GM-CSF and GM-CSF βc receptor in adult patients with pulmonary alveolar proteinosis

B. Bewig*, X-D. Wang*, D. Kirsten*, K. Dalhoff†, H. Schäfer*


ABSTRACT: Pulmonary alveolar proteinosis (PAP) is a rare disorder of unknown origin characterized by alveolar fillings with periodic acid-Schiff (PAS)-positive material mainly consisting of phospholipids. Mice defective in the granulocyte-macrophage colony-stimulating factor (GM-CSF) gene or the GM-CSF/interleukin (IL)-3/IL-5-receptor common β chain (βc) demonstrate a pathology resembling PAP. A recent study revealed defects in the βc chain of the GM-CSF receptor in four out of eight paediatric patients. This study investigates the role of the GM-CSF coding region and components of the GM-CSF receptor in adult patients.

Four adult patients with proven PAP were analysed for GM-CSF and GM-CSF-βc receptor in regard to protein level, messenger ribonucleic acid (mRNA) expression and sequence composition.

None of the adult patients displayed the mutation at position 1,835 of the βc-receptor previously described in paediatric patients. Expression of the βc receptor was found to be normal on the surface of peripheral blood cells. In three out of four patients GM-CSF release from blood cells failed to respond adequately to lipopolysaccharide (LPS). In one of these patients a heterozygous mutation was found in the GM-CSF complementary deoxyribonucleic acid (cDNA) from thymine (T) to cytosine (C) at position 382 of the published sequence putatively causing a change in the protein from isoleucine to threonine at position 117.

This study indicates that the mutation of the β chain receptors found in some of the paediatric patients suffering from pulmonary alveolar proteinosis is not a common problem in adult patients.


Pulmonary alveolar proteinosis (PAP) in adult patients is a rare disorder of unknown origin. The disease is characterized by the accumulation of periodic acid-Schiff (PAS)-positive material in the alveoli of the lung. This material consists of phospholipids and proteins derived from surfactant proteins [1, 2]. PAP has been observed “idiopathic” or in association with haematological malignancies, infections or exposure to inhaled chemicals. The diagnosis of PAP is based on bronchoalveolar lavage findings or histology [3].

Abnormal composition of alveolar proteins in PAP suggests involvement of surfactant production or surfactant metabolism in the development of the disease. Surfactant, a mixture of phospholipids and associated surfactant proteins (SP-A, SP-B, SP-C, SP-D), is synthesized and secreted by type II pneumocytes. Clearance of surfactant is achieved by recycling and degradation in alveolar macrophages and in type II pneumocytes [4, 5]. Congenital PAP has been attributed to diminished levels of surfactant protein B caused by a frameshift mutation (121ins2) resulting in functional and quantitative disturbance of SP-A and -C [6, 7]. In adults, these mutations in the surfactant coding sequences have not been found. However, abnormal macrophage function was identified in patients with PAP in 1979 suggesting PAP in adults is caused by defective clearance of surfactant in alveolar macrophages rather than by abnormal production [8–10]. Recent studies suggest granulocyte-macrophage colony-stimulating factor (GM-CSF) and GM-CSF-receptor to be involved in the metabolism of SP and lipids [11–14]. Mice defective in the GM-CSF gene or the GM-CSF/interleukin (IL)-3/IL-5-receptor common β chain (βc) demonstrate a pathology resembling PAP with increased amounts of surfactant in the alveoli and in the macrophages. Similar to adult humans, no accumulation of surfactant or its precursor was detected in the type II pneumocytes of the mice. These observations indicate a role of GM-CSF or its receptor in processing surfactant within alveolar macrophages.

In humans defects in the GM-CSF receptor have been described in four out of eight paediatric patients [15]. However, no abnormalities in the GM-CSF gene have been described yet, although a report suggests missing protein in the presence of normal amounts of GM-CSF messenger ribonucleic acid (mRNA) [16]. The authors analysed GM-CSF and GM-CSF receptor for protein levels, mRNA expression and sequence composition in four adult patients with PAP.

