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Human β-Defensin-1 Is a Salt-Sensitive Antibiotic in Lung That Is Inactivated in Cystic Fibrosis

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Summary

A human bronchial xenograft model was used to characterize the molecular basis for the previously described defect in bacterial killing that is present in the cystic fibrosis (CF) lung. Airway surface fluid from CF grafts contained abnormally high NaCl and failed to kill bacteria, defects that were corrected with adenoviral vectors. A full-length clone for the only known human β-defensin (i.e., *hBD-1*) was isolated. This gene is expressed throughout the respiratory epithelia of non-CF and CF lungs, and its protein product shows salt-dependent antimicrobial activity to P. aeruginosa. Antisense oligonucleotides to hBD-1 ablated the antimicrobial activity in airway surface fluid from non-CF grafts. These data suggest that hBD-1 plays an important role in innate immunity that is compromised in CF by its salt-dependent inactivation.

Introduction

Study of the primary molecular and cellular defects responsible for cystic fibrosis (CF) lung disease has been complicated by the secondary abnormalities that result from chronic respiratory infections. One hypothesis is that ion transport abnormalities in the respiratory-conducting epithelia lead to dehydration of mucus, inspissation of secretions, and diminished clearance of inhaled pathogens (Welsh et al., 1995). Another model is that the macromolecular composition of mucus in the CF airway is abnormal, affecting both its rheologic properties and affinity for microorganisms (Cheng et al., 1989; Tang et al., 1995; Pier et al., 1996). A more recent hypothesis suggests the primary event in pathogenesis is colonization of the airway with pathogenic bacteria that initiate cycles of infection, inflammation, airway obstruction, and destruction of lung architecture. In this model, mucus represents the debris of chronic inflammation and cell destruction composed of cellular DNA and cytoskeletal proteins. Recent studies of CF infants who underwent bronchoscopy in the first months of life revealed evidence of inflammation and bacterial colonization before onset of clinically overt disease (Armstrong et al., 1995, 1996).

Smith et al. (1996) recently presented data that support a defect in host defense in the CF lung. Using primary cultures of human airway epithelial cells, they showed that the airway surface fluid (ASF) from normal respiratory epithelia was capable of killing pathogens such as P. aeruginosa, whereas the ASF from CF cells was not. Diluting normal ASF into hypertonic solution eliminated antibacterial activity, while diluting CF ASF with hypotonic solutions increased this activity. This suggested that an antibacterial substance is present in ASF that is reversibly inactivated in the high salt milieu of CF. They proposed a model whereby the defect in the CF gene product, the cystic fibrosis transmembrane conductance regulator (CFTR), leads to elevated NaCl in ASF, which inactivates antimicrobial molecules. This model would resemble the absorptive epithelia of sweat glands that result in elevated levels of Na and Cl in sweat when CFTR is deficient (Quinton, 1983). Direct measurements of the ionic composition of human ASF support this hypothesis. Gilljam et al. (1989) showed an increase in Cl from bronchial secretions from 85 ± 54 mM in non-CF to 170 ± 79 mM in CF. Joris et al. (1993) measured Na and Cl concentration from non-CF tracheal ASF to be 82 ± 6 mM and 84 ± 9 mM, respectively, which increased to 121 ± 4 mM and 129 ± 5 mM in CF. The nature of these salt-sensitive bactericidal molecules in ASF was not described by Smith et al. (1996).

In evaluating the biology of the antimicrobial activity normally present in ASF, a variety of molecules should be considered, including inorganic molecules (e.g. hydrogen peroxide and nitric oxide), enzymes (e.g. proteases and neuraminidases), and cytotoxic peptides, such as the antimicrobial peptides called defensins. This family of small structurally similar peptides contributes to innate immunity in a wide variety of invertebrate and vertebrate organisms (Ganz and Lehrer, 1994, 1995). The α-defensins, which are found exclusively in mammals (including humans), play a major role in host defense at multiple sites, with the highest expression noted in neutrophil granules and Paneth cells of the gut. β-defensins have been described in a broader array of organisms and sites.

Zasloff and colleagues isolated the only known airway epithelial defensins, tracheal antimicrobial peptide (TAP) and lingual antimicrobial peptide (LAP), which are members of the β-defensin family. TAP was purified from cow trachea and shown to have bactericidal activity against a broad array of organisms (Diamond et al., 1991). It is expressed at high levels in columnar epithelia of the proximal conducting airway. A similar but distinct molecule called LAP was isolated from cow tongue (Schonwetter et al., 1995). Genes encoding these peptides are upregulated at a transcriptional level in response to bacterial LPS and inflammatory cytokines such as TNFα (Diamond et al., 1996; Russell et al., 1996). Activation of LAP expression is observed in vivo at actual sites of inflammation. These two molecules are not stored in intracellular granules, which clearly distinguishes them from known α-defensins.

The goal of this study was to define the molecular

basis of the antimicrobial activity in human ASF that is defective in CF.

