Original Article

P. aeruginosa in the paranasal sinuses and transplanted lungs have similar adaptive mutations as isolates from chronically infected CF lungs☆

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Abstract

Background: Pseudomonas aeruginosa cells are present as biofilms in the paranasal sinuses and the lungs of chronically infected cystic fibrosis (CF) patients. Since different inflammatory responses and selective antibiotic pressures are acting in the sinuses compared with the lungs, we compared the adaptive profiles of mucoid and non-mucoid isolates from the two locations.

Methods: We studied the genetic basis of phenotypic diversification and gene expression profiles in sequential lung and sinus P. aeruginosa isolates from four chronically infected CF patients, including pre- and post-lung transplantation isolates.

Results: The same phenotypes caused by similar mutations and similar gene expression profiles were found in mucoid and non-mucoid isolates from the paranasal sinuses and from the lungs before and after transplantation.

Conclusion: Bilateral exchange of P. aeruginosa isolates between the paranasal sinuses and the lungs occurs in chronically infected patients and extensive sinus surgery before the lung transplantation might prevent infection of the new lung.

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1. Introduction

During the chronic lung infection in patients with cystic fibrosis (CF), Pseudomonas aeruginosa can survive for long periods under the challenging selective pressure imposed by the immune system and antibiotic treatment. This is thought to be due to the biofilm mode of growth [1] and adaptive evolution mediated by genetic variations [2]. The adapted phenotypes are characterized by increased alginate production and occurrence of mucoid variants [3], loss of quorum-sensing (QS) [2,4], motility [5], effector proteins of the type III secretion system [6] and loss of the O-antigen components of the lipopolysaccharide [7], reduced virulence [8], reduced capacity of the non-mucoid phenotype for in vitro biofilm formation in contrast to the mucoid which maintain this capacity [9–11] and increased antibiotic resistance of both phenotypes [12]. These adaptive patterns are found in P. aeruginosa infecting different CF patients, showing a parallel evolution of the microorganisms during the chronic infection [13,14].

There is increasing evidence that the sinuses can be bacterial foci for P. aeruginosa in CF patients [15]. In the early stages of
the lung colonization, the paranasal sinuses are considered to be sites of adaptation, where the bacteria get ready to colonize and infect the lungs. In these stages the migration mainly goes from the upper to the lower airways [16].

In the later stages of the infection, the paranasal sinuses have been shown to be a reservoir for bacteria that re-colonize the lung allograft with the same bacterial clone in patients who have been lung transplanted [17,18].

The host immune response and the massive antibiotic treatment represent important selective pressures, which influence the bacterial evolution in the CF airways. It has been shown that the large number of polymorphonuclear leukocytes (PMNs) surrounding the biofilms in the lungs could play an important role in the bacterial evolution in the CF lungs, leading to mucoid conversion [19] and through oxidative radical damage to increased mutability [20,21]. However, in addition to impaired penetration of antibiotics, the infection in the paranasal sinuses is accompanied by a reduced PMN response due to high local levels of secretory IgA [22]. These differences in the selective pressures acting in the paranasal sinuses and the lungs might influence the bacterial evolution differently in the two locations [23,16].

Phenotypic characterization, sequencing and gene expression analyses have previously been used to investigate the evolution and adaptation of *P. aeruginosa* during infection of CF airways [14,16,24]. However, a detailed phenotypic characterization of the sinus and lung *P. aeruginosa* isolates from chronically infected patients has not been investigated before. In the present study, we characterize the adaptive profile of sequential *P. aeruginosa* isolates from the upper and lower airways in four patients with chronic lung infection, three of which have undergone lung transplantation (LTX) previous to the sinus surgery.

2. Material and methods

2.1. Patients

Four chronically infected CF women: CF 1, CF 2, CF 3 and CF 4 (two being homozygous and two heterozygous for the ΔF508 mutation), three of which underwent LTX before sinus surgery (CF 1, CF 3 and CF 4) have been included in the study. The chronic lung infection is defined as growth of *P. aeruginosa* in six consecutive monthly sputum samples, or less if there are two or more precipitating antibodies [25,26].