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Materials and methods

Patients

Four patients were available for analysis (PAPa, PAPb, PAPc, PAPd). All patients had clinical manifestations of the disease (table 1). In one patient histology revealed pulmonary alveolar proteinosis associated with interstitial inflammation (PAPc). Up to seven persons without evidence of PAP were investigated as control subjects. All patients gave their written consent to participate in this study. The study was performed in accordance to the Helsinki declaration of 1964.

Isolation of mononuclear cells from peripheral blood

Venous blood was taken from patients in ethylene diamine tetra-acetic acid (EDTA)-anticoagulated tubes and diluted with phosphate-buffered saline (PBS). Peripheral blood mononuclear cells (PBMCs) were separated by density gradient centrifugation over Ficoll-Hypaque separating solution (density 1.077) at 500 × g at 20 °C for 20 min. PBMCs were recovered from the interphase between plasma and Ficoll-Hypaque by needle aspiration and washed twice in PBS.

Harvesting bronchoalveolar lavage cells

Bronchoalveolar lavage (BAL) or whole lung lavage were performed therapeutically for removal of phospholipids and proteins from the alveolar spaces using prewarmed sodium chloride (0.9%) or standard Ringer solution. Lavage fluid was gathered and cell pellets were collected by centrifugation at 300 × g for 5 min. Cells were resuspended in PBS or Dulbecco’s minimum essential medium (MEM) and grown on culture dishes. Alveolar macrophages were selected by washing due to their increased adherence to surfaces compared to leukocytes.

Table 1. – Clinical details of pulmonary alveolar proteinosis (PAP)

<table>
<thead>
<tr>
<th></th>
<th>PAPc</th>
<th>PAPb</th>
<th>PAPc</th>
<th>PAPd</th>
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<tbody>
<tr>
<td>Sex</td>
<td>M</td>
<td>M</td>
<td>F</td>
<td>M</td>
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<tr>
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<td>35</td>
<td>22</td>
<td>45</td>
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<tr>
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<td>D, C</td>
<td>D, C</td>
<td>D, C</td>
</tr>
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<td>BI</td>
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<tr>
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<td>PR, DDC</td>
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<td>BAL +</td>
<td>TMB</td>
<td>BAL +</td>
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<tr>
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<td>TMB</td>
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<td>Macrophage %</td>
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<td>78</td>
<td>73</td>
<td>61</td>
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<td>4</td>
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<td></td>
<td>P\textsubscript{a}CO\textsubscript{2} mmHg</td>
<td>30</td>
<td>37</td>
<td>34</td>
</tr>
</tbody>
</table>

M: male; F: female; D: dyspnoea; C: cough; Cr: crackles; CP: chest pain; BI: bilateral infiltrates; PR: pulmonary restriction; DDC: decreased diffusion capacity; BAL: bronchoalveolar lavage; TMB: transbronchial lung biopsy; P\textsubscript{a}O\textsubscript{2}: arterial oxygen tension; P\textsubscript{a}CO\textsubscript{2}: arterial carbon dioxide tension.

Cell culture and stimulation

Isolated PBMCs and lavage cells were resuspended in 37 °C prewarmed Dulbecco’s MEM (Life Technologies GmbH, Eggenstein, Germany) supplemented with 10% foetal calf serum (FCS; Life Technologies GmbH) and 100 U·mL\textsuperscript{-1} penicillin (Life Technologies GmbH) and 100 μg·mL\textsuperscript{-1} streptomycin (Life Technologies GmbH) to a concentration of 1 × 10\textsuperscript{6} cells·mL\textsuperscript{-1}. For reverse transcriptase-polymerase chain reaction (RT-PCR) analysis of GM-CSF mRNA cells were stimulated with lipopolysaccharide (LPS; Sigma, St. Louis, MO, USA) at a concentration of 2 μg·mL\textsuperscript{-1} in a humidified chamber at 37 °C for 0, 4, 12 or 24 h. For protein analysis cells were incubated with LPS for 24 h. For RT-PCR of the β receptor mRNA, cells were processed without cultivation and without stimulation.

