Transfer of a cathelicidin peptide antibiotic gene restores bacterial killing in a cystic fibrosis xenograft model

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Received for publication January 20, 1999, and accepted March 5, 1999.

Recent studies suggest that the gene defect in cystic fibrosis (CF) leads to a breach in innate immunity. We describe a novel genetic strategy for reversing the CF-specific defect of antimicrobial activity by transferring a gene encoding a secreted cathelicidin peptide antibiotic into the airway epithelium grown in a human bronchial xenograft model. The airway surface fluid (ASF) from CF xenografts failed to kill Pseudomonas aeruginosa or Staphylococcus aureus. Partial reconstitution of CF transmembrane conductance regulator expression after adenovirus-mediated gene transfer restored the antimicrobial activity of ASF from CF xenografts to normal levels. Exposure of CF xenografts to an adenovirus expressing the human cathelicidin LL-37/hCAP-18 increased levels of this peptide in the ASF three- to fourfold above the normal concentrations, which were equivalent in ASF from CF and normal xenografts before gene transfer. The increase of LL-37 was sufficient to restore bacterial killing to normal levels. The data presented describe an alternative genetic approach to the treatment of CF based on enhanced expression of an endogenous antimicrobial peptide and provide strong evidence that expression of antimicrobial peptides indeed protects against bacterial infection.


Introduction
Cystic fibrosis (CF) is caused by mutations in the gene coding for the CF transmembrane conductance regulator (CFTR); these mutations result in chronic respiratory infections with accompanying severe inflammation (1). CFTR is mainly recognized as a low-conductance chloride channel expressed predominately in the serous gland cells and the surface epithelial cells of human airways (2). Current attempts to treat CF range from repeated courses of antibiotic therapy to suppress bacterial infections to correction of the primary genetic defect by CFTR gene transfer (3).

A better understanding of the link between the defect in chloride conductance and the chronic airway infection and inflammation is important in designing alternative genetic and pharmacologic therapies. Insight into this conundrum was provided by Smith et al. (4), who demonstrated diminished ability of the CF airway surface to kill bacteria, an ability that was restored by lowering the salt concentration of the airway surface fluid (ASF). One hypothesis is that the abnormal milieu of the CF airways inactivates antimicrobial molecules that are secreted into the ASF, such as lysozyme, lactoferrin, surfactant proteins, and secretory phospholipase A2. We and others recently discovered a family of epithelial-derived antimicrobial peptides expressed in the human lung that contribute to innate immunity. These include human β-defensins 1 and 2 (5–8) and the cathelicidin LL-37/hCAP-18 (9), all of which are variably inactivated by salt. Antimicrobial peptides belong to the innate host defense systems of various animals. Functional analysis of purified peptides and proteins in vitro does not necessarily reflect the complexity of component interactions, such as synergism and antagonism between multiple substances. The host defense defect in CF airways has been studied mainly in experimental model systems (4, 6, 8). The host defense system has evolved families of peptides and proteins with overlapping functions, suggesting a biologic redundancy that complicates the functional analysis of individual components. In fact, the evidence that antimicrobial peptides actually contribute to innate immunity in vivo is largely indirect. Genetic approaches, such as analysis of knockout animals or depletion of substances by antisense techniques (6), may help clarify these issues. Another approach is the overexpression of endogenous antimicrobials to analyze the outcome on host defense functions, such as bacterial killing. We describe in this report a novel strategy for reversing the bacterial killing defect in CF by overexpression of an antimicrobial peptide after adenovirus-mediated gene transfer to surface epithelial cells. The linear peptide antibiotic LL-37/hCAP-18 was selected for gene transfer because it is endogenously expressed in the airways and has a broad spectrum of activity (10). These results also provide direct evidence that expression and secretion of mammalian antimicrobial peptides protect against bacterial colonization and infection.