2.2. Bacteria and culture conditions

The samples from the paranasal sinuses were taken during Functional Endoscopic Sinus Surgery (FESS) as previously described [23]. Sputum samples were obtained by expectoration and investigated as previously described including Gram-staining and microscopy to confirm the origin from the lower airways by identification of cylinder epithelial cells and inflammatory cells and absence of squamous epithelial cells [27]. *P. aeruginosa* was the only bacterial pathogen cultured from the samples. Mucoid and non-mucoid isolates were considered paired when simultaneously present in a sample.

Fifty-two sequential paired mucoid and non-mucoid *P. aeruginosa* isolates from the lungs and paranasal sinuses of the four CF patients were included in this study. Fifteen of them represented mucoid and non-mucoid pairs from the paranasal sinuses. Four pairs of mucoid and non-mucoid isolates from sputum samples (eight isolates in total) collected close to the time of sinus surgery were chosen for comparison to the sinus samples (a total of twenty-three isolates represent sino-pulmonary samples).

The remaining samples represented sequential lung isolates collected before and after sinus surgery from the bacterial collection available at the Department of Clinical Microbiological, Rigshospitalet. A complete list of all isolates is presented in Supplementary Table 1. Prior to phenotypic analyses (see later), all isolates were grown in LB media, at 37 °C.

2.3. *P. aeruginosa* genotyping

The relatedness of the isolated strains and identification of genotypes were assessed by Pulsed-field gel electrophoresis (PFGE). For the PFGE analysis, bacterial DNA was digested with the restriction enzyme Spe I (BioLabs, Inc., New England) and PFGE was carried out by contour-clamped homogeneous electric field electrophoresis (CHEF-DR III apparatus, Bio-Rad, Munich). Evaluation of similarity was done according to Tenover et al. [28].

2.4. Determination of protease activity

Supernatant from an overnight culture (50 µl) was applied to skim milk plates followed by overnight incubation at 37 °C. The diameter of the clearing zone surrounding the inoculation spot was measured indicating the ability of isolates to produce proteases.

2.5. Motility assays

2.5.1. Swimming

Cells from one colony were inoculated by use of a sterile toothpick on tryptone plates containing 1% tryptone, 0.5% NaCl and 0.3% Bacto agar. The swimming zone was measured after 24 h incubation at 25 °C.

2.5.2. Swarming

Swarm plates were composed of 0.5% Bacto agar and 8 g nutrient broth/L and 5 g dextrose/L. The plates were dried for 2 h at room temperature. Cells from one colony were inoculated on the surface of the plates. The plates were incubated at 30 °C for 24 h, and the surface locomotion of the bacteria was observed.

2.6. Determination of antibiotic susceptibility

The minimal inhibitory concentration (MIC) was determined on 10^5 CFU/ml of *P. aeruginosa* by using the E-test system (Biomerieux, Marcy-l’Etoile, France) according to instructions of the manufacturer.
2.7. Measurements of the frequencies of mutants of 
P. aeruginosa isolates

The mutation frequencies were investigated on rifampicin (300 mg/L) and streptomycin (500 mg/L) containing LB plates as previously described [20]. An isolate was considered hypermutable when the mutation frequency was 20 fold higher than the mutation frequency of the reference strain PAO1 (≥3×10⁻⁷).

2.8. Sequence of mucA, algT, lasR, rpoN, mutS and mutL and PCR for deletions in region PA1428-PA1431 (lasR) and PA4120-PA4122 (hpc operon)

To investigate the genetic background of alginate production, changes in QS regulation and hypermutability, the alginate regulatory genes mucA and algT, the gene encoding the QS-regulator LasR and genes involved in DNA repair mutS and mutL have been sequenced using previously published primer sets [14]. In one of the dominant clones present among patients attending the Copenhagen CF Center (called DK_2 [27]), changes in the aminoacid sequence of RpoN as well as deletions in PA1428-1431 and PA 4120-PA4122 genes have previously been detected [13]. PA1428-1431 includes the lasR gene (PA1430) and PA 4120-PA4122 contains the transcriptional regulator of hpc operon encoding genes involved in the catabolism of 4-hydroxyphenylacetic acid (4-HPC) [13]. Therefore, in isolates belonging to DK_2 clone from CF 3 and CF 4, the rpoN gene has been sequenced using previously published primer sets and the deletions in PA4120-PA4122 and PA1428-1431 were investigated by PCR as previously described [13]. The sequencing results were compared to strain PAO1 sequence (http://www.pseudomonas.com/) with DNASIS Max version 2.0 (Hitachi software Engineering), in order to determine sequence variations.