Protein analysis

GM-CSF protein quantification. GM-CSF levels were determined in plasma, supernatants of cultured PBMCs and cultured BAL cells using an enzyme-linked immunosorbent assay (GM-CSF ELISA; Immunotech, Marseille, France) following the instructions of the manufacturer. Briefly, microtitre plates coated with mouse anti-human GM-CSF monoclonal antibody were incubated with 50 μL of serum, supernatant or control at room temperature for 2 h. After washing a biotinylated second antibody against mouse anti-human GM-CSF antibody was added together with streptavidin-conjugated peroxidase. After incubation at room temperature for 30 min and washing, a chromogenic substrate was added. Extinction was taken at 450 nm using a BioRad EIA reader (Bio-Rad Laboratories GmbH, München, Germany). Concentration of GM-CSF was determined using a standard curve. The sensitivity of the assay was 5 pg·mL\textsuperscript{-1}.

Flow cytometry analysis of GM-CSF/IL-3/IL-5 receptor common β chain expression. GM-CSF/IL-3/IL-5 receptor common β chain expression was analysed by flow cytometry as previously described [15]. Two hundred microlitres of whole blood was incubated with a monoclonal mouse anti-human GM-CSF/IL-3/IL-5 receptor β antibody (Genzyme, Cambridge, MA, USA) at room temperature for 30 min. Red blood cells were lysed by fluorescence-activated cell sorter (FACS) lysing solution (Becton Dickinson, Heidelberg, Germany). Remaining leukocytes were collected by centrifugation and washed with PBS (GibcoBRL). Staining was accomplished using 4 μL of fluorescein isothiocyanate (FITC)-conjugated second antibody (goat anti-mouse immunoglobulin (Ig)-FITC; Becton Dickinson). Fluorescence was detected by FACScan flow cytometer (Becton Dickinson). At least 10,000 cells were analysed in each sample. Results from fluorescence intensity analysis represent percentage of cells expressing β receptor.

Ribonucleic acid extraction. BAL cells or PBMCs were used for RNA extraction applying an RNA isolation kit (High pure RNA isolation kit; Boehringer Mannheim, Mannheim, Germany). Briefly, 1–4 × 10\textsuperscript{6} unstimulated or LPS-stimulated cells in a volume of 200 μL were denatured in 400 μL lysis-buffer and added to a fibre fleece in a filter tube. After centrifugation deoxyribonucleic acid (DNase) 1
First-stranded complementary deoxyribonucleic acid synthesis. Total RNA was used for first-stranded complementary deoxyribonucleic acid (cDNA) in a reverse transcription system (Reverse transcription system, Promega, Madison, WI, USA) following the instructions of the manufacturer. Briefly, a 20 μL reaction mixture containing 1 μg total RNA, 0.5 μg oligo(dexoxythymidine (dT))15 primer, 1 mM each deoxyribonucleotide (dNTP), 15 U avian myeloblastoses virus (AMV) reverse transcriptase, 20 U ribonuclease inhibitor (rRNasin), 5 mM MgCl2, and 1 × reverse transcription buffer (10 mM Tris-HCl, 50 mM KCl and 0.1% Triton X-100) was incubated at 42 °C for 15 min, followed by an incubation at 99 °C for 5 min. The reaction was stopped by 5 min incubation at 5 °C.

Polymerase chain reaction. First stranded cDNAs were amplified in a thermocycler (Perkin Elmer Biosystems, Foster City, CA, USA) by PCR as described previously. A reaction volume of 40 μL containing 1 × PCR buffer (10 mM Tris-HCl, 50 mM KCl and 1.2% agarose gel in 1 × TAE buffer) was added. Visualization was followed by 30 cycles of denaturation at 95 °C, annealing at 56 °C, and 72 °C for 10 min at the end of 30 cycles.

**Primer.** GM-CSF cDNA (536 base pair (bp) amplification product): sense primer: 5'-GTT TCT CGG CCC TCC TTC GCT-3' (position 18–37); F1 antisense: 5'-GTT TCT CTT CGC CCC TCC TTC GCT-3' (position 1,019–998); F2 sense: 5'-CCC ACG GCC AAT ACA TTCG TC-3' (position 972–991); F2 antisense: 5'-CCA GAT GTG GGA TCA CAG AC-3' (position 1,680–1,661); F3 sense: 5'-GAG GTG TCA CCT CTC ACC AT-3' (position 1,625–1,644); F3 antisense: 5'-GAG CTC ATG GTG ATA GAC GCC AC-3' (position 2,124–2,105); F4 sense: 5'-GAG CTC ATG GTG ATA GAC GCC AC-3' (position 2,098–2,117); F4 antisense: 5'-AGT AGG CCG CGG GGA AG-3' (position 2,784–2,765).