Results

A Salt-Sensitive Bactericidal Activity Exists in ASF of Human Xenografts

To evaluate further the molecular basis of the defect in bacterial killing in CF ASF, we used an authentic model for CF lung biology based on the growth of human bronchial xenografts in *nu/nu* mice (Engelhardt et al., 1992b, 1993a; Goldman et al., 1995; Zhang et al., 1996). Primary cultures of epithelial cells derived from the proximal airways of CF and non-CF patients were seeded into denuded rat trachea and implanted subcutaneously into *nu/nu* mice with the proximal and distal ends open to the surface via ligated tubing. Within two weeks, a pseudostratified epithelium of human origin was established. Quantitative morphometric analysis of transmission electron micrographs indicated that the epithelium generated on the xenograft was indistinguishable in organization, cell type distribution, and ultrastructure when compared to the epithelium of the native bronchus from which the epithelial cells were derived (Engelhardt et al., 1992b, 1993a; Zhang et al., 1996). Submucosal glands containing ducts with serous and mucus tubules are partially formed (Engelhardt et al., 1995). Previous studies evaluated the ion-conductive properties of the xenograft epithelia through the measurement of surface voltage in response to modulators of ion transport. In these studies, we showed functional properties of these epithelia were identical to those observed in the human nasal and intrapulmonary airways (Goldman et al., 1995). The most discriminating measurement between non-CF and CF was the change in voltage measured in response to a decrease in luminal Cl and activation with cAMP.

A number of xenografts were established for characterization of the antimicrobial defect in ASF, including 12 grafts from 6 non-CF patients and 12 grafts from 3 CF patients. Results of the studies are presented in Figure 1. Non-CF grafts were distinguished from CF grafts based on the measure of ΔV_T in response to low chloride and cAMP (non-CF: -14.3 ± 1.1 mV; CF: -0.3 ± 0.3 mV). ASF from xenografts was obtained for antimicrobial assays and ion measurements by expelling the luminal contents with air, followed by a brief centrifugation to remove mucus. Direct ionic measurements of non-CF ASF revealed both the Na and Cl concentrations to be 83 ± 3 mM. Antimicrobial activity was measured by incubating ASF (30 μl) with 10^3 P. aeruginosa for 2 hr at 37°C prior to a quantitative assessment of bacterial viability using standard colony counts. ASF from all non-CF xenografts completely killed bacteria. This bacterial activity was inactivated in high salt and reducing agents, and fractionated through gel filtration with an apparent molecular weight less than 10 (data not shown). Similar studies performed in CF xenografts revealed a significant increase in Na and Cl content in ASF to 178 ± 9 mM and 172 ± 9 mM, respectively. ASF from all CF xenografts failed to kill P. aeruginosa. Diluting CF ASF in hypotonic solution reconstituted the bactericidal activity (data not shown).

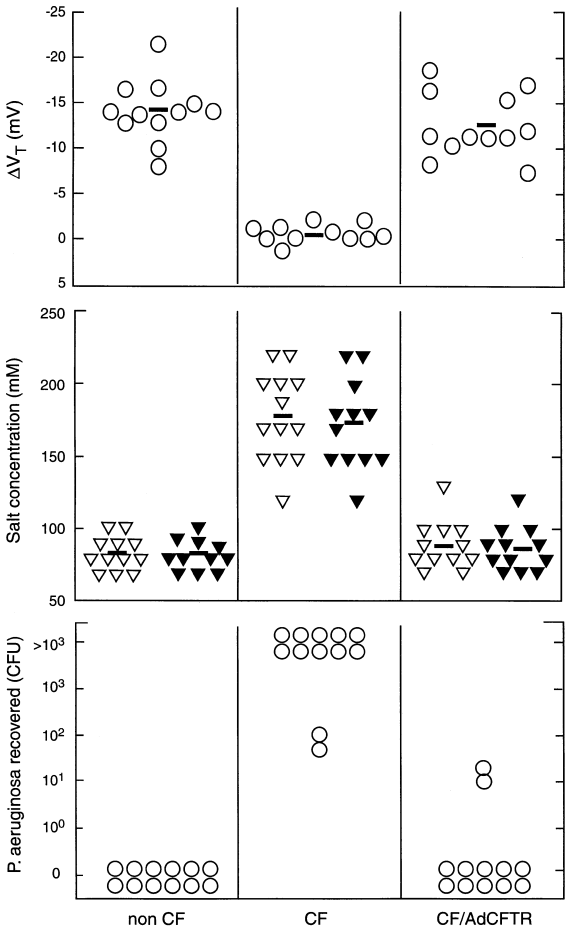

Figure 1. Correction of the CF Defect in Xenografts Normalizes ASF Salt Concentration and Restores Its Bactericidal Activity

ASF was collected from non-CF (left), CF (middle), and CF bronchial xenografts treated with a recombinant adenovirus expressing CFTR (right). In this fluid, sodium (open inverted triangle) and chloride (closed inverted triangle) concentrations were measured and antibacterial liquid broth assays performed against 10^3 CFU of P. aeruginosa (middle and bottom rows, respectively). Each point shown represents results from an individual graft ($n = 12$). Transepithelial potential difference measurements were obtained from xenografts before and after gene transfer. The data presented (top row) show the change in transepithelial potential difference in amiloride-treated epithelium in response to both chloride substitution and cAMP agonist treatment. As controls, non-CF ($n = 6$) and CF xenografts ($n = 6$) were treated with a recombinant adenovirus expressing β-galactosidase. The ion measurements and antibacterial properties of ASF from grafts treated with the β-galactosidase vector were similar to untreated controls (data not shown).

