

Human β -Defensin 2 Is a Salt-sensitive Peptide Antibiotic Expressed in Human Lung

Robert Bals,* Xiaorong Wang,* Zhongren Wu,* Theresa Freeman,* Vineet Bafna,† Michael Zasloff,§ and James M. Wilson*

*Institute for Human Gene Therapy, Department of Medicine and Molecular and Cellular Engineering, The Wistar Institute, Philadelphia, Pennsylvania 19104; †Bioinformatics Division, SmithKline Beecham Pharmaceuticals, King of Prussia, Pennsylvania 19406; and

§Magainin Pharmaceutical, Plymouth Meeting, Pennsylvania 19662

Abstract

Previous studies have implicated the novel peptide antibiotic human β -defensin 1 (hBD-1) in the pathogenesis of cystic fibrosis. We describe in this report the isolation and characterization of the second member of this defensin family, human β -defensin 2 (hBD-2). A cDNA for hBD-2 was identified by homology to hBD-1. hBD-2 is expressed diffusely throughout epithelia of many organs, including the lung, where it is found in the surface epithelia and serous cells of the submucosal glands. A specific antibody made of recombinant peptide detected hBD-2 in airway surface fluid of human lung. The fully processed peptide has broad antibacterial activity against many organisms, which is salt sensitive and synergistic with lysozyme and lactoferrin. These data suggest the existence of a family of β -defensin molecules on mucosal surfaces that in the aggregate contributes to normal host defense. (*J. Clin. Invest.* 1998. 102:874–880.)

Key words: peptide antibiotics • cystic fibrosis • lung infection • mucosal immunity

Introduction

Antimicrobial peptides play an important role in host defense at mucosal surfaces. The β -defensins are small, cysteine-rich, cationic peptides expressed by a number of epithelia (1). The first such peptide, called tracheal antimicrobial peptide (TAP),¹ was isolated from cow trachea (2); it is expressed throughout the surface epithelia of the cow lung where it is believed to contribute to host defense (3). A homologous peptide called lingual antimicrobial peptide was subsequently purified from cow tongue and shown to express in multiple epithelia, including those of the lung (4). Expression of these defensin genes is regulated in response to infection and inflammation (5).

Recent studies suggest that the pulmonary consequences of cystic fibrosis (CF) may initiate through an early breach in innate immunity. Children with this disease are born with normal pulmonary function. Soon after birth their respiratory tract becomes colonized with bacterial pathogens such as *Pseudomonas aeruginosa* leading to chronic respiratory inflammation (6, 7). A direct link between the genetic defect in CF and host defense was provided by Smith et al., who demonstrated strong antibacterial activity in airway surface fluid from normal lung epithelia that was substantially reduced in the high salt environment of the CF airway surface fluid (8). They speculated that the primary genetic defect in CF increases the salt content of fluid lining the airway (9, 10), which reversibly inactivates antimicrobial molecules (8).

We proposed a model for CF pathogenesis, in which human β -defensins secreted by airway epithelia onto the airway surface are inactivated in the milieu of CF (11). The first characterized human β -defensin (hBD-1), was originally isolated from large volumes of hemodialysate (12). We showed that hBD-1 is also expressed in the surface epithelia and submucosal glands of both normal and CF lungs (11). A synthetic hBD-1 peptide showed broad activity against a number of bacterial organisms that was diminished by high salt (11). Inhibition of hBD-1 expression in normal bronchial xenografts by antisense oligonucleotides partially compromised the ability of epithelia to secrete antimicrobial activity, thereby providing a direct link between the expression of an antibiotic peptide and host defense in the lung (11).

Defensins often exist as families of homologous genes with complementary and/or redundant activity (1). An example is the large family of enteric defensin genes expressed in Paneth cells of the mouse intestine (13). The goal of this study was to identify other members of the β -defensin family in humans and evaluate their role in lung host defense.

Methods

Isolation and characterization of a cDNA clone for human β -defensin 2 (hBD-2). The β -defensin specific cysteine pattern was used to perform a “basic local alignment search tool” search at an expressed sequence tag database (SmithKline Beecham, Inc., King of Prussia, PA) and three overlapping sequences were found. Primers for the 3' region were designed from the database sequences as follows: CCT TTC TGA ATC CGC ATC AGC CAC AG (H2 rapid amplification of cDNA ends [RACE] 1) and GTC GCA CGT CTC TGA TGA GGG AG (H2 RACE 2), and used in RACE with cDNA from human lung as template (Marathon-Ready cDNA; Clontech, Palo Alto, CA). PCR products between 200 and 550 bp were cloned into pGEM-T (pGEM-T Cloning Systems; Promega, Madison, WI) and sequenced using an Applied Biosystems Model 373 fluorescent DNA sequencer (Applied Biosystems, Foster City, CA). Primers for the 5' region of the cDNA were designed from the sequence determined (CCA GGT GTT TTT GGT GGT ATA GG [H2 P1], GGT GAA GCT CCC AGC CAT CAG C [H2 P2]) and together with the RACE primers

Address correspondence to James M. Wilson, 3601 Spruce Street, 204 Wistar Institute, Philadelphia, PA 19104-4268. Phone: 215-898-3000; FAX: 215-898-6588; E-mail: jurmu@wista.wistar.upenn.edu

Received for publication 5 December 1997 and accepted in revised form 7 July 1998.

1. *Abbreviations used in this paper:* CF, cystic fibrosis; hBD-1, human β -defensin 1; hBD-2, human β -defensin 2; RACE, rapid amplification of cDNA ends; TAP, tracheal antimicrobial peptide; TFA, trifluoroacetic acid.