Amplified products were subcloned into pcCR2.1 using the TA cloning kit following the instructions of the manufacturer (Invitrogen, San Diego, CA, USA). Briefly, 10 μL reaction mixture containing 0.6 μM Tris-HCl, 6 mM MgCl2, 5 mM NaCl, 7 mM β-mercaptoethanol, 0.1 mM adenosine triphosphate (ATP), 2 mM dithiothreitol, 1 mM spermidine, 0.1 mg·mL⁻¹ bovine serum albumin (BSA), pH 7.5, 1 μL PCR product, 2 μL pCR2.1 vector (25 ng·μL⁻¹), 1 μL T4 DNA ligase and distilled water was incubated at 14 °C overnight.

pCR2.1 vector containing the amplified PCR product was used for transformation of Escherichia coli competent cells (INV-640; Invitrogen, San Diego, CA, USA). Briefly, 2 μL of 0.5 mM β-mercaptoethanol and 2 μL of the ligase reaction were transferred to a vial of competent cells. After incubation on ice for 30 min, cells were exposed to 42 °C for 30 min and then cooled down on ice again. Two hundred and fifty microlitres of 37 °C prewarmed SOC medium (2% tryptone, 0.5% yeast extract, 10 mM NaCl, KCl, 10 mM MgCl2, 20 mM glucose pH 7.0) were added to the cells followed by an incubation at 37 °C for 1 h. One hundred and fifty microlitres cell culture from each transformation was spread on a Luria-Bertani (LB: 1% tryptone, 0.5% yeast extract, 10 mM NaCl, KCl, 10 mM MgCl2, 20 mM glucose pH 7.0) agar plate. The plate was incubated at 37 °C overnight and at 4 °C for 2 h. Five to 10 white colonies were picked from each plate and transferred to 5 mL LB medium containing ampicillin (50 μg·mL⁻¹) for overnight growth at 37 °C.

Cultured E. coli were used for DNA extraction applying the QIAprep miniprep protocol (Qiagen, Hilden, Germany). Briefly, bacterial cells were pelleted by centrifugation at 1,200 × g for 5 min, resuspended in 250 μL buffer, and lysed with 250 μL lysis buffer before a final denaturation buffer was added. After centrifugation at 10,000 × g for 10 min the supernatant was applied to a spin column capturing the DNA during a further centrifugation step. Two more washings were performed before finally DNA was eluted in 50 μL of buffer (10 mM Tris HCl, pH 8.5).

DNA concentration was determined spectrophotometrically.

Final sequencing was performed by the Sanger dideoxy-meditated chain termination method utilizing the SequiTherm EXCEL II Long Read DNA sequencing kit (Epicentre Technologies, Madison, WI, USA). The procedure followed the modified instructions of the manufacturer. Instead of radiolabelled probes fluorophore labelled sequencing primers were used. The primers (M13 forward primer: 5'-AGG GTT TTC CCA CAG TCG ATC TTG-3' and M13 reverse primer: 5'-GAG CGG ATA ACA ATT TCA CAC AGG-3' (MWG Biotech, Ebersberg, Germany) were labelled with IRD800 dye at the 5' ends.

Gel electrophoresis and detection of fluorescence was accomplished by DNA sequencer (DNA sequencer 4,000
concentrations (675–2,100 pg/mL) one of the PAP patients (PAPc) had significant GM-CSF production (one control subject). Both patients had slightly higher GM-CSF levels without stimulation. Upon challenge with LPS samples from all control subjects and five controls and from all patients with alveolar proteinosis had undetectable levels of GM-CSF in plasma (data not shown).

Results

GM-CSF protein in plasma and supernatant of cultured peripheral blood mononuclear cells

Concentration of GM-CSF in plasma was determined by enzyme linked immunosorbent assay (ELISA)-technique (sensitivity 5 pg·mL\(^{-1}\)). In one out of seven control subjects GM-CSF was detected at a concentration of 9 pg·mL\(^{-1}\), all other control subjects and all four patients with alveolar proteinosis had undetectable levels of GM-CSF in plasma (data not shown).