Methods
Recombinant adenoviruses: The structure and production of the adenoviral vector H5.020.CBCFTR have been described previously (11). The vector expresses a human CFTR cDNA from a chicken β-actin promoter enhanced by sequences of the immediate early gene of cytomegalovirus (CMV). Sequences spanning E1 and E3
are deleted. The vectors H5.020.CMVLL-37 and H5.020.CMVPG3 were generated using the same viral backbone as used for H5.020.CBCFTR deleted in E1 and E3 genes and express the cDNAs of the cathelicidins LL-37/hCAP-18 from human and protegin 3 from pig, respectively. The recombinant viruses were produced by cotransfection and screening for recombinant plaques as described for H5.020.CBCFTR (11). Both transgenes are driven from the 5′ flanking region of the immediate early gene of CMV. The cDNA for LL-37/hCAP-18 and protegin 3 were obtained from G.H. Gudmundsson (Karolinska Institute, Stockholm, Sweden) and R. Lehrer (University of California–Los Angeles, Los Angeles, California, USA), respectively. The vectors coding for antimicrobial peptides were intensively analyzed to determine whether their use results in secretion of antimicrobially active substances (data not shown). Infection of various cultivated cell types (293, HeLa, Calu-3, airway epithelial cells in transwell culture) with H5.020.CMVLL-37 revealed the presence of LL-37 in the supernatant of most transduced cells. In no case could we detect any antimicrobial activity or any other secretory product after infection with H5.020.CMVPG3. Therefore, H5.020.CMVPG3 was used as control vector in the present study.

Human bronchial xenografts. Human bronchial xenografts were prepared as described earlier using cells from normal individuals and patients with CF (5, 9, 12). In short, respiratory primary cells removed from a human bronchus by digestion with protease 14 (Sigma Chemical Co., St. Louis, Missouri, USA) and maintained in culture for five to seven days were seeded (2 × 10^6 cells per graft) in 30 μl of hormonally defined growth medium (Cloneics; BioWhittaker Inc. Walkersville, Maryland, USA) in tracheas obtained from CO2-asphyxiated Fisher 344 rats, from which the epithelium was denuded by three rounds of freeze thawing. These tracheas were ligated to plastic tubing, implanted subcutaneously in the flanks of nu/nu BALB/c mice, and maintained for three weeks to allow maturation of a fully differentiated epithelium. For infection of differentiated xenografts, 5 × 10^9 particles of the virus (H5.020.CMVLL-37, H5.020.CMVPG3, or H5.020.CBCFTR) were suspended in 50 μl of PBS and applied into the tubing system of normal and CF xenografts. After 12 h of incubation, the xenografts were flushed with sterile PBS. Beginning five days after the application of the virus, the ASF produced by the xenografts was collected and analyzed for antimicrobial activity and presence of LL-37/hCAP-18.