J. Clin. Invest.

© The American Society for Clinical Investigation, Inc.

0021-9738/98/09/0874/07 \$2.00

Volume 102, Number 5, September 1998, 874–880

<http://www.jci.org>

applied in amplifications using cDNA from human lung as template (Quick-clone cDNA; Clontech). A PCR product of ~440 bp was isolated, cloned into pGEM-T, and sequenced.

Dot blot analysis. [³²P]dCTP random primer-labeled probes of hBD-2 and ubiquitin cDNA were hybridized separately to a nylon filter with dotted mRNAs from 50 different human organs (Human RNA Master Blot; Clontech). After washes at high stringency conditions, the signals were quantified using a Phosphorimager 445 SI (Molecular Dynamics, Sunnyvale, CA). The expression data of hBD-2 were normalized using hybridization to the housekeeping gene ubiquitin.

Genomic localization. A genomic sequence containing the intron-exon 2 border was amplified (Human GenomeWalker Kit; Clontech), cloned, and sequenced. Two gene specific primers were then used to screen a genomic bacterial artificial chromosome library (Genome Systems, Inc., St. Louis, MO). The digoxigenin-labeled DNA of a positive clone was used as probe for fluorescent in situ hybridization of normal human metaphase chromosomes. The chromosome of interest was identified by analysis of the 4', 6-diamidino-2'-phenylindole dihydrochloride (DAPI)-banding patterns and by cohybridization of the hBD-2 specific probe with a probe specific for the centromere of chromosome 8 (GenomeSystems, Inc.).

In situ hybridization. Various human tissues were embedded in OCT (Tissue-Tek; Miles Laboratories, Inc., Naperville, IL), cryosectioned (6 μm), mounted on slides, and fixed in 4% paraformaldehyde in PBS (pH 7.4, 4 h, 4°C). After dehydration, sections were treated with 10 μg/ml proteinase K (30 min, 30°C), fixed in 4% paraformaldehyde in PBS, treated with acetic anhydride, and dehydrated through ethanol. Prehybridization was performed for 4 h at 54°C in 10 mM Tris (pH 8.0), 50% formamide, 2.5×Denhardt's solution, 0.6 M NaCl, 1 mM EDTA, 0.1% SDS, 500 μg/ml tRNA, and 10 mM dithiothreitol. RNase control sections were treated with 200 μg/ml RNase A for 1 h at 37°C before the prehybridization step. Sections were hybridized in the prehybridization solution (16 h, 54°C) using digoxigenin-labeled antisense or sense probes synthesized by in vitro transcription of full-length hBD-2 cDNA. Sections were incubated with antibodies against digoxigenin conjugated with alkaline phosphatase followed by a solution of nitro-blue tetrazolium/5-bromo-4-chloro-3-indolyl-phosphate. For colocalization with lysozyme, the hybridized sections were initially incubated with antibodies against digoxigenin conjugated with Texas red followed by incubation with 28 μg/ml rabbit anti-human lysozyme (Dako Co., Carpinteria, CA) (in PBS for 90 min at room temperature). After washes, the sections were incubated with FITC-conjugated anti-rabbit IgG antibody (goat) (ZYMED Laboratories, Inc., San Francisco, CA), and covered with mounting medium containing 1.5 μg/ml DAPI (Boehringer Mannheim, Indianapolis, IN).

Production of hBD-2 by recombinant baculovirus and isolation of hBD-1 from urine. The full-length hBD-2 cDNA (WT hBD-2) and a mutated form of hBD-2 (G26M hBD-2) were prepared using gene-specific primers in standard PCR or PCR-directed mutagenesis (14), and ligated with the transfer vector pBAC-1 (Novagen Inc., Madison, WI). The recombinant transfer plasmids were cotransfected with the linearized baculovirus DNA (BacVector-3000; Novagen, Inc.) into Sf9 cells and recombinant viral plaques were purified individually and amplified (15). Sf9 cells were grown in serum-free medium (SF-900; GIBCO BRL, Gaithersburg, MD) in suspension (27°C, 110 rpm). Cells were infected with recombinant virus at approximately one moi and diluted to 10⁶ cells/ml. The medium was collected 60–72 h after infection by centrifugation, adjusted to pH 5.8, and chromatographed on a cation-exchange column (2.5 × 10 cm, carboxymethyl cellulose) equilibrated with ammonium acetate (32 mM, pH 5.8). After washing with ammonium acetate, a one-step elution was performed using 50 ml of 0.8 M NaCl in 32-mM ammonium acetate/20% acetonitrile. For the mutated peptide, an overnight cleavage reaction in 0.25 M CNBr/1% trifluoroacetic acid (TFA) was performed. WT hBD-2 and G26M hBD-2 were further purified on a 0.5 × 25 cm Dynamax-300Å C-18 RP-HPLC column (Rainin Instrument Co., Woburn, MA) using a lin-

ear gradient of acetonitrile with 0.1% TFA. Fractions were dried, resuspended in 50 μl distilled water, and tested for antimicrobial activity in agarose diffusion assays (see below). Purified peptides were characterized by mass spectrometry (Voyager BioSpectrometry Workstation; PerSeptive Biosystems, Framingham, MA), capillary zone electrophoresis (270A-HT Capillary Electrophoresis System; Applied Biosystems), and NH₂-terminal amino acid analysis by Edman degradation (D. Speicher, Wistar Institute, Philadelphia, PA). Using synthetic hBD-1 as standard, the concentrations of the purified peptides were determined by capillary zone electrophoresis. The 44-amino acid isoform of hBD-1 was purified from human urine by cation-exchange chromatography and subsequent RP-HPLC, and analyzed by CZB, NH₂-terminal sequencing, and mass spectrometry, as described for the isolation of hBD-2 from the baculovirus system. A similar procedure for purification of hBD-1 from urine was reported recently (16).