PBMCs were separated and grown in cell culture in the presence or absence of LPS. The supernatant was collected for GM-CSF determination. Only samples from two control subjects secreted GM-CSF at a measurable amount (8.8 and 19.2 pg·mL\(^{-1}\) supernatant). All other samples from five controls and from all patients with PAP were negative for GM-CSF without stimulation (table 2). After stimulation of separated PBMCs with LPS for 24 h GM-CSF was detectable in all control subjects. The concentration was between 35 and 165 pg GM-CSF·mL\(^{-1}\) cell supernatant. In one of the patients suffering from PAP (PAPc) GM-CSF was found within the same range of concentration (115 pg·mL\(^{-1}\)). However, two other patients with PAP produced only low level GM-CSF (11 and 5.2 pg·mL\(^{-1}\) respectively) and in the third patient no GM-CSF was detectable (table 2).

GM-CSF release from bronchoalveolar lavage cells

Alveolar macrophages from BAL were cultivated and grown with or without LPS for 24 h. The supernatant was analysed for GM-CSF by the ELISA-technique. Two patients with PAP were available for this analysis. They were compared with four control lavages (table 3). If the cells were not stimulated with LPS control subjects had no (three control subjects) or low level (8 pg·mL\(^{-1}\) ) GM-CSF production (one control subject). Both patients had slightly higher GM-CSF levels without stimulation. Upon challenge with LPS samples from all control subjects and one of the PAP patients (PAPc) had significant GM-CSF concentrations (675–2,100 pg·mL\(^{-1}\)). The other PAP patient had only minimal GM-CSF levels in the BAL cell supernatant (32.5 pg·mL\(^{-1}\)).

GM-CSF messenger ribonucleic acid expression in peripheral blood mononuclear cell

PBMCs were isolated and incubated in the presence or absence of LPS. After RNA extraction from PBMCs cDNA was transcribed by reverse transcriptase and amplified by PCR. The PCR product was visualized by ethidium bromide staining after gel electrophoresis. A representative gel is shown in figure 1.

In none of the control subjects and none of the patients with PAP were any GM-CSF cDNA PCR products detectable directly after isolation. Weak bands were detectable after 4–12 h of incubation (no stimulation with LPS) with a decrease after further incubation. Strongest bands were obtained after 4 h of incubation in the presence of LPS indicating highest amounts of GM-CSF cDNA. The time course of appearance and the strength of bands was not different between control subjects and patients with PAP.

GM-CSF messenger ribonucleic acid expression in alveolar macrophages

GM-CSF was determined in supernatants of cultured alveolar macrophages in the presence or absence of LPS according to the methods applied for PBMCs (fig. 2). No GM-CSF cDNA was detectable by PCR in unstimulated cells (four control subjects and two patients with PAP). After stimulation with LPS for 4 h strong bands representing presence of GM-CSF cDNA were found in all samples.

Table 2. – Granulocyte - macrophage colony - stimulating factor (GM-CSF) release from peripheral blood mononuclear cells in patients with pulmonary alveolar proteinosis (PAP) and control subjects

<table>
<thead>
<tr>
<th>Subjects</th>
<th>GM-CSF pg·mL(^{-1})</th>
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<tbody>
<tr>
<td></td>
<td>LPS</td>
</tr>
<tr>
<td>PAPa</td>
<td>&lt;5</td>
</tr>
<tr>
<td>PAPb</td>
<td>&lt;5</td>
</tr>
<tr>
<td>PAPc</td>
<td>&lt;5</td>
</tr>
<tr>
<td>PAPd</td>
<td>&lt;5</td>
</tr>
<tr>
<td>Control 1</td>
<td>9</td>
</tr>
<tr>
<td>Control 2</td>
<td>&lt;5</td>
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<td>Control 3</td>
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<td>Control 4</td>
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<td>Control 5</td>
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<tr>
<td>Control 6</td>
<td>&lt;5</td>
</tr>
<tr>
<td>Control 7</td>
<td>&lt;5</td>
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</tbody>
</table>

LPS: lipopolysaccharide.