The relationship between ionic composition and bactericidal properties of ASF and the epithelial expression of CFTR was further studied in CF xenografts treated with adenoviral vectors containing a cDNA encoding CFTR. E1-deleted adenoviral vectors expressing CFTR or β-galactosidase were instilled into the lumen of CF xenografts. In situ hybridization revealed transduction and high-level recombinant CFTR expression in 9%–15% of surface epithelial cells (Figure 2). *CFTR* gene transfer corrected the conductive properties of the CF epithelia ($\Delta V_T = -12.7 \pm 0.3$ in response to low chloride

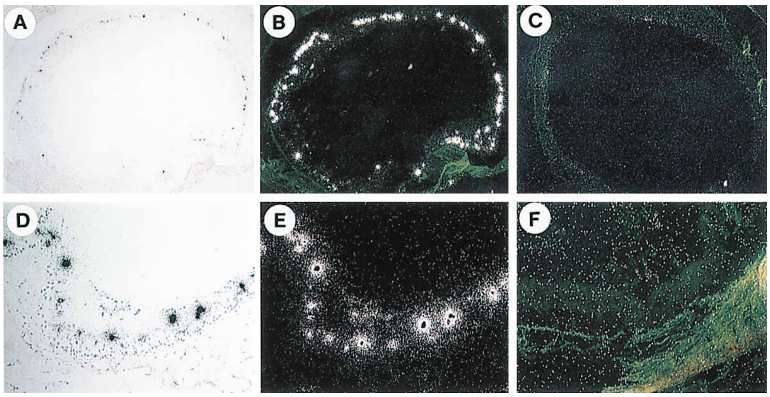

Figure 2. Analysis of CF Xenografts for Expression of Recombinant mRNA Encoding CFTR

Gene transfer to CF bronchial xenografts treated with a recombinant adenovirus expressing CFTR was analyzed by in situ hybridization. Antisense- (B and E) and sense- (C and F) probed sections of xenograft tissues were exposed to photoemulsion for one week. Bright-field (A and D) and dark-field (B and E) photomicrographs of the same section are shown. The efficiency of gene transfer was determined by dividing the number of positive signals by the total number of cells present in multiple sections from an individual graft. Magnification: (A–C) 4×; (D–F) 15×.

and cAMP) and normalized ASF Na (89 ± 5 mM) and Cl (87 ± 4 mM) (see Figure 1). ASF from the CFTR-corrected CF xenografts was capable of complete killing of P. aeruginosa in 10 out of 12 grafts, with partial killing in the remaining 2 (Figure 1). CF xenografts treated with similar doses of LacZ virus were indistinguishable from untreated grafts (data not shown).

Human β-Defensin-1 Is a Salt-Sensitive Antibiotic Expressed in Human Airway

Previous studies by Bensch et al. (1995) identified a peptide in hemofiltrate of patients with end-stage renal disease that has structural similarities to previously described β-defensin peptides of other organisms. A partial cDNA for this peptide was identified from mRNA isolated from kidney. Based on these modest sequence homologies, they named the peptide human β-defensin-1 (hBD-1).

We cloned the full-length cDNA encoding hBD-1 from mRNA derived from cultured human bronchial epithelial primary cells (Figure 3). This cDNA encodes an open reading frame of 68 amino acids in length with the characteristic features of a β-defensin, including a prepropeptide and six appropriately spaced cysteines. hBD-1 shows homology with the two previously described epithelial defensins from cow, i.e., TAP and LAP (Diamond et al., 1991; Schonwetter et al., 1995).

The homology of hBD-1 to TAP and its cloning from mRNA of a human airway cell-line suggested it may play a role in host defenses within human lung. Expression of hBD-1 was evaluated in tissue from human lung by in situ hybridization. Figure 4 presents a series of micrographs of non-CF and CF lung tissues hybridized to antisense and sense probes of hBD-1. High-level RNA for hBD-1 was present throughout the superficial conducting airway of non-CF lung from proximal bronchi (Figures 4A–4C) through distal bronchioles (Figures 4D–4F). Expression was also detected throughout the epithelia of submucosal glands (Figures 4A and 4B) and alveolar cells (Figures 4D and 4E). This differs from the expression of TAP in bovine lung that was primarily detected in proximal airway (Diamond et al., 1991). A similar distribution of hBD-1 expression was demonstrated in the CF lung (proximal airway, Figures 4G–4I; distal airway, Figures 4J–4L). Hybridization of serial sections with the sense probe failed to demonstrate specific signals, confirming the specificity of the assay (Figure 4, right column).

To test the hypothesis that hBD-1 is a salt-sensitive antibiotic, a synthetic form of the mature peptide was made, retaining the three disulfide bridges (i.e., Cys_5–Cys_{34}, Cys_{12}–Cys_{27}, and Cys_{17}–Cys_{35}). In the presence of low NaCl, synthetic hBD-1 demonstrated potent bactericidal activity to a broad array of organisms, including P. aeruginosa and E. coli (Figure 5 and data not shown). The impact of NaCl concentration on hBD-1 was evaluated by incubating the synthetic peptide with 5×10^4 colony-forming units of P. aeruginosa in the presence of varying NaCl concentrations (Figure 5). The antimicrobial activity of hBD-1 exhibits a dramatic salt dependence characterized by a sharp loss of activity as the salt concentration increases from 50 mM to 125 mM. Bacterial killing was observed in low salt with concentrations of hBD-1 varying between 60–500 μg/ml. This titration of hBD-1 activity occurs in the range of NaCl that distinguishes non-CF from CF ASF in native human proximal lung and the bronchial xenograft model.

