs through the blood in chronically infected CF patients.

**Methods:** Blood and sputum samples from 37 stable, chronically (CF+P) and 6 non-infected (CF/C) CF patients without exacerbations were compared using FACS, leukocyte counting, and ELISA. Within the CF+P patients, the blood parameters were compared to the lung function (FEV1 and FVC) and to the sputum. Similar measurements were performed on 15 chronically infected CF patients before and after elective antibiotic treatment.

**Results:** In the CF+P patients the concentration of G-CSF in the sera and PMNs in the blood was increased and correlated to poor lung function. However, only the concentration of G-CSF in the sera was correlated to the concentration of TNF-α in the sputum. After the antibiotic treatment, the lung function was improved and the concentration of PMNs in the blood and G-CSF in the sera was reduced.

**Conclusion:** G-CSF in the sera may contribute to the pulmonary inflammation in CF patients with chronic *P. aeruginosa* lung infection by regulating the number of PMNs available for migration and may be considered as an indicator of clinical status.

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**Keywords:** *Pseudomonas aeruginosa*; Cystic fibrosis; Lung infection; G-CSF; PMNs

1. Introduction

Cystic fibrosis (CF) is the most common lethal disorder with autosomal recessive heredity in Caucasians [1]. The disease is caused by mutations in a single gene of chromosome 7, which encodes the CF transmembrane regulator gene (CFTR) [2]. CFTR is an epithelial chloride channel and mutations affect apical ion and water transport leading to viscous sputum and impaired mucociliary clearance in the CF airways [3].

The majority of patients with CF acquire chronic *Pseudomonas aeruginosa* lung infection after a period of intermittent infections [4]. Although the host factors responsible for the establishment of the chronic infection are not yet clear the chronic infection is characterized by biofilm formation [4] and the presence of numerous polymorphonuclear leukocytes (PMNs) in the lungs surrounding the microcolonies [4,5]. Despite the various antibacterial defense mechanisms of the PMNs the bacteria persist. Resistance to the PMNs is provided by formation of mucoid biofilm [6,7], but the antibacterial activity of the PMNs may also be inhibited by Quorum Sensing—regulated virulence factors [8] and local hypoxia in the CF lungs [9]. Instead of clearing the bacteria, proteolytic enzymes [10] and superoxide [11] released by the PMNs...
are suspected to cause the progressive lung tissue damage responsible for the premature death in CF [4]. In addition, the persistent accumulation of PMNs in the lungs may obstruct the airways [12] and the DNA from necrotic PMNs increases the viscosity of the sputum [13,14]. More than $10^8$ PMNs per ml sputum may be found in the lungs of CF patients with chronic *P. aeruginosa* lung infection [12] and considering the short life span of the PMNs, the normal pool of circulating PMNs may be insufficient to sustain the persistent high number of PMNs in the lungs. Thus, a supply of PMNs from the bone marrow is expected to be mobilized into the blood in order to migrate into the infected lungs. In this study, we hypothesized that the migration of PMNs is detectable in the blood by the number and activation of the PMNs and by the concentration of cytokines regulating the migration via the blood of CF patients with chronic *P. aeruginosa* lung infection.

The experiment was carried out by comparing blood samples from stable, chronically infected CF patients without exacerbations and non-infected CF patients using flow cytometry, leukocyte counting, and ELISA. In addition, we estimated the relation between the lung function, the concentration of PMNs and the proinflammatory cytokines in the blood of CF patients with chronic *P. aeruginosa* lung infection. The finding from these groups of patients were further verified by comparing the same parameters in stable, chronically infected CF patients without exacerbations before and after attenuation of the lung inflammation by elective antibiotic treatment [15,16].

2. Materials and methods

2.1. Patients

Cross-sectional: 43 patients with CF were included. The diagnosis of CF was based on abnormal sweat electrolytes, characteristic clinical features, and detection of specific mutations. The CF patients were divided in two groups:

A) Thirty-seven stable CF patients (24 males and 13 females, median age 26, range 16–38 years) with chronic *P. aeruginosa* lung infection (CF + P) without exacerbations receiving 2 weeks elective courses of i.v. anti-pseudomonal antimicrobial treatment on a regular basis every third month [17]. Chronic infection was defined as ≥6 months of continuous colonization or shorter, but with 2 or more precipitins against *P. aeruginosa* [18]. In order to minimize variation patients were included 1 to 2 months after the i.v. elective treatment.