Preparation of antibodies against hBD-2. Antiserum against hBD-2 purified from the recombinant baculovirus system was obtained by a standard immunization scheme (Research Genetics, Huntsville, AL). 100 μg of peptide was coupled to keyhole limpet hemocyanin and injected into two rabbits. Injections were repeated after two and seven wk using the same dose. The titer against hBD-2 as assayed by ELISA increased from < 50 (preimmune) to > 100,000 (10 wk) for both animals. An IgG fraction was prepared by purification on a protein A column (HiTrap Protein A; Pharmacia Biotech, Piscataway, NJ). A similarly purified antibody fraction from the preserum of the rabbits was used as negative control in all experiments. The hBD-2-specific antibody did not cross-react with human serum proteins or other cationic, antimicrobial proteins, or peptides, such as lysozyme, lactoferrin, hBD-1, or LL-37/hCAP-18 (data not shown).

Western blot analysis of airway surface fluid from human lung and human bronchial xenografts. The airways of explanted lungs were lavaged using 150 ml of PBS. Furthermore, airway surface fluid was expelled with air from a human bronchial xenograft model as previously described (11). In short, respiratory primary cells removed from human bronchus by digestion with protease 14 (Sigma Chemical Co., St. Louis, MO) and maintained in culture for 5–7 d were seeded (2 × 10⁶ cells per graft, in 30 μl of hormonally defined growth medium [Clonetics, San Diego, CA]) 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 3 wk to allow for maturation of a fully differentiated epithelium. The collected liquids from lung and xenografts were extracted in acetonitrile (final concentration 60%) and TFA (final concentration 1%) overnight at room temperature. After lyophilization, the substance was resuspended in water, cleared by centrifugation at 10,000 g for 20 min, and further purified on Ultrasphere C-18 RP-HPLC columns (4.6 mm × 25 cm or 10 mm × 25 cm) from Beckman (Fullerton, CA), using a linear gradient of acetonitrile with 0.1% TFA. Fractions were dried by vacuum centrifugation, resuspended in 50 μl distilled water, and tested for antimicrobial activity in agarose diffusion assays (see below). From each fraction, 2 μl was dotted onto a nitrocellulose membrane and immunolabeling was performed using the polyclonal rabbit antibody to hBD-2 (1:500) followed by a monoclonal peroxidase-conjugated anti-rabbit antibody from goat (Sigma Chemical Co.). After washes, bound antibodies were visualized by using chemiluminescent substrate (ECL Western blotting detection kit; Amersham, Arlington Heights, IL) and exposure to X-ray films. Positive fractions were used in a SDS-PAGE using a 15% tris-tricine running gel. After electrophoretic separation, the proteins were blotted onto nitrocellulose and hBD-2 immunoreactive bands visualized as described above.

Antimicrobial assays. To screen chromatography fractions for antibacterial activity, each 2 μl were applied on the top of 0.7% agarose containing *Escherichia coli* D31 (5 × 10⁸cfu/10 ml), and incubated for 12 h at 37°C. Fractions with antimicrobial activity were determined visually. The isolated peptides were used in antibacterial microdi-

lution assays (17) against the following bacteria: *E. coli* D31, *E. coli* ATCC 25922, *P. aeruginosa* ATCC 27853, *Enterococcus faecalis* ATCC 29212, and *S. aureus* ATCC 29213. For minimal inhibitory concentration (MIC) testing, twofold serial dilutions of peptides were prepared in half-strength Mueller-Hinton broth. Inocula of $\sim 10^5$ cfu bacteria growing in log phase were added to each well. After 12 h of shaking (37°C, 200 rpm), bacterial growth was determined by visual analysis and the OD 595 nm. The MIC was defined as the lowest antimicrobial concentration at which there was no visible turbidity or increase of the OD 595 after 12 h (17). For analyzing the dependency of antimicrobial activity of WT hBD-2 on the concentration of NaCl, *E. coli* D31 was used in microdilution assays combining 250 $\mu\text{g/ml}$ of peptide with different concentrations of NaCl in quarter-strength LB medium. To test whether hBD-2 acts synergistically with other antimicrobial substances found in mucosal secretions, we performed antibacterial assays combining serial dilutions of WT hBD-2 with human lysozyme (500 $\mu\text{g/ml}$; Sigma Chemical Co.) and lactoferrin (1 mg/ml, 250 $\mu\text{g/ml}$ for *S. aureus* ATCC 29213; Sigma Chemical Co.), applying concentrations that do not show any effect on the bacterial growth if used without hBD-2.

Results

The β -defensin-specific cysteine pattern was used to search the expressed sequence tag database developed by SmithKline

Beecham and Human Genome Sciences. Three overlapping sequences were found in a cDNA library of human lung. Alignment of these clones revealed that the open reading frame of this cDNA, hBD-2, showed 39% identity with hBD-1 and 43% identity with TAP, including the conserved six β -defensin specific cysteine residues. A full-length cDNA for hBD-2 was isolated by RACE-PCR, the open reading frame of which is identical to a novel peptide antibiotic recently purified from human skin (18). A bacterial artificial chromosome spanning the entire hBD-2 gene was isolated and used to determine its localization within the human genome. Fluorescent in situ hybridization of human metaphase spreads localized the hBD-2 gene to 8p22–23 immediately contiguous to the hBD-1 gene (19). This suggests that the genes for both human β -defensins arose by gene duplication of a progenitor.