Antisense Inhibition of hBD-1 in Non-CF Bronchial Xenografts Ablates Antimicrobial Activity in ASF

Important to an understanding of CF lung pathogenesis is an identification of a molecule in ASF that confers microbial killing in non-CF lung and is inactive in CF. The tissue distribution of hBD-1 expression and its salt-sensitivity to bacterial killing suggested it may play a role in ASF-mediated host defense. In situ hybridization demonstrated high-level and diffuse hBD-1 expression in epithelia from both non-CF (Figures 4M–4O) and CF (Figures 4P–4R) xenografts that was indistinguishable from native tissue. The role of hBD-1 in microbial killing was further studied by genetically ablating its expression with a 21-mer phosphorothioate oligonucleotide antisense DNA specific to the 5′ region of mRNA that encodes hBD-1. Controls included 1 nonsense oligonucleotide containing a scrambled sequence and 2 mismatched oligonucleotides identical to the antisense oligo at 19 of 21 (mismatch 1) and 14 of 21 (mismatch 2) nucleotides. The oligonucleotides were instilled into the lumen of non-CF xenografts, and three days later ASF was harvested for microbial killing assays (Figure 6A) prior to removal of the xenografts for isolation of

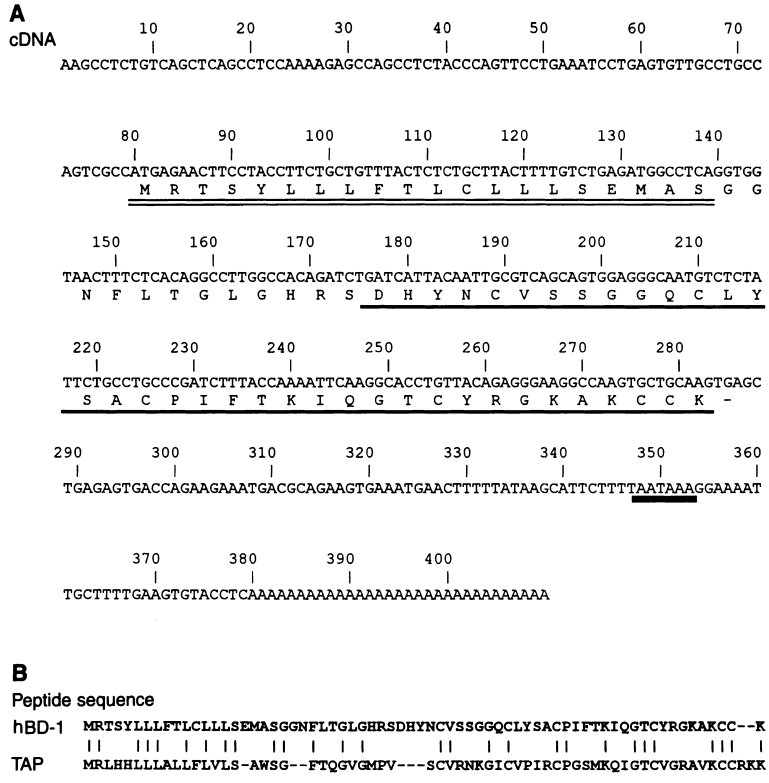

Figure 3. cDNA and Peptide Sequences of hBD-1

(A) Complementary DNA and deduced amino acid sequences of hBD-1. The double underline indicates a putative signal sequence; the solid underline indicates a mature peptide; the dash represents the termination codon; and the bold underline marks the polyadenylation signal.

(B) Putative prepropeptide sequences of hBD-1 and tracheal antimicrobial peptide (TAP) are shown, along with their homology.

RNA for RT-PCR analysis of hBD-1 expression (Figure 6B). RNA for hBD-1 was detected by RT-PCR at equivalent levels in all non-CF xenografts that were either untreated or preincubated with nonsense, mismatch 1, or mismatch 2 oligonucleotides. Preincubation of non-CF xenografts with these control nucleotides had no effect on the ASF antimicrobial activity; eight out of nine ASF samples completely killed bacteria while one sample yielded only 20 colonies. Preincubation of non-CF xenografts with the hBD-1 antisense oligonucleotide substantially diminished RNA for hBD-1 in six out of eight xenografts, each of which failed to completely kill bacteria; full antimicrobial activity was retained in two antisense-treated xenografts that were the same grafts in which the RNA for hBD-1 was not decreased.

Discussion

Chronic respiratory infections with pathogens such as P. aeruginosa and S. aureus characterize the pulmonary manifestations of CF (Welsh et al., 1995). One explanation for this aspect of the disease was recently suggested by Smith et al. (1996), who demonstrated a salt-sensitive antimicrobial activity in the ASF of non-CF-cultured cells that was defective in CF. We describe in this study the characterization of a novel antimicrobial peptide, called human β-defensin-1 (hBD-1), which is expressed in human airway epithelial cells and confers salt-dependent antimicrobial activity in ASF.

hBD-1, isolated from hemodialysate of humans with end-stage renal failure, was classified as a β-defensin antimicrobial peptide based on sequence homology to other nonhuman β-defensins (Bensch et al., 1995). Analysis of synthetic hBD-1 in our study confirmed that it has broad-spectrum antimicrobial activity against gram-negative organisms (i.e., P. aeruginosa and E. coli) at concentrations similar to what has been described for most classical defensins (i.e., 10–100 μg/ml; Ganz and Lehrer, 1994). hBD-1 showed structural homology with the bovine β-defensin TAP, although its distribution of expression within human lung is different (Diamond et al., 1991). The gene encoding hBD-1 is diffusely expressed at high levels throughout the conducting and respiratory airway from the mainstem bronchus to alveoli, including the epithelia of the submucosal glands, whereas expression of the *TAP* gene is restricted to proximal superficial epithelia.