Table 3. – Granulocyte - macrophage colony - stimulating factor (GM-CSF) release from bronchoalveolar lavage cells in patients with pulmonary alveolar proteinosis (PAP) and control subjects

<table>
<thead>
<tr>
<th>Subjects</th>
<th>GM-CSF pg·mL(^{-1})</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>LPS</td>
</tr>
<tr>
<td>PAPc</td>
<td>27</td>
</tr>
<tr>
<td>PAPd</td>
<td>19</td>
</tr>
<tr>
<td>Control 1</td>
<td>&lt;5</td>
</tr>
<tr>
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<tr>
<td>Control 3</td>
<td>&lt;5</td>
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<tr>
<td>Control 4</td>
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</tbody>
</table>

LPS: lipopolysaccharide.
Sequence analysis of GM-CSF complementary deoxyribonucleic acid

All patients with PAP and two control subjects were analysed for sequence integrity of the cDNA. In one patient (PAPa) a point mutation was found from thymine (T) to cytosine (C) at position 382 of the published sequence (fig. 3). This mutation putatively causes a change in the amino acid sequence at position 117 from isoleucine to threonine. Repeated analysis of completely independent clones (total of 10) from this patient revealed presence of both wildtype cDNA and the mutated form as well, suggesting a heterozygous genomic mutation. Analysis of all other control subjects and patients with PAP revealed no differences from the published sequence.

GM-CSF/interleukin-3/-5 β chain receptor protein

GM-CSF/IL-3/IL-5 βc chain receptor expression was assessed on PBMCs and polymorphonucleated cells (PMNCs) by flow cytometry using a specific monoclonal antibody directed against GM-CSF/IL-3/IL-5 βc receptor (figs 4 and 5). All four patients with PAP and all control subjects showed normal expression of the receptor on PBMCs (56–92% of cells positive) and PMNCs (66.1–98.2% of cells positive).

GM-CSF/interleukin-3/-5 β chain receptor messenger ribonucleic acid

cDNA obtained from PBMCs and BAL cells was amplified by PCR spanning the region 1,625–2,124 bp of the GM-CSF/IL-3/IL-5 βc receptor. The 500 bp amplification product was analysed by gel electrophoresis and visualized after ethidium bromide staining by ultraviolet illumination (fig. 6). In all samples (control subjects and patients with PAP) similar bands of the amplification product were detectable demonstrating presence of GM-CSF/IL-3/IL-5 βc receptor cDNA.
Sequence analysis of GM-CSF/interleukin-3/-5 β chain receptor complementary deoxyribonucleic acid

GM-CSF/IL-3/IL-5 βc receptor cDNA sequencing was performed on all patients with PAP. Two patients (PAPc and PAPd) had mutations at the position 1,972 of the published sequence. However, this change in cDNA is not associated with a change in the coding for amino acids. No abnormalities in the sequence of the GM-CSF/IL-3/IL-5 βc receptor were found in patient PAPA and PAPb. Especially no mutation was found at position 1,835.

Discussion

This study indicates that the mutation of the GM-CSF βc receptor found in some paediatric patients suffering from PAP is not a common problem in adult patients. Recent studies have revealed the importance of GM-CSF in the regulation of surfactant balance, which may play a pathogenetic role in PAP. Transgenic mice lacking GM-CSF or the common β subunit (βc) of the GM-CSF receptor develop a lung pathology resembling PAP [11–14]. PAP can be treated successfully, when GM-CSF production is restored in the GM-CSF knockout mouse [17–19] or when bone marrow cells expressing the βc chain are transplanted to the GM-CSF receptor knockout mouse [20, 21].

In paediatric patients with intact SP-B a defect in the expression of the βc receptor was found in four out of eight cases [15]. In one of these patients a mutation was detected at position 1,835 of the genomic DNA (from C to adenine (A)) causing a change of codon 602 from proline to threonine, a motif possibly of importance for the integrity of the βc protein. Paediatric patients suffering from PAP related to acute myeloid leukaemia were lacking the βc chain on their myeloid blasts [22]. After high dose chemotherapy the defective βc clone was eliminated and patients recovered from PAP symptoms. Defects in the βc...
chain may also be responsible for recurrence of alveolar proteinosis after lung transplantation [23].

In the current study none of the patients displayed abnormalities in the βc expression on the cell surface and no mutation was found at the previously described locus. Two patients had mutations at position 1,972 of the published βc sequence, which was not associated with a change in the coding for amino acids. This change at the genomic level appears to be a nonrelevant functional polymorphism.