The distribution of hBD-2 expression was evaluated by dot blot hybridization using a commercially available filter that contains RNA from 50 different human tissues. The resulting hybridization signal was quantified on the phosphoimager and normalized to expression of ubiquitin. Fig. 1 presents the relative expression of hBD-2 in all organs that showed a signal above background except for brain, which is an example of a negative result. Other negative tissues are listed in the figure legend. The highest expression was found in lung with substan-

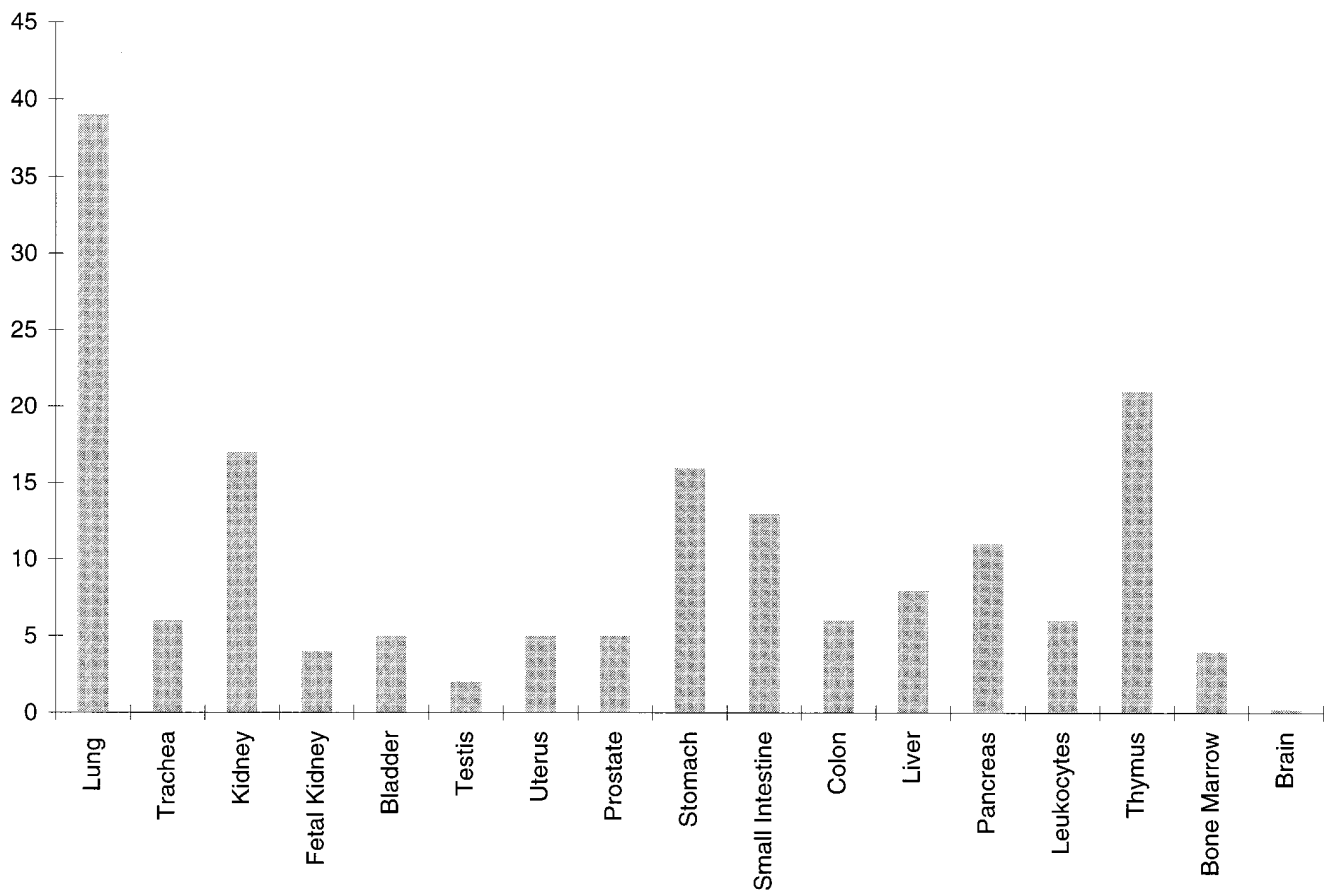


Figure 1. Tissue distribution of hBD-2 expression. RNAs from 50 human tissues were blotted onto a filter that was hybridized to an hBD-2 specific probe. The signal was quantified and normalized to expression of ubiquitin. This figure presents the relative expression of hBD-2 normalized to ubiquitin in all tissues that demonstrated signal above background. An example of a negative tissue is brain. Negative tissues were brain (different parts), heart, and mammary gland. Data are plotted as ratios of phosphoimager signal of hBD-2 over that obtained for ubiquitin multiplied by 10,000 (this normalization factor accounts for the fact that ubiquitin is consistently expressed at levels much higher than hBD-2).