Several observations support the hypothesis that hBD-1 is involved in innate immunity in the lung and that its activity is compromised in CF. The gene encoding hBD-1 is expressed at high levels in most surface epithelial cells throughout the lung. Secretion of hBD-1 into the airway lumen would lead to wide distribution of highly concentrated antibiotic peptide in ASF, the volume of distribution of which has been estimated to be no greater than 8–10 ml spread over 2 m^2 of airway surface (Boucher, 1994). In addition, the impact of NaCl on hBD-1 function correlates with the NaCl-dependent antimicrobial activity in ASF measured by Smith et al. (1996) and in our xenograft model. Furthermore, the change in NaCl that occurs in ASF of native lung as a result of CFTR deficiency (i.e., 80 mM NaCl in non-CF versus 120–170 mM in CF [Gilljam et al., 1989; Joris et al., 1993]) spans the range of NaCl that inactivates hBD-1

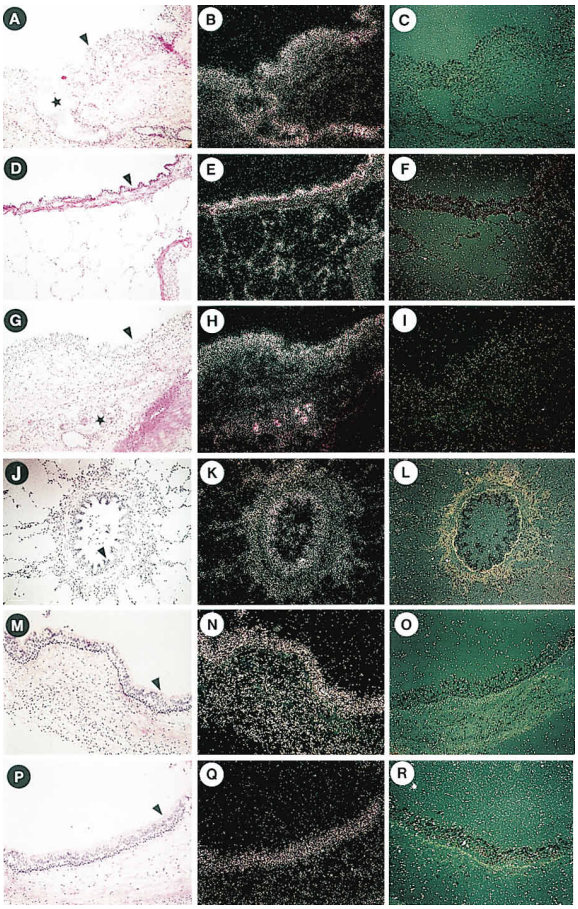

Figure 4. Detection of mRNA Encoding hBD-1 in Normal and CF Pulmonary Tissues

Antisense (middle column) and sense (right column) oligonucleotides to the gene encoding hBD-1 were probed to sections of human lung tissues and were exposed to photoemulsion for six days. Proximal (A–C and G–I) and distal noncartilagenous airways (D–F and J–L) are shown from normal (top rows [A–F]) and CF (middle rows [G–L]) lungs. Representative sections from non-CF (M–O) and CF xenografts (P–R) are also shown (bottom rows). Bright-field (left column) and dark-field (middle column) photomicrographs of the same region are presented. mRNA encoding hBD-1 was not seen in serial sections hybridized with the antisense probe after treatment of the section with RNase, confirming the specificity of the assay. Arrowheads indicate epithelium; stars denote submucosal glands. Magnification: proximal airway, 8×; distal airway and xenografts, 12.5×.

(i.e., from 50–125 mM). In fact, hBD-1 is partially inactivated at the NaCl concentration of non-CF ASF, suggesting that any perturbation in ASF NaCl concentration will have a direct effect on hBD-1 function. The most compelling evidence for the role of hBD-1 in CF lung pathogenesis was provided in antisense experiments, where specific ablation of hBD-1 function in non-CF xenografts abolished bactericidal activity in ASF. The specificity of this assay was illustrated in control experiments in which mismatches in only 2 of 21 nucleotides of the antisense oligonucleotide failed to inhibit hBD-1 activity.

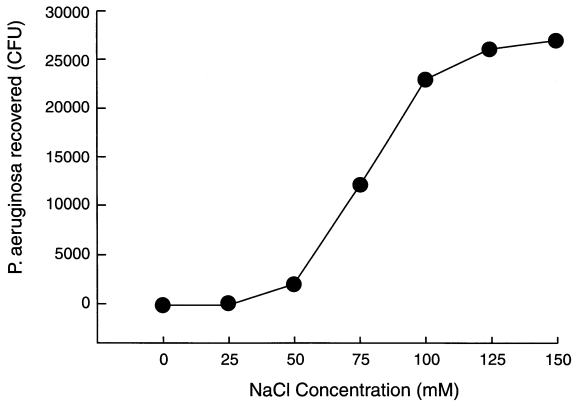

Figure 5. The Activity of hBD-1 Peptide Is Salt Dependent

Synthetic hBD-1 was incubated with 5×10^4 colony-forming units of P. aeruginosa in 100 μl of 10 mM phosphate buffer (pH 7.4) and the indicated concentrations of NaCl. Reactions were then incubated at 37°C for 20 min. Serial dilutions were plated and colony counts performed the following day. Similar bacterial killing in low NaCl solution was observed with hBD-1 concentrations ranging from 60 to 500 μg/ml.