B) Six CF patients (3 males and 3 females, median age 19, range 16–29 years) without chronic *P. aeruginosa* lung infection and no other signs of lung infection (CF–P).

C) Seven normal volunteers (3 males and 4 females, median age 24, range 18–37 years).

Longitudinal: Fifteen stable CF patients (9 males and 6 females, median age 30, range 17–52 years) with chronic *P. aeruginosa* lung infection without exacerbations before and after regular elective course of i.v. anti-pseudomonal antimicrobial treatment with an aminoglycoside and a β-lactam for 2 weeks.

2.2. Sampling

Sera and heparinized peripheral blood samples were obtained by venous puncture. Sera was collected in VACUTAINERS (367615, Becton Dickinson, Copenhagen, Denmark). Anti-coagulated blood was collected in VACUTAINERS (368484, Becton Dickinson). Sputum samples were centrifuged (8500 ×g, 4 h, 4 °C). The supernatant was stored at −80 °C until measurement of cytokines.

2.3. Leukocyte counting

The concentration of leukocytes in the blood was estimated by routine counting in the central laboratory.

2.4. Staining for flow cytometry of PMNs and activation markers

Erythrocytes were removed by adding cold lysing-buffer (8.02 g/l NH₄Cl, 0.84 g/l NaHCO₃, 0.37 g/l EDTA in MilliQ water). The remaining leukocytes were washed in cold phosphate buffered saline (PBS) without Ca²⁺ and Mg²⁺ before staining. All antibodies were purchased from Pharmingen (San Diego, CA, US). Lysed blood was double stained with the PMN phenotypic surface marker, monoclonal phycoerythrin conjugated mouse anti-CD15 antibody (IgG₃, κ, clone: HI98). To estimate the activation of circulating PMNs during the reduction of the lung inflammation by the elective antibiotic treatment, PMN activation markers were stained with monoclonal fluorescein isothiocyanate conjugated mouse anti-CD16 antibody (IgG₁, κ, clone: 3G8) or anti-CD62L antibody (IgG₁, κ, clone: Dreg 56). The cells were incubated on ice in the dark for 30 min and washed once in cold PBS. The samples were analyzed using a FACSort (Becton Dickinson) equipped with a 15 mW argon-ion laser tuned at 488 nm for excitation. Light scatter and logarithmically amplified fluorescence parameters from 10,000 events were recorded in list mode after gating on forward light scatter to avoid debris, cell aggregates and bacteria. The instrument was calibrated using Calibrite (Becton Dickinson) and the specific fluorescence was calculated by subtracting the fluorescence intensity from samples stained with irrelevant isotypic control antibodies.

2.5. Cytokines

The concentration of G-CSF and IL-8 in the sera was measured by Quantikine (R&D Systems, Abingdon, UK).
and the concentration of TNF-α by OptEIA (Pharmingen) ELISA kits according to the instructions of the manufacturer. Samples were measured in duplicates. The sensitivity was less than 1 pg/ml for all three cytokines. The range of the G-CSF standard curve was 1.25–80 pg/ml, the range of the IL-8 standard curve was 31.2–2000 pg/ml, and the range of the TNF-α standard curve was 7.8–500 pg/ml. To minimize variation, each entire set of samples for ELISA was run on the same day by the same person. Based on the standard curves, the CV did not exceed 20%.

2.6. Lung function

Lung function was assessed by spirometry (Pneumotach, Dräger, Germany). Forced vital capacity (FVC) and forced expiratory volume in 1 s (FEV1) was recorded. Both parameters were expressed as percentage of predicted values corrected for gender and height [19].

2.7. Statistics

Data were analyzed by Mann–Whitney test, Spearman Rank test, and Wilcoxon signed pair-differences rank test using Staview 4.51 (Abacus Concepts, Inc., Berkeley, CA, US) software for Macintosh. $P \leq 0.05$ was considered statistically significant.

3. Results

3.1. PMNs in the blood (Fig. 1)

A higher concentration of neutrophils was seen in the blood of the CF+P patients ($p < 0.02$; Fig. 1). The concentration of PMNs in the blood was obtained from 31 of the 37 CF+P patients and in all 6 CF–P.