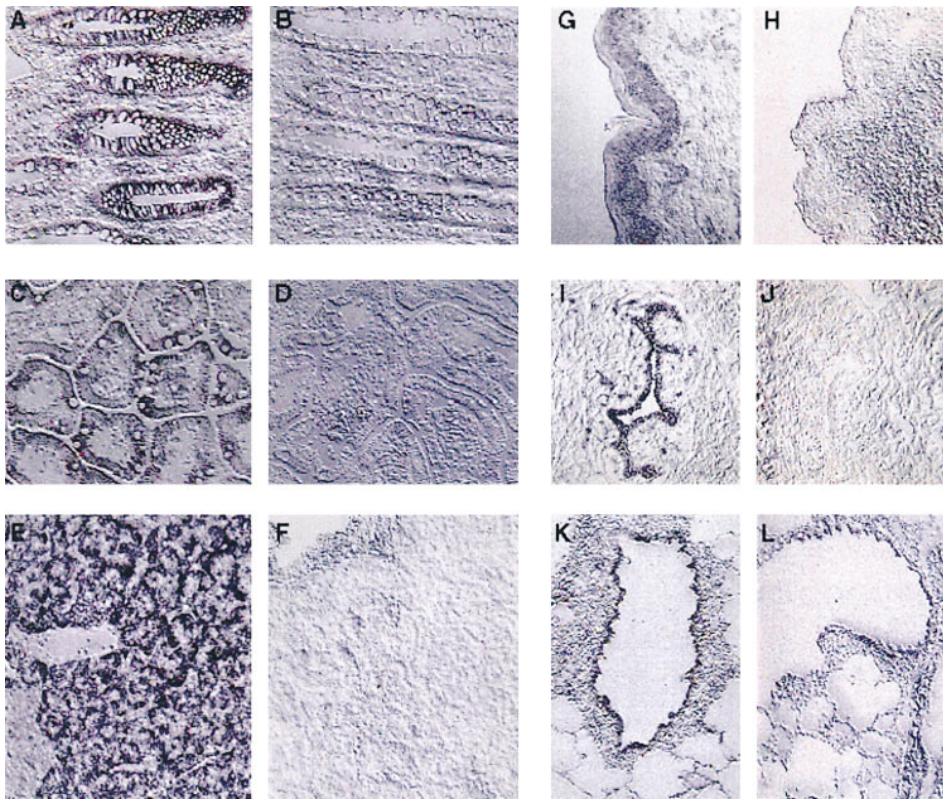


Figure 2. In situ hybridization of hBD-2 in various organs. Surgical specimens or tissues harvested at transplant were frozen in OCT and analyzed by in situ hybridization using alkaline phosphatase conjugated antibodies. Micrographs of tissues hybridized to both the antisense (A, C, E, G, I, and K) and sense probe (B, D, F, H, J, and L) are shown. The following tissues were analyzed: colon (A and B), small intestine (C and D), pancreas (E and F), skin (G and H), breast (I and J), and distal lung (K and L). Magnification $\times 50$.

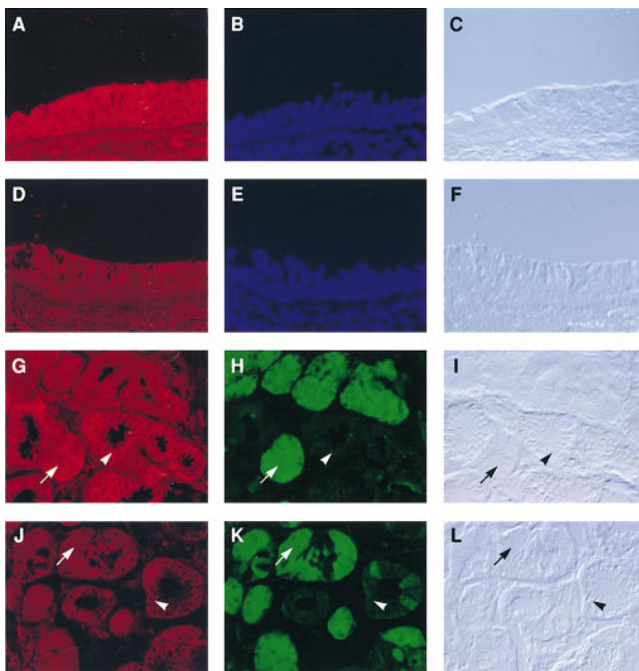


Figure 3. In situ hybridization of hBD-2 in proximal lung. Tissue samples were frozen in OCT and evaluated by fluorescent in situ hybridization using probes to hBD-2 labeled with Texas red. Proximal airway: (A–C) antisense probe; and (D–F) sense probe. Visualization of the hBD-2 probe labeled with Texas red is in A and D; staining of nuclei with DAPI (i.e., blue fluorescence) is in B and E; and Nomarski images are in C and F. Submucosal glands: (G–I) antisense probe; and (J–L) sense probe. Visualization of the hBD-2 probe labeled with Texas red is in G and J; immunohistochemical detection of lysozyme with antibody labeled with FITC is in H and K; and Nomarski images

tial levels of RNA demonstrated in the urogenital system, intestinal tract, and hematopoietic system (leukocytes, thymus, and bone marrow). Selected tissues were analyzed by in situ hybridization with an antisense probe to hBD-2 labeled with digoxigenin and detected with alkaline phosphatase–conjugated antibodies (Fig. 2). hBD-2 RNA was found in epithelial cells throughout the colon (Fig. 2 A) and small intestine (Fig. 2 C); a signal was diffusely present throughout the acinar cells of the pancreas (Fig. 2 E), epithelial cells of the skin (Fig. 2 G), and ducts of the female breast (Fig. 2 I). The specificity of this assay was confirmed in serial sections hybridized with the sense probe (Fig. 2, B, D, F, H, and J) or RNase pretreated sections hybridized with the antisense probe (data not shown). The higher sensitivity of in situ hybridization detected expression of hBD-2 in breast, whereas the signal in the dot blot hybridization was low.