2.9. Gene expression analysis

The gene expression profile was investigated in mucoid and non-mucoid isolates representing sino-pulmonary samples. The isolates included are shown in Supplementary Tables 1 and 4. The strains were cultured in LB to an OD₆₀₀ = 1.0. Four ml of each culture was harvested and RNA isolation and purification were performed using RNeasy Mini Kit (Qiagen, Germany). RQ1 RNase-free DNAse (Promega) was added to remove contaminating DNA. The experiment was run in triplicates.

Processing of the P. aeruginosa GeneChip (Affymetrix) was performed at the Department of Clinical Biochemistry, Microarray Core Unit, Rigshospitalet, University of Copenhagen, Denmark.

The gene expression analysis was done by ArrayStar v.5 Software (DNASTAR). The data sets were normalized using Robust Multiarray Analysis method. The data were clustered by the hierarchical method. Differences in gene expression between isolates from upper and lower airways were tested using Student’s t-test with FDR (Benjamini–Hochberg) correction for multiple testing. Genes were considered differently expressed if the fold-change was higher than 2-fold with confidence of p<0.05.

2.10. Statistical analysis

The description and analysis of the phenotypic data were carried out using StatViewH 5.0.1. software. The data are given as means±SDs. The unpaired t test was used for comparison of the data for mucoid and non-mucoid isolates from sinus and lung of the sino-pulmonary samples.

3. Results

3.1. Clonal distribution

Isolates collected from the sinuses and lungs from patient CF 1 belonged to DK_1 clone, from patient CF 3 and CF 4 to DK_2 clone and from patient CF 2 to a unique clone. DK_1 and DK_2 clones are two of the dominant clones among the 21 different P. aeruginosa clones that have been identified among chronically infected CF patients at Copenhagen CF Centre [27,29,30].

3.2. Phenotypes

All isolates, from the paranasal sinuses and lower airways showed adapted phenotypes with lack of protease activity and decreased motility compared with the wild-type P. aeruginosa (Supplementary Table 2). In three patients (CF 1, CF 2 and CF 3) mutator isolates were identified in non-mucoid or mucoid or both morphotypes as shown by the mutations identified in mut genes (Table 1). In the case of CF 1 and CF 3, the hypermutable isolates were identified in the CF lungs before transplantation, as well as in the allograft, and in the paranasal sinuses (Supplementary Table 1).

Due to possible differences in the selective pressures in the upper and lower parts of the united airways, we would have expected differences in the phenotypes of the mucoid and non-mucoid isolates from paranasal sinuses and lungs. However, no statistically significant difference between swimming, swarming motility and mutation frequencies was observed between mucoid or non-mucoid isolates from the sino-pulmonary samples (Supplementary Table 2). A tendency towards higher MIC values of all antibiotics in lung isolates compared to sinus isolates has been observed, though the differences did not reach the statistical significance with the exception of ceftazidime; MIC values (mean±SD) in non-mucoid sinus and lung isolates were 5.2±5 and 128.4±147.3 μg/ml, respectively, p=0.03 (Supplementary Table 3).

3.3. Genetic basis of adaptation

Sequence analysis of mucA, algT and lasR identified the types of mutations present in mucoid and non-mucoid P. aeruginosa isolates (Table 1). These genotypes were found in the two morphotypes from sinus and lung and the same mutations were identified in lung isolates obtained prior to FESS and after
Table 1
The type of non-synonymous mutations in mucA, algT, lasR, mutS or mutL and the presence of ΔPA1428-31 and ΔPA4120-22 in the DK_2 clone (see Material and methods) in P. aeruginosa isolates from lung and paranasal sinuses in each of the four patients. The mutations are presented as the type of base change and the position represents the base number from the gene start. NC=no change means no aminoacid change compared to the sequence of the reference strain PAO1.

A full list of the identified mutations in all the isolates is presented in Supplementary Table 1.