3.2. Cytokines in sera (Fig. 2)

The concentration of G-CSF was increased in the sera of the CF+P patients ($p < 0.001$; Fig. 2). In contrast, the concentration of IL-8 was higher in the sera of the CF-P patients ($p < 0.006$). The concentration of cytokines in sera was measured in all CF patients.

3.3. Lung inflammation and G-CSF in the blood (Fig. 3)

In 31 CF+P patients sputum samples were obtained allowing the concentration of TNF-α in the sputum to be used as a marker of lung inflammation (Fig. 3). The concentration of G-CSF in the sera was correlated to the

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Fig. 1. The concentration of PMNs in the blood of CF patients with chronic *P. aeruginosa* lung infection ($n = 31$) and in non-infected CF patients ($n = 6$). The lines show the medians. Normal reference interval: 1.8–7.4 × 10⁹ PMNs pr l. Data were analyzed by Mann–Whitney test.

Fig. 2. This figure shows the concentration of G-CSF and IL-8 in the sera of CF patients with chronic *P. aeruginosa* lung infection ($n = 37$) and in non-infected CF patients ($n = 6$). The concentration of cytokines was measured with ELIZA as pg/ml. (A) G-CSF. (B) IL-8. The lines show the medians. Data were analyzed by Mann–Whitney test.
concentration of TNF-α in the sputum ($p < 0.006$, Rho = 0.475). No significant correlation could be demonstrated between the concentration of TNF-α in the sputum and the concentration of PMNs in the blood or the concentration of IL-8 in the sera (data not shown).

3.4. Lung function and PMNs and cytokines in the blood (Fig. 4)

In the CF+P patients, the concentration of PMNs in the blood was inversely correlated to the FEV1 ($p < 0.03$, Rho = −0.400) and to the FVC ($p < 0.03$, Rho = −0.421). Estimations of lung function and the concentration of PMNs in the blood were performed on 29 of the 37 CF+P patients (Fig. 4).

The concentration of G-CSF in the sera was inversely correlated to FEV1 ($p < 0.009$, Rho = −0.458) and FVC ($p < 0.02$, Rho = −0.400). Lung function and the concentration of G-CSF in sera was measured in 34 of the 37 CF+P patients.

3.5. Antibiotic treatment and lung function (Fig. 5)

After elective antibiotic treatment the lung function was measured as FEV1 and FVC. Both FEV1 and FVC were
increased ($p<0.05$; Fig. 5). Only 14 of the 15 patients in elective anti-biotic treatment were included. One patient was unable to perform the lung function test before treatment.

3.6. Antibiotic treatment and G-CSF and PMNs in the blood (Fig. 6)

Antibiotic treatment may reduce the inflammatory parameters, including the number of PMNs, in the lungs [15,16] (Fig. 6). After the course of treatment, a reduction of the concentration of G-CSF in the sera ($p<0.05$) and the concentration of PMNs in the blood ($p<0.05$) was seen.

3.7. Antibiotic treatment and CD16 and CD62L expression on PMNs (Fig. 7)

As a measure of PMN activation the expression of CD16 ($p<0.01$) and CD62L was included (Fig. 7). An increased expression of CD16 ($p<0.01$) and CD62L ($p<0.03$) was observed on the PMNs in the peripheral blood from the 15 patients after elective antibiotic treatment.

4. Discussion

G-CSF production is primarily confined to monocytes/macrophages, fibroblasts, and endothelial cells [20] and is enhanced in response to bacterial products and inflammatory mediators [21–23], such as tumor necrois factor (TNF)-α [24]. During intrapulmonary infections G-CSF production is increased exclusively in the lungs from where G-CSF is released to the circulation resulting in a non-compartmentalized distribution [25]. In addition, a pulmonary inflammatory response is accompanied by an increased concentration of G-CSF in the blood in humans after bronchial endotoxin instillation [26], indicating a non-compartmentalized distribution of G-CSF during local inflammation in contrast to, e.g. TNF-α and IL-8. In this study, the G-CSF concentration in the sera was correlated to the concentration of the inflammatory marker, TNF-α, in the sputum in the CF+P patients. Thus, the increased G-CSF concentration in the sera of the CF+P patients may probably be derived from the inflammatory response of the infected lung tissue.