Expression of hBD-2 in proximal human lung was studied by fluorescent in situ hybridization. A diffuse hybridization signal to the antisense probe was demonstrated throughout the superficial epithelia of the conducting airway (Fig. 3, A–C), as well as secretory tubules of the submucosal glands (Fig. 3, G–I). The highest expression of hBD-2 in submucosal glands occurs in the serous tubules as documented by colocalization of the in situ signal with immunohistochemical detection of the serous cell–specific marker lysozyme (Fig. 3, G–I, arrows). Detectable, but less hBD-2 RNA is found in the mucous tubules (Fig. 3, G–I, arrowheads). Specificity of hBD-2 detection in proximal lung was confirmed in sections hybridized to a sense

are in I and L. Arrows indicate serous cells and arrowheads, mucous cells. Magnification $\times 100$.

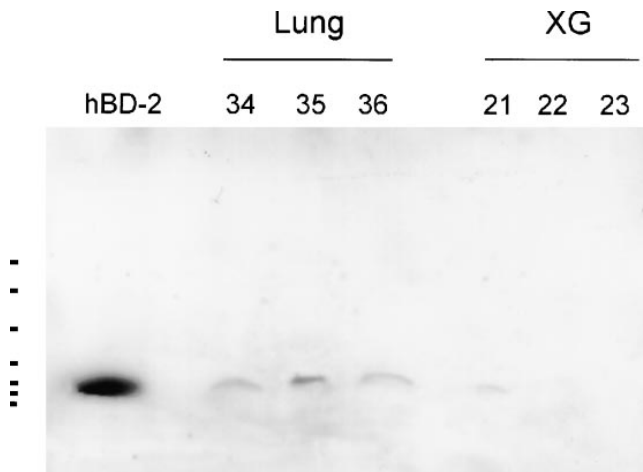


Figure 4. Western blot analysis of HPLC-purified airway surface fluid. Airway surface fluid from human lung and a human bronchial xenograft model was extracted, lyophilized, resuspended in water, and fractionated over an HPLC column. Fractions that showed antimicrobial activity and positive immunoreactivity with the anti-hBD-2 antibody in dot blot analysis were analyzed by Western blot. This figure shows the results of the Western blot for fractions 34–36 from human lung (*Lung*) and 21–23 from a human bronchial xenograft (*XG*). The immunopositive substance from airway surface fluid comigrated with 40 ng of purified hBD-2 (41-amino acid isoform, *hBD-2*). Bars along the left border indicate molecular weight markers of 2.3, 3.4, 6.5, 14.3, 21.5, 30.0, and 46.0 kD.

probe (Fig. 3, *D–F* and *J–L*). Expression of hBD-2 in distal lung was best accomplished using alkaline phosphatase for detection because of high autofluorescence. Lower but detectable levels of hBD-2 are found in most cells of the distal-con-

ducting airway (Fig. 2 *K*); signal was even less in serial sections of lung hybridized to a sense probe (Fig. 2 *L*).

Expression of hBD-2 was also observed in surface epithelial cells of human bronchus grown in an immune deficient mouse (i.e., human bronchial xenograft, data not shown).

Additional studies were performed to determine if hBD-2 peptide is secreted onto the airway surface. Human lungs were derived from potential donor organs that were deemed unsuitable after harvest. Lungs were lavaged and fluid was recovered for partial purification of hBD-2 by organic extraction and reverse phase HPLC. Fractions demonstrating antimicrobial activity were further analyzed by gel electrophoresis and Western blot using the specific antibody generated to recombinant-derived hBD-2 (see next section). Fig. 4 shows the presence of immunoreactive peptide in fractions 34–36 that comigrates with recombinant hBD-2 (41-amino acid isoform). Diminished but detectable hBD-2 was seen in airway surface fluid of human bronchial xenografts (Fig. 4). Only the 41-amino acid isoform of hBD-2 could be detected in airway secretions. The 38-amino acid isoform also secreted from the insect cells (see below) was not found in the expected fractions 23–26.

hBD-2 peptide was isolated for biochemical studies using a baculovirus expression system (15). Sf9 cells were infected with a recombinant baculovirus containing full-length cDNA for hBD-2 called WT hBD-2. The resulting supernatants were subjected to sequential fractionations on cation exchange and reverse phase chromatography. The primary peaks of antimicrobial activity were analyzed by capillary zone electrophoresis, NH₂-terminal Edman degradation, and mass spectroscopy. These studies revealed a homogenous preparation of the mature peptide with a molecular weight of 4,328 spanning the 41-COOH-terminal amino acids of hBD-2 in the HPLC-fractions 34–36 (Fig. 5). This corresponds to the peptide isolated from human psoriatic skin (18). A smaller version of this peptide of