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<tr>
<th>Combination of mutations</th>
<th>Morphotype</th>
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<td>C352T</td>
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<tr>
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<th>NC or G92A</th>
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<td>lasR</td>
<td>C538T</td>
<td>NC</td>
<td>C292T</td>
<td>C292T</td>
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<tr>
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<td>mutS: ΔA2429</td>
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| rpoN        | | | | | | | | |

| ΔPA4120-22  | | | | | | | | |

| ΔPA1428-31  | | | | | | | | |

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3.4. Gene expression profiles

Hierarchical clustering of the gene expression data of the sinus samples (Supplementary Table 1). For isolates belonging to the DK_2 clone, single nucleotide polymorphism (SNP) data were also investigated as these previously showed the clonal evolution of the CF lung and in the allograft (Supplementary Table 1).
Affimetrix chip showed that the mucoid isolates from the lungs and sinuses clustered together and separate from the non-mucoid isolates (Fig. 1). Interestingly, the mucoid isolates of CF 3 and CF 4, which belong to clone DK_2, were clustered together, confirming the parallel evolution of this clone in two different patients. When gene expression of all sinus samples was compared to gene expression data of all the lung samples no genes with statistically significant differential expression were found between isolates from sinuses and lungs (Supplementary Fig. 1). The number of genes differentially expressed at 2 fold and 95% confidence interval between the mucoid and non-mucoid isolates in the paranasal sinuses were 152 and 147 in the lungs, respectively showing the different phenotypes expressed by these two morphotypes in the airways (Supplementary Fig. 2) confirming previous studies showing that the two phenotypes follow different evolutionary pathways due to adaptation to different niches in the lungs [10,33,34].

Comparisons of the sinus and lung isolates within individual patients showed a variable number of differentially expressed genes. Interestingly, up-regulation in the sinus isolates of nar genes involved in nitrogen metabolism and anaerobic respiration was observed in CF 3 and up-regulation of the genes involved in arginine metabolism was observed in CF 2. Up-regulation in the sinus isolates of genes involved in type VI secretion and pyochelin biosynthesis was observed in CF 1 (Table 2). Thus, it looks like adaptation to the paranasal sinuses does not occur through common pathways but through various pathways as identified in the four CF patients.

![Dendogram (Heat Map)](Fig. 1. Dendogram (Heat Map) of the hierarchical clustering of the gene expression of all 5886 genes in *P. aeruginosa* mucoid and non-mucoid sinus and lung isolates. The dendogram uses color to display the expression levels of many genes across the different isolates. Each row corresponds to one specific gene, and each column represents one isolate. The dendogram shows that the mucoid isolates from four different CF patients belonging to three different clones (DK_1, DK_2 and a unique clone) are clustering together and therefore have similar gene expression profiles in the sinuses and lungs. The non-mucoid isolates are clustering together and separate from the mucoid isolates. No non-mucoid isolates were available from the lung for CF 4 and the lung mucoid isolate of CF 3 reverted to non-mucoid phenotype and therefore these are not represented in the figure.)
A full list of genes with significantly different expression in mucoid and non-mucoid isolates from the sinus compared to the lung is presented in Supplementary Table 4.

4. Discussion

It has previously been shown that *P. aeruginosa* form biofilms in the sinuses in chronically infected patients similar to the biofilms in the lungs but with an important difference in the inflammatory response: there were only very few PMNs around the biofilms in the sinuses in contrast to the significant amount of PMNs surrounding the biofilms in the lungs [23]. This was attributed to the non-inflammatory secretory IgA response, which may prevent local and systemic inflammation and recognition of microorganisms [22,23]. The different inflammatory conditions that characterize the upper and lower parts of the united airway probably cause different selective pressures for the evolutionary process of the bacteria residing in the two locations. This might be reflected in different evolutionary pathways followed by the bacteria in the sinuses and lungs [16]. However, we found that the genetic basis of adaptation and the subsequent gene expression phenotypes were similar in sinus and lung isolates from chronically infected patients. This could be interpreted as either I. the entire adaptive process occurs in the sinuses and move from the sinuses to the lungs. Different selection pressures could cause different gene expression phenotypes and different evolutionary pathways followed by the bacteria in the sinuses and lungs [16].

The similar combination of mutations in several genes identified in the isolates from the sinuses and lungs suggests their occurrence in the lung with later migration to the sinuses. The similar adaptive profile of the mucoid and non-mucoid isolates encountered in the right and left parts of the paranasal sinuses (CF 3, CF 4) is in favor for a movement of bacteria from the lungs to the sinuses. It has been shown that in the early intermittently colonization stage, the bacteria evolve different in the right and the left side of the paranasal sinuses [16] and move from the sinuses to the lungs.