Our data suggest that the bactericidal activity in human airway to some gram-negative organisms is primarily constituted through the action of a single antimicrobial peptide, hBD-1. This is interesting in that many defensins exist as families of genes whose products serve complementary and sometimes redundant functions, although this type of genetic program differs substantially between species. For example, there are four α-defensins in human polymorphonuclear neutrophils (Ganz and Lehrer, 1994, 1995), whereas no α-defensin protein has been identified in murine polymorphonuclear neutrophils (Eisenhauer and Lehrer, 1992). The enteric defensins exist as a family of up to 16 genes in the mouse (Ouellette et al., 1989; Huttner et al., 1994), whereas only two human homologs have been identified in human gut (Jones and Bevins, 1992, 1993; Mallow et al., 1996). While our data suggest a primary role for hBD-1 in lung against some bacterial pathogens, they do not rule out a role for other antimicrobial substances in the lung. The fact that antisense inhibition of hBD-1 does not fully prevent microbial killing is consistent with either incomplete inhibition of hBD-1 or the existence of other bactericidal molecules. Furthermore, other defensins with complementary functions may play a more significant role against fungal, viral, and other bacterial pathogens.

The question remains as to the contribution of hBD-1 inactivation to the pathogenesis of CF. Its role in the development of disease in extrapulmonary organs such as liver, pancreas, and the male reproductive tract is not obvious, since infection does not routinely occur at these sites. The story in lung is substantially different, where colonization with bacterial pathogens and indices of chronic infection are established in the first four months of life in what appears to be an otherwise normal pulmonary system (Armstrong et al., 1995, 1996). The hypothesis that emerges from this work is that a breach

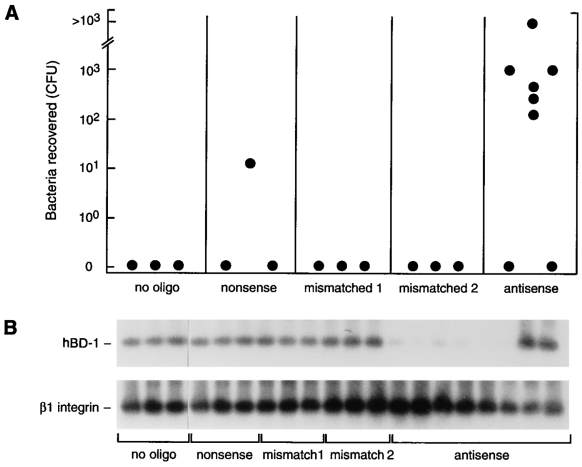

Figure 6. Specific Down-Regulation of RNA for hBD-1 by an Antisense Oligonucleotide

(A) Airway surface fluid was collected from non-CF xenografts before and after the instillation of phosphorothioate oligonucleotides. Antibacterial activity was uniformly present in the ASF from all grafts before the administration of any oligonucleotide (data not shown). Antibacterial broth assays were performed against P. aeruginosa (10^3 CFU) by incubating the organisms with 30 μl of ASF for 2 hr at 37°C, plating serial dilutions of the mixture, and completing colony counts the following day. Each point shown represents results from an individual graft.

(B) RT-PCR analysis for RNA encoding hBD-1 and β1-integrin subunit from xenografts treated with phosphorothioate oligonucleotides. Four days after the local administration of the oligonucleotide, grafts were explanted and RNA isolated. RT-PCR was performed using hBD-1 or β1-integrin subunit-specific primers, and an aliquot of the reaction mixture blotted to a nitrocellulose membrane and hybridized with specific probes.

in innate immunity occurs due to the inactivation of hBD-1 in the high salt environment of the CF ASF, which leads to colonization in the airway with microbial pathogens and chronic pulmonary inflammation. Other components of innate immunity and antigen-specific immunity are mobilized without apparent success in clearing of bacteria. Major components of the mucus within the airway are derived from debris of the ongoing inflammatory process, including DNA and cytoskeletal proteins of inflammatory cells. Pharmacologic agents that dissipate the secondary components of mucus, such as DNase for DNA and gelsolin for actin, are in fact being evaluated for treatment of the obstructive components of CF lung disease (Fuchs et al., 1994; Vasconcellos et al., 1994). One issue not resolved is whether primary defects in mucus biochemistry and rheology also contribute to obstructive lung disease and infection.

Further consideration of this model will require a better understanding of the perturbations in ion and water transport that occur at the airway surface in CF. Quantitative measures of electrolytes in ASF are difficult, although there has been a consistent trend toward elevated NaCl in CF in a variety of models, including in vitro cultures (Smith et al., 1996), human bronchial xenografts (this study), and native human airway (Gilljam et al., 1989; Joris et al., 1993). It is possible the human airway exhibits a functional similarity to the sweat gland, where Cl is secreted in the lumen in the distal coil and absorbed in the proximal duct. In the CF sweat gland, Cl absorption is defective, thus leading to elevated NaCl in sweat (Quinton, 1983). In extending this model to lung, it will be important to identify the site(s) of CFTR-independent Cl secretion into the airway. One possibility is the submucosal glands, which express high levels of CFTR and have substantial secretory capacity (Engelhardt et al., 1992a). Finally, it will be important to reconcile the elevated NaCl in CF ASF with the finding of sodium hyperabsorption that characterizes the CF defect in polarized epithelia (Willumsen and Boucher, 1991; Goldman et al., 1995).