G-CSF is a major mobilizer of PMNs from the bone marrow [27–29]. Accordingly, the increased concentration of G-CSF in the sera of the CF+P patients provides an explanation for the increased concentration of PMNs in the blood. In addition, after antibiotic treatment a reduction of both the concentration of PMNs and G-CSF in the blood was seen.

Apart from modulating the production and mobilization of PMNs in the bone marrow, G-CSF may affect the surface expression of adhesion molecules and Fc-γ receptors on the PMNs [30,31]. In this respect, the increased expression of CD16 and CD62L on the circulating PMNs from CF+P
patients after antibiotic treatment may be caused by the reduced concentration of G-CSF in the sera. The direct influence of the i.v. antibiotic treatment on the PMNs was also considered. Though a direct effect of the antibiotics cannot be ruled out in this study, the below proposed direct effects of antibiotics was not seen. The ability of gentamycin to reduce PMN chemokinesis [32] was not reflected in the results. On the contrary, despite that a reduced PMN chemokinesis predicts a longer transit time in circulation leading to an increased concentration of PMNs, a lower concentration of PMNs was observed after antibiotic treatment. The capability of aminoglycosides to affect the phagocytic activity [33] and respiratory burst on the PMNs [34,35] may change the fractions of PMNs with phagocytic activity and respiratory burst of the PMNs. However, changed fractions of PMNs with phagocytic activity and respiratory burst were not found (data not shown).

The concentration of PMNs in the blood did not correlate to the concentration of G-CSF in the sera nor to the concentration of TNF-α in the sputum in the CF+P patients. This may be explained by individual responsiveness to G-CSF and neutrophil transit-time in the blood. Moreover, G-CSF may affect the lung inflammation by inducing a Th-2 dominated immune response [36].

The inverse relation of G-CSF in the sera to the lung function may reflect the dependency of pulmonary G-CSF production and release in response to inflammatory mediators and bacterial products [21–24] suggesting that G-CSF has an impact on the neutrophil dominated lung inflammation by regulating the number of PMNs available for migration in parallel with casein induced inflammation of the peritoneal cavity [27]. The involvement of PMN migration in the lung inflammation is indicated by the inverse relation of the concentration of PMNs in the blood to the lung function. Thus, being released from the chronically infected lungs, G-CSF may sustain the persistent PMN dominated response by stimulating the production and mobilization of PMNs in the bone marrow. Furthermore, the postponing of PMN apoptosis by G-CSF [23], may also increase the number of PMNs in the lungs.

Increased pulmonary concentration of IL-8 is a characteristic of CF patients with chronic P. aeruginosa lung infection [37,38]. The reduced neutrophil influx by neutralization of IL-8 by anti-IL-8 antibodies has shown that IL-8 is a part of the chemotactic gradient that attracts the PMNs to the site of inflammation [39,40]. Surprisingly, the results in this study show a higher IL-8 concentration in the blood of the non-infected CF patients. However, lung bacteriology did not relate to the serum concentration of IL-8 in another study of CF patients [41,42] and IL-8 measurement in the sera may be difficult to interpret due to the presence of autoantibodies [43].

The effect of IL-8 in the circulation on the number of PMNs released from the bone marrow is a paradox. IL-8 increases the mobilization of PMNs [44], but inhibits the neutrophoiesis in the marrow [45,46]. This suggests that IL-8 in the blood may not be able to sustain a persistent migration of PMNs to the lungs. Accordingly, the results of this study show no correlation of the IL-8 concentration in the sera to the concentration of PMNs in the blood nor to the lung function. Therefore the proinflammatory effect of IL-8 in the CF+P patients is likely to be confined to the lungs.

In conclusion, the actual extent of inflammation may be reflected in the G-CSF concentration in the sera in stable, chronically infected CF patients without exacerbations. It is widely recognized that C-reactive protein (CRP) may serve as a useful systemic marker of lung inflammation during exacerbations in chronically infected CF patients. However, CRP has not been useful for assessing the inflammation in stable, chronically infected CF patients without exacerbations receiving elective 2 week courses of antibiotics every third month as only a few of these patients had increased CRP and it was only a modest increase [47,48]. The present study suggests that the concentration of G-CSF in circulation may be an alternative to CRP to be considered for estimating the ongoing lung inflammation in stable chronically infected CF patients without exacerbations.

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References