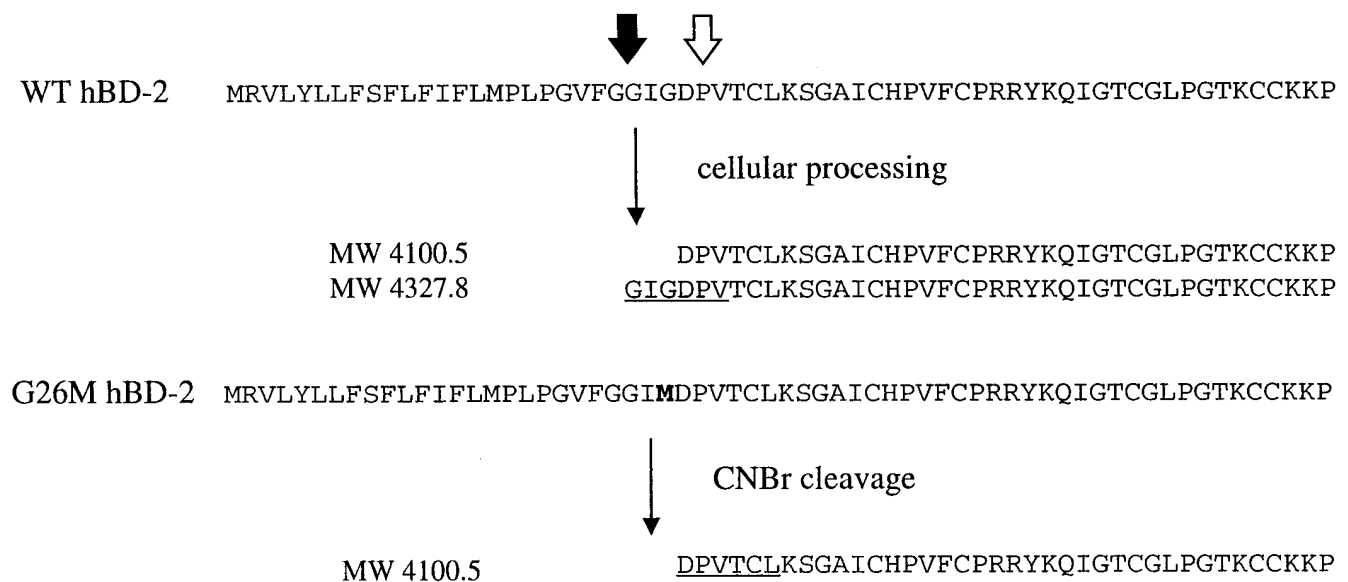


Figure 5. Human β -defensin 2 isolated from the recombinant baculovirus system. Two different constructs were used to generate recombinant baculovirus: WT hBD-2 corresponds to the full-length cDNA of hBD-2, whereas for G26M hBD-2, the glycine-26 was substituted with methionine by PCR-based mutagenesis. For WT hBD-2, two products were secreted into the culture medium, one longer peptide (*filled arrow*, 41 amino acids, predicted MW 4327.8), which was more abundant, and a shorter, less abundant, peptide (*empty arrow*, 38 amino acids, predicted MW 4100.5). The CNBr cleavage of the G26M hBD-2 resulted in a peptide of 38-amino acids length (predicted MW 4100.5). For both versions of hBD-2, the NH₂-terminal amino acid sequences were determined by Edman degradation (*underlined*).

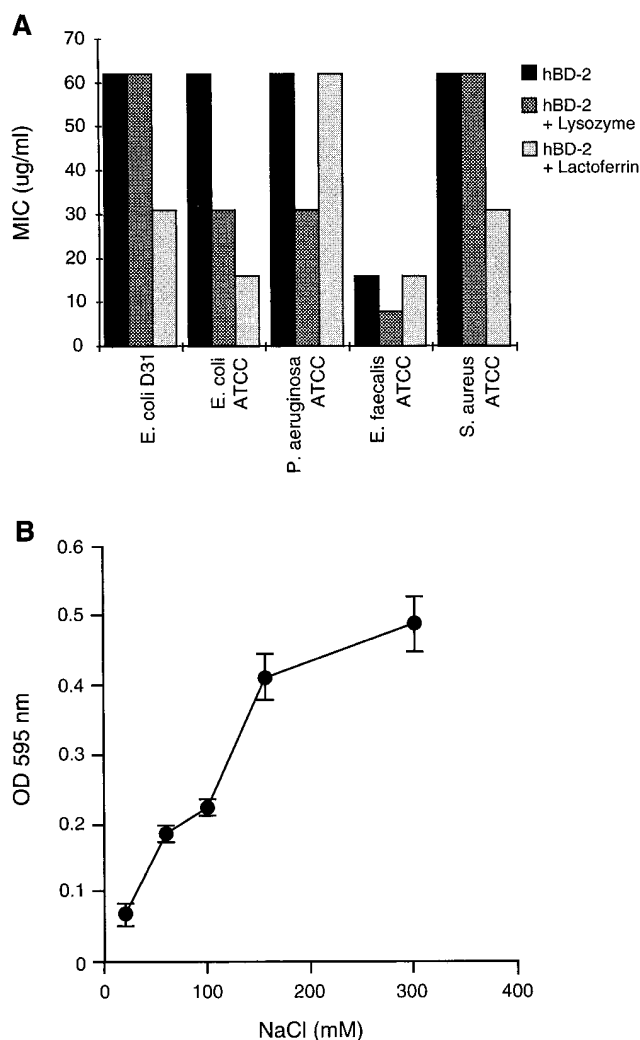


Figure 6. Functional analysis of recombinant hBD-2 peptide. Broth cultures of bacteria were established at low density and various antimicrobial proteins were added. Growth of bacteria under these conditions was studied by measuring the OD of the culture at 595 nm. (A) The MIC for hBD-2 was measured against the indicated bacteria. Analyses were performed in the presence of hBD-2 alone or in combination with non-bacteriostatic concentrations of lysozyme and lactoferrin. (B) The activity of hBD-2 against *E. coli* was measured as a function of sodium chloride concentration.