The putative role of hBD-1 in lung pathogenesis of CF has implications in the development of therapy. This model supports the rationale for gene therapy in which reconstitution of CFTR function normalizes the ionic milieu of the ASF. Indeed, we show in the xenograft model that as little as ~10% gene transfer leads to a diminution in ASF NaCl and reconstitution of antimicrobial activity. Likewise, pharmacologic approaches for enhancing chloride transport through the mutant CFTR or alternative chloride channels should be useful if ASF NaCl is corrected. Pharmacologic administration of β-defensin into the airway would be therapeutic only if a salt-insensitive peptide were created.

Experimental Procedures

Recombinant Adenoviruses

The structure, production, and measurement of titer of E1-deleted viruses that express β-galactosidase (CMV promoter in a sub360 backbone) and *CFTR* (CMV-enhanced β-actin promoter in a dl7001 backbone) have been described previously (Engelhardt et al., 1993b). The viruses were titred as previously described (Engelhardt et al., 1993b).

Generation of Bronchial Xenografts

Human bronchial tissues were obtained from explanted or donor lungs at the time of lung transplantation, and bronchial xenografts were prepared as previously described (Engelhardt et al., 1992b, 1993a; Goldman et al., 1995). Surface epithelial cells were removed by incubating the bronchus in protease 14 (Sigma), plated in primary culture, and maintained for 5–7 days prior to release with trypsin. Donor rat tracheas were harvested from CO_2-asphyxiated Fisher 344 rats (200 g), from which the epithelium was denuded by three rounds of freeze thawing. These tracheas were ligated to tubing at both ends after seeding of 2×10^6 primary bronchial epithelial cells in 30 μl of hormonally defined growth medium (Clonetics). Grafts were then implanted subcutaneously in the flanks of male *nu/nu* BALB/c mice and maintained for three weeks in vivo to allow for maturation of a fully differentiated bronchial epithelium. Virus was instilled into the graft lumen (5×10^{10} pfu/ml, 100 μl) and expelled with air 6 hr later. Samples from three CF lungs (ΔF508 homozygotes) and six non-CF lungs were used to generate xenografts for these studies.

Transepithelial Potential Difference Measurements

Xenografts were analyzed for changes in potential difference (PD) in response to perfusion with different solutions before and after gene transfer as described previously (Goldman et al., 1995). In preparation for these measurements, animals were anesthetized with intraperitoneally administered ketamine/xylazine (100 μl, 10% v/v) in phosphate-buffered saline (PBS) (pH 7.4). Agar bridges were prepared by filling 21-gauge butterfly needles and tubing with 1 M KCl in 4% agar. The reference bridge was implanted subcutaneously in the flank of the mouse while the exploring electrode bridge was in contact with a reservoir of buffered solution being delivered at a constant rate of 2 ml/min by a syringe pump. Each bridge was connected by a calomel half-cell to a voltmeter. Measurements were

recorded every 10 s by a computer interfaced with the voltmeter. Prior to their use, pairs of agar bridges were kept in a common reservoir of buffered solution and only the electrodes that differed less than 0.2 mV were used for analysis. Each recording measured voltage as a function of time in response to the sequential perfusion of the following: (i) HEPES phosphate-buffered ringers solution (HPBR) containing 10 mM HEPES (pH 7.4), 140 mM NaCl, 5 mM KCl, 1.2 mM $MgSO_4$, 1.2 mM Ca gluconate, 2.4 mM K_2HPO_4, and 0.4 mM KH_2PO_4; (ii) HPBR with 100 μM amiloride; (iii) chloride-free HPBR (gluconate replaces chloride) with 100 μM amiloride; (iv) chloride-free HPBR with 100 μM amiloride, 200 μM 8-cpt cAMP, and 100 μM forskolin; and (v) return to HPBR.

Isolation of the cDNA Encoding hBD-1

The cDNA encoding hBD-1 was amplified from primary human bronchial epithelial cell poly(A)$^+$ RNA by 5′ and 3′ RACE (Frohman et al., 1988). The primers used were derived from the nucleotide sequence of hBD-1 (Bensch et al., 1995). Amplification products were subcloned into a Bluescript vector (Stratagene) and sequenced. Multiple clones were analyzed to verify the nucleotide sequence, and a full-length cDNA was reconstructed.