**Figure 1**
Antimicrobial activity of ASF from human bronchial xenografts against *Pseudomonas aeruginosa* PAO1 (a) and *Staphylococcus aureus* ATCC 29213 (b). Xenografts were prepared with normal cells (NL) or CF cells (CF) and were partly treated with the adenoviral vectors H5.020.CBCFTR (AdCFTR), H5.020.CMVLL-37 (AdLL-37), or H5.020.CMVPG3 (AdPG3). Arrows indicate the numbers of CFU in the inoculum (open arrow) or in the NB positive control (filled arrow).
Characterization of ASF. ASF was recovered from xenografts and was extracted and lyophilized as described (5, 9, 12). This treatment has been shown to increase the recovery of cationic antimicrobial substances, such as antimicrobial peptides, lysozyme, and lactoferrin, and to improve the sensitivity of the antimicrobial assay. After reconstitution to the original volume and adjustment of the pH to neutral, the antimicrobial activity of the fluid was determined against *Pseudomonas aeruginosa* PAO1 and *Staphylococcus aureus* ATCC 29213. The bacteria were grown as described (5, 9). A total of 200 CFU of bacteria in 5 μl were inoculated into 30 μl of ASF and incubated for two hours. After diluting the volume to 200 μl with nutrient broth (NB), one quarter was plated onto NB agar plates and colonies were counted after overnight incubation. Each experiment was repeated at least three times, using between 10 and 30 individual xenografts for one experimental group. The number of CFU inoculated was determined by serial plating onto NB agar plates. As positive control, NB medium was used instead of samples and treated identically. Additionally, synthetic LL-37 (Louisiana State University Medical Center, Core Laboratories, New Orleans, Louisiana, USA) at final concentrations of 1, 10, and 100 μg/ml was added to CF ASF samples that were then treated like other samples. Data are displayed as means ± SEM and were compared using ANOVA and post hoc Student’s t tests. Concentrations of LL-37/hCAP-18 were determined using a quantitative dot blot assay. Three microliters of the reconstituted ASF was dotted onto nitrocellulose and detected as described previously using a polyclonal antibody against LL-37/hCAP-18 (9). The concentration was determined by quantification of the signal intensity using the AlphaImager 2000 Analysis System (Alpha Innotec, San Leandro, California, USA) and comparison with signals from known amounts of synthetic peptide. Immunoblots of ASF were prepared after separation of the ASF using reverse-phase HPLC as described (9). For immunohistochemistry, xenograft tissues were formalin-fixed and paraffin-embedded using standard procedures. After sectioning, the tissue was stained using the polyclonal rabbit anti–LL-37 antibody (1:1,000, overnight, 4 °C) followed by a peroxidase-conjugated secondary antibody (sheep anti-rabbit) and DAB/H2O2 as substrate.
Results
Our studies were performed in a human bronchial xenograft model in which primary epithelial cells from normal or CF bronchi are seeded onto rat trachea and grown subcutaneously in the flanks of nu/nu BALB/c mice and maintained for three weeks to allow matura-
tion of a fully differentiated respiratory epithelium. ASF recovered from normal xenografts killed *P. aeruginosa* PAO1 and *S. aureus* ATCC 29213 more effectively than did ASF from CF xenografts (Figure 1). To determine whether deficient antimicrobial activity is due to decreased concentration of LL-37/hCAP-18, the concentration of this peptide was determined using a specific and sensitive dot blot assay. Endogenous LL-37/hCAP-18 protein was detected at low but equivalent levels in ASF of normal and CF xenografts (Figure 2c).

The xenograft model was used to evaluate the outcome of primary genetic reconstitution (i.e., CFTR gene transfer) versus augmentation of antimicrobial peptide expression (i.e., LL-37/hCAP-18 gene transfer). Instillation of an adeno-
viral vector expressing CFTR into a CF xenograft resulted in enhanced killing activity of the ASF against *P. aeruginosa* and *S. aureus*, as described previously (4, 6), without affecting LL-37/hCAP-18 expression (Figure 1 and Figure 2c). Exposure of CF xenografts to the LL-37/hCAP-18 vector led to a three- to fourfold increase in the concentration of this peptide in the ASF, coincident with a normalization of killing against *P. aeruginosa* and *S. aureus* (Figure 1 and Figure 2, c and d). The molecular weights of endogenous and recombi-
nantly derived LL-37/hCAP-18 peptides were indistin-
guishable (Figure 2d). Immunohistochemical analysis revealed increased expression of the peptide in xenografts treated with the adenoviral vector coding for LL-37/hCAP-18 compared with treatment with the CFTR vector (Figure 2, a and b). Exposure of xenografts to a vector that carried the cDNA of protegrin 3 failed to correct the killing defect (Figure 1), confirming that the increased antimicrobial activity after LL-37/hCAP-18 gene transfer is not a nonspecific consequence of aden-
viral gene transfer and depends on the elevated con-
centration of LL-37/hCAP-18 in the ASF. Infection of cultured cells with H5.020.CMV did not result in the secretion of an antimicrobially active substance. Consistent with these observations, the addition of syn-
thetic LL-37 to CF ASF samples showed a dose-depend-
ent increase of antimicrobial activity, with almost com-
plete killing of *P. aeruginosa* and *S. aureus* at a final concentration of 10 μg/ml (data not shown).