PAP may be caused by the lack of GM-CSF protein. A recent observation indicates absence of the protein release while the GM-CSF gene expression was normal in one adult patient with PAP [16]. Tchou-Wong et al. [16] suggest an inhibitory effect of IL-10 on GM-CSF secretion by altering translation or protein processing. However, changes in the structure of the GM-CSF molecule which may impair GM-CSF function have not been excluded. The current authors have addressed this question with this study.

In three out of four patients (PAPa, PAPb, PAPd) GM-CSF release failed to respond adequately to LPS as observed in the previously described patient. BAL cells were analysed from one of these three patients and diminished GM-CSF levels were also found here. Induction of GM-CSF mRNA was normal in all patients and control subjects. The kinetics of GM-CSF mRNA after treatment with LPS is similar to that observed in peritoneal mouse macrophages. No abnormalities have been found on the transcriptional level in the patient described by Tchou-Wong et al. [16].

Sequence analysis revealed a point mutation in one of the patients, whose clinical presentation at the time of diagnosis was not apparently different from the other patients. This mutation represents a change from T to C at position 382 of the published GM-CSF cDNA which will result in a change in amino acid 117 from isoleucine to threonine. Repeated analyses of completely independent clones (total of 10) demonstrated both mutated and wild-type forms of gene indicating heterozygous genomic mutation. This patient displayed the lowest level of GM-CSF release in response to LPS stimulation.

Since isoleucine is hydrophobic and threonine is hydrophilic the mutation may cause changes in the polarity of the protein resulting in diminished stability or impaired function. However, the relevance of the mutation concerning the development of PAP remains uncertain because this region of the GM-CSF gene is not well preserved, e.g.

Fig. 5. – Flow cytometry analysis of granulocyte-macrophage colony-stimulating factor (GM-CSF) interleukin (IL)-3/IL-5 β chain (βc) expression on peripheral blood mononuclear cells (PBMC). Intensity of fluorescence is shown on the x-axis, relative cell number is represented on the y-axis. Light areas represent gated PBMCs, shaded area indicate cells after binding to the GM-CSF/IL-3/IL-5 βc receptor antibody. a) Control subject, b) patient with pulmonary alveolar proteinosis (PAP).

Fig. 6. – Gel electrophoresis of polymerase chain reaction (PCR) amplification products granulocyte-macrophage colony-stimulating factor (GM-CSF) interleukin (IL)-3/IL-5 β chain (βc) receptor, 500 base-pairs (bp)) from complementary deoxyribonucleic acid (cDNA) of a patient with pulmonary alveolar proteinosis (PAP). Ribonucleic acid (RNA) was isolated from peripheral blood mononuclear cells (PBMCs) or bronchoalveolar lavage (BAL) cells grown in the absence or presence of lipopolysaccharide (LPS) (2 μg·mL⁻¹). cDNA was obtained by reverse transcriptase. M: molecular weight marker; lane 1: PBMCs from a control subject; lane 2: PBMCs from a PAP patient; lane 3: BAL cells from a control subject; lane 4: BAL cells from a PAP patient.
porcine GM-CSF consists of serine at this position, another hydrophilic amino acid. Low levels of GM-CSF even after stimulation were observed in two other patients, although the GM-CSF cDNA was intact. Therefore, future studies will have to analyse factors influencing translation and stability of the protein.

Of certain interest are the findings observed in the patient PAPEc emphasizing the heterogeneity of PAP. The patient suffered from histologically proven PAP in association with interstitial inflammation. All analyses of GM-CSF and GM-CSF receptor β chain including the transcriptional and protein level, and sequencing were normal in the samples suggesting a pathway for the development of PAP not involving GM-CSF or the βc receptor.

Careful evaluation of patients with pulmonary alveolar proteinosis is necessary before a specific treatment can be applied. Diminished levels of granulocyte-macrophage colony-stimulating factor in patients with pulmonary alveolar proteinosis may justify substitution therapy and beneficial effects have been described in a case report [24]. However, causes of pulmonary alveolar proteinosis seem to be variable and optimal therapy besides whole lung lavage depends on the underlying defect.

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References