In Situ Analyses

To perform cytochemical analyses, the xenografts were explanted, embedded in OCT (Tissue-TCK, Miles Inc.), and cryosectioned. Tissue sections (6 μm) were mounted on slides and fixed in 4% paraformaldehyde in PBS (pH 7.4) for 4 hr. Following fixation, sections were dehydrated through graded concentrations of ethanol. Sections were then desiccated overnight under vacuum and stored at −20°C. The following day, sections were treated with 10 μg/ml proteinase K for 30 min at 30°C, rinsed twice in 0.2× SSC for 30 s each time, fixed in 4% paraformaldehyde in PBS, rinsed twice in 0.1 M triethanolamine (pH 8.0) for 4 min each time, incubated in 0.25% acetic anhydride for 10 min at room temperature, and then dehydrated through graded concentrations of ethanol. Sections were again dried under vacuum before prehybridization for 4 hr at 54°C in 10 mM Tris (pH 8.0), 50% formamide, 2.5× Denhardt's, 0.6M NaCl, 1 mM EDTA (pH 8.0), 0.1% SDS, 500 μg/ml tRNA, and 10 mM dithiothreitol (DTT). RNase control sections were treated with 200 μg/ml RNase A for 1 hr at 37°C before the prehybridization step. Sections were then hybridized with 5×10^6 cpm/ml ^{35}S antisense or sense probes for 16 hr at 54°C. Probes were synthesized with the Promega in vitro transcription system using ^{35}S UTP and ^{35}S CTP. cRNA probes for hBD-1 were generated from the T7 and T3 RNA polymerase promoters of full-length cDNA encoding hBD-1 and were subcloned into BlueScript (Stratagene). Probes for CFTR were synthesized from the T7 and SP6 RNA polymerase promoters of a PCR template derived from the cDNA for CFTR. Following hybridization, slides were washed in 4× SSC for 20 min (four changes) at room temperature, RNase (20 μg/ml) for 30 min at 37°C, 2× SSC/1 mM DTT at room temperature for 10 min (two changes), and finally, three 15 min washes in 0.5× SSC/1 mM DTT at 54°C. Slides were dehydrated through ethanol and air dried before dipping in photoemulsion (Kodak). Slides were developed and analyzed by bright- and dark-field microscopy using a Microphot-FXA Nikon microscope. The efficiency of adenovirus-mediated *CFTR* gene transfer to xenografts was determined by dividing the number of positive signals by the total number of cells present in 20 sections derived from multiple blocks of each treated graft. Expression of hBD-1 was evaluated in xenografts from uninfected samples.

Analyses of ASF from Non-CF and CF Xenografts

To obtain sufficient volumes of ASF for study, airway secretions in xenografts were allowed to accumulate for at least one week and then collected by expulsion with air. After a brief centrifugation ($10^4 \times$ g) to remove the mucus, the supernatant was recovered for antibacterial assays and ion measurements. Sodium and chloride concentrations in ASF were measured using ion-specific glass microelectrodes (Londonderry, NH) calibrated to prepared standards. Antibacterial studies were performed as previously described (Harwig et al., 1994; Smith et al., 1996). Single colonies of bacteria, P. aeruginosa (ATCC# 39324), or a clinical isolate were inoculated into LB broth and cultured overnight at 37°C. An aliquot of this culture was transferred to fresh LB and incubated for an additional 2–3 hr at 37°C to obtain mid-logarithmic-phase cells. The organisms were washed with 10 mM sodium phosphate buffer (pH 7.4), and the concentration of colony-forming units (CFU) per ml^{-1} quantitated by measuring its absorbance at 620 nm. ASF (30 μl) was mixed with 10^3 bacterial CFU and incubated for 2 hr at 37°C. Serial dilutions were then plated and colony counts completed the following day. Analyses of airway surface fluid were conducted from at least 12 individual non-CF and CF xenografts, before and after gene transfer. Identical studies were also performed on six non-CF xenografts treated with a recombinant adenovirus expressing β-galactosidase.

Peptide Synthesis

A peptide corresponding to the mature form of hBD-1 was synthesized by the solid phase methodology. The regioselective formation of the three disulfide bridges, Cys_5–Cys_{34} (I), Cys_{12}–Cys_{27} (II), and Cys_{17}–Cys_{35} (III), was carried out following published procedures (Kellenberger et al., 1995). The disulfide bridge I was formed first, followed by the disulfide bridges II and III. The peptide was characterized by mass spectral high performance liquid chromatographic and capillary zone electrophoretic analyses. A detailed discussion of the synthesis, purification, and characterization of this molecule will be published elsewhere.

Oligonucleotide Delivery to Xenografts and Molecular Analysis

Phosphorothioate oligonucleotides consisted of 21-mer analogs to the 5′ end of the *hBD-1* gene. Mismatches are underlined. Antisense 5′-CAGAAGGTAGGAAGTTCTCAT-3′, nonsense 5′-TACAGAGGTG CTCACTGGGTA-3′, mismatched 1 5′-CAGAAGGTAGGAAGTTGT CTT-3′, and mismatched 2 5′-TCTAAGGTAGGGAGTTCTTTG-3′ phosphorothioate oligonucleotides were instilled into non-CF xenografts at a concentration of 20 μM oligonucleotide in 70 μM phosphate buffer (pH 7.4). Any remaining solution was expelled with air the following day. The ability of ASF to kill bacteria was measured before and then three days after oligonucleotide delivery. Antibacterial broth assays were performed as previously described. Grafts were harvested four days after oligonucleotide administration and total RNA was prepared using RNAzol B (Tel-Test, Inc). RT-PCR was performed on 1 μg of template RNA using the Titan RT-PCR system (Boehringer Mannheim) and primer sets specific for hBD-1 or β1-integrin subunit. Aliquots of the PCR reactions were resolved on a 1% agarose gel, blotted to nitrocellulose, and hybridized to specific ^{32}P random primer-labeled probes. The cDNA for the human β1-integrin subunit was kindly provided by Dr. Clayton Buck (The Wistar Institute, Philadelphia, PA).

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