4,100 molecular weight, missing the three NH₂-terminal amino acids, was identified in fractions 23–26 (Fig. 5). Both isoforms of hBD-2 produced by the recombinant baculovirus system were immunoreactive with the specific antibody. Larger quantities of this 38-amino acid form of hBD-2 were isolated from a separate recombinant baculovirus, which incorporated a methionine immediately before the NH₂-terminal amino acid of the mature peptide (Fig. 5, G26M hBD-2). Cyanogen bromide digestion of a partially purified preparation of G26M hBD-2 released substantial quantities of the 38-amino acid peptide. This smaller version of hBD-2 corresponds to that predicted from the structure of mature TAP and hBD-1 (2, 12). Both the 38- and 41-amino acid versions of these mature peptides demonstrated similar functional characteristics in terms of bacterial killing against *E. coli* D31 (MIC for both WT and G26M

hBD-2 was 62 μg/ml). Subsequent studies were performed with the 41-amino acid mature peptide. The data presented indicated that the structure of hBD-2 is similar to other β-defensins including three disulfide bonds. As in skin (18), the 41-amino acid isoform seems to be the predominant form in the airway surface fluid.

Recombinant derived hBD-2 demonstrated detectable bacterial killing against a number of gram negative and gram positive organisms. The MIC for hBD-2 was 62 μg/ml for *E. coli*, *P. aeruginosa*, and *S. aureus* and 15 μg/ml for *E. faecalis* (Fig. 6 A), which compares favorably to the activity of a Magainin peptide (MIC against *E. coli* D31 15 μg/ml, *E. coli* ATCC 25922 15 μg/ml, *P. aeruginosa* ATCC 27853 8 μg/ml, *E. faecalis* ATCC 29212 15 μg/ml, *S. aureus* ATCC 29213 125 μg/ml), and the 44-amino acid isoform of hBD-1 isolated from urine (MIC against *E. coli* D31 31 μg/ml). Activity of hBD-2 against *E. coli* was sensitive to the concentration of sodium chloride in the assay. The ability of hBD-2 to inhibit growth of *E. coli* diminished eightfold when salt concentration was increased from a 20 mM to 150 mM (Fig. 6 B).

Additional experiments were performed to evaluate potential synergism between hBD-2 and lysozyme and lactoferrin, which are other antimicrobial proteins found in the proximal human airway (20). MICs were measured for hBD-2 in the presence of nonbacteriostatic concentrations of lysozyme and lactoferrin. The MICs for hBD-2 against *E. coli* and *S. aureus* were decreased two- to fourfold in the presence of lactoferrin; synergism was also noted with lysozyme against *E. coli*, *P. aeruginosa*, and *E. faecalis*.

Discussion

We show in this study that hBD-2 is an antibiotic peptide with broad spectrum activity that is expressed throughout the epithelia of many organs and secreted onto the airway surface. The finding of a second epithelial derived β-defensin in humans supports the notion of gene families that contribute to host defense. Localization of the hBD-2 gene immediately adjacent to that of hBD-1 (19) is consistent with gene duplication in the genesis of these loci. The relative contribution each human β-defensin peptide plays in pulmonary host defense is unclear and likely to be complex. We have confirmed synergistic effects of this peptide antibiotic with other antimicrobial molecules such as lysozyme and lactoferrin.

The particular role hBD-2 plays in CF remains speculative. It demonstrates similar sensitivity to salt as was described for hBD-1 (11) suggesting it may be inactivated in the reported high salt environment of the CF airway surface (6, 7). Confirmation of this hypothesis has been complicated by difficulties in accurately measuring the ionic composition of the airway surface fluid, which in some studies appears identical between CF and non-CF (21). It is possible that the CF genetic defect may also lead to a primary abnormality in biogenesis of functional antimicrobial molecules. Nevertheless, the broad epithelial distribution of hBD-2 throughout many organs suggests it plays an important systemic role in innate immunity.

Acknowledgments

We acknowledge the contribution of the Cell Morphology Core of the Institute of Human Gene Therapy, especially Shijun Yang. We appreciate the helpful discussions with Mitchell Goldman.

Robert Bals is a recipient of a fellowship of the Deutsche Forschungsgemeinschaft. This work was supported by the Cystic Fibrosis Foundation and the National Institutes of Health (R01 HL49040-06 and P30 DK47757-04) as well as Genovo, Inc., a biotechnology company that J.M. Wilson founded and has equity in.

References

1. Ganz, T., and R.I. Lehrer. 1994. Defensins. *Immunology*. 6:584–589.
2. Diamond, G., M. Zasloff, H. Eck, M. Brasseur, W.L. Maloy, and C. Bevins. 1991. Tracheal antimicrobial peptide, cysteine-rich peptide from mammalian tracheal mucosa: peptide isolation and cloning of a cDNA. *Proc. Natl. Acad. Sci. USA*. 88:3952–3956.
3. Diamond, G., D.E. Jones, and C.L. Bevins. 1993. Airway epithelial cells are the site of expression of a mammalian antimicrobial peptide gene. *Proc. Natl. Acad. Sci. USA*. 90:4596–4600.
4. Schonwetter, B.S., E.D. Stolzenberg, and M.A. Zasloff. 1995. Epithelial antibiotics induced at sites of inflammation. *Science*. 267:1645–1648.
5. Russell, J.P., G. Diamond, A.P. Tarver, T.F. Scanlin, and C.L. Bevins. 1996. Coordinate induction of two antibiotic genes in tracheal epithelial cells exposed to the inflammatory mediators lipopolysaccharide and tumor necrosis factor α . *Infect. Immunol.* 64:1565–1568.
6. Armstrong, D.S., K. Grimwood, R. Carzino, J.B. Carlin, A. Olinsky, and P.D. Phelan. 1995. Lower respiratory infection and inflammation in infants with newly diagnosed cystic fibrosis. *Br. Med. J.* 310:1571–1572.
7. Armstrong, D.S., K. Grimwood, and J.H.B. Carlin. 1996. Bronchoalveolar lavage or oropharyngeal cultures