Discussion
An initial step in the development of CF lung disease is the colonization of the respiratory system after birth as a result of defects in innate immunity (13). A substantial and persistent inflammatory response develops, but is incapable of clearing the infection. The defect of host defense is closely linked to the deficiency in CFTR, as cor-ection of the genetic defect in cultured CF cells results in restoration of the antimicrobial activity (4, 6). Two fam-
ilies of antimicrobial peptides, the defensins and the cathelicidins, have been described to be part of the human pulmonary host defense system; hBD-1, hBD-2, and LL-37/hCAP-18 are produced in epithelial cells and secreted into the ASF (5–9). Evidence that mammalian antimicrobial peptides actually contribute to innate immunity in vivo is largely based on in vitro activity of purified substances and, therefore, is largely indirect.

Using a human bronchial xenograft model, we showed in the present study that LL-37/hCAP-18 is equally abundant in ASF from normal and CF xenografts, ruling out the possibility that decreased concentrations of this pep-
tide contribute to the host defense defect found in CF.

Correction of the genetic defect of the CF cells by CFTR gene transfer resulted in correction of the killing defect without altering the concentrations of LL-
37/hCAP-18. Overexpression of this peptide using recombinant adenovirus resulted in increased concentra-
tions in the ASF and restoration of the defect of antimi-
nergic activity in ASF from CF xenografts. We esti-
mate that the concentration of endogenous LL-
37/hCAP-18 in ASF of normal and CF xenografts is 1.7–1.8 μg/ml. This is slightly less than the minimal inhibitory concentration (MIC) of synthetic peptide against *P. aeruginosa* in the presence of lactoferrin, which is 8 μg/ml using in vitro assays (9). The increased concentra-
tion of LL-37/hCAP-18 from 1.8 to 6.7 μg/ml achieved by gene transfer is in the range of the MIC, sug-
gesting that it could be biologically meaningful. This effect is likely to be even more biologically meaningful because LL-37/hCAP-18 acts synergistically with other secretory antimicrobial substances, such as lysozyme and lactoferrin (9). The results obtained from the exper-
iments in which addition of synthetic LL-37 to CF sam-
ples restored killing activity at a final concentration of 10 μg/ml support these assumptions. The biologic effect seen in this study was not due to a nonspecific response to viral infection, as the application of a control vector did not show any impact on antimicrobial function.

This study demonstrates an alternative genetic approa-
ach to the treatment of CF based on enhanced expression of an endogenous broad-spectrum peptide antibiotic. Whether this novel approach is applicable for treatment of patients cannot be answered from our results, obtained from a model system for CF airways and in vitro antimicrobial assays. The fact, however, that the CF-specific defects in bacterial killing can be reversed by overexpression of endogenous antibiotics supports the hypothesis that a lack of antimicrobial activity con-
tributes to CF lung disease. Furthermore, it underscores the potential for augmentation of the innate immune sys-
tem of the airways for prevention or treatment of airway infection in CF. Our innovative approach has several potential advantages over gene replacement with CFTR.

Reconstitution of innate immunity across the epitheli-
um may be possible with suboptimal gene transfer because the transgene product is secreted. For this or any other gene theray to be successful, a vector must be identified that efficiently and stably transduces airway epithelial cells. The peptide antibiotic cDNAs are much smaller than CFTR, enabling the use of vectors with limited capacity, such as adeno-associated virus. In addition, the data presented here also provide direct evidence that expression of antimicrobial peptides indeed protects against bacterial colonization and infection. This genet-
ic approach of overexpressing a specific host defense substance highlights the biologic role that antimicrobial peptides have in protecting internal or external body surfaces from colonization and infection.

Acknowledgments
This work was supported by the National Institutes of Health (P30 DK-47757 and R01 HL-49040), Cystic Fibrosis Foundation, and Genovo Inc., a company J.M. Wilson founded and has equity in. R. Bals was a recipient of a fellowship of The Deutsche Forschungsgemeinschaft.