The specific combination of mutations in the investigated genes characterizing the mucoid or non-mucoid isolates was maintained for long period in the lungs and sinuses (Table 1 and Supplementary Table 1). This dynamic supports the different evolutionary pathways followed by the two morphotypes [10]. It has been proposed that the mucoid isolates are adapted to the aerobic niches, where they are protected from the mutagenic effect of reactive oxygen species by alginate, while the non-mucoid isolates are adapted to the anaerobic niches [33,34].
It has been shown that anaerobe niches are present in the sinuses [38], similar to the anaerobic conditions met in the sputum [39] from where both mucoid and non-mucoid isolates are frequently isolated.

After reaching the sinuses during episodes of upper respiratory viral infections, the lung-adapted isolates will probably reside in the paranasal sinuses from where they can migrate downwards to the lung, through drainage and aspiration [16,23].

While residing in the upper part of the united airways, the microorganisms might elicit adaptive responses to the specific selective pressures encountered in the sinuses.

For example, the higher susceptibility to cefazidime of the sinus isolates compared with the lung isolates can be explained by the fluctuations in the bacterial subpopulations that occur during antibiotic treatment [40] suggesting a lower selective pressure exerted by beta-lactam antibiotics in the sinuses compared with the lungs. From our analysis of the gene expression results, we could not identify a common pathway of adaptation to the paranasal sinuses in these isolates from the chronically infected patients. However, the up-regulation in the sinus isolates of operons involved in anaerobic respiration or arginine fermentation in CF 3 and CF 2 might be interpreted as adaptive responses of P. aeruginosa to the anaerobic environment described in the sinuses [38]. The down-regulation in isolates of CF 1 of the type VI secretion system and pyochelin genes in the lung compared with the sinus isolates might reflect the difference in the inflammatory response between the two locations of the united airways [22,23]. It has been shown that the siderophore pyochelin is down regulated in the presence of hydrogen peroxide [41] probably as a protective mechanism against reactive oxygen species produced by Fenton reaction, which involves iron. Down-regulation of type VI secretion system in the lung would lead to decrease virulence factor productions and subsequent lack of PMN recruitment to the site of infection.

In the three patients, who had undergone lung transplantation, the isolates that infected the allograft had similar adaptive patterns as isolates collected before the transplant from the CF infected lungs as well as the isolates from the paranasal sinuses of the patients. This suggests that the transplanted lungs have become infected with already adapted P. aeruginosa isolate that probably hide mainly in the sinuses. Therefore, extensive sinus surgery and postoperative treatment before or in relation to LTX might have preventive effects on the post-transplant infection. This was shown by Holzmann et al. [42] who did extensive surgery and thorough follow-up, finding a lower incidence of tracheobronchitis and pneumonia after LTX and that eradication of the sinus bacteria was possible. Recently, Vital et al. [43] showed that sinus surgery and daily nasal douching reduced P. aeruginosa in lung transplant recipients and absence of post-transplant colonization with P. aeruginosa had a positive impact on post-transplant survival and development of bronchiolitis obliterans. Conflicting results showing no effect of sinus surgery on the recolonization of the lung allograft in CF patients have been published [44,45]. However, in these retrospective studies there was a lack of post-surgical treatment of the sinuses and the sinus surgery was not as extensive as in the studies reporting positive results.

We are convinced that the sinuses can be a reservoir for CF pathogens and that these bacteria can drain/migrate/be aspirated to the lower airways in LTX CF patients [16,46]; it is more debatable what can be done to prevent it. Vital et al. [43] suggests sinus surgery after recovery from LTX (mean days after LTX 36) combined with nasal irrigations and IV and nebulized antibiotic therapy. Mainz et al. [47] found an effect of ongoing Colomycin 1 MIU with a Pari Sinus™ nebulizer.

In conclusion, in chronically infected CF patients the P. aeruginosa isolates from sinuses and lungs show the same phenotype caused by similar mutations in global gene regulators suggesting a bidirectional movement between the two compartments of the united airways.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.jcf.2013.02.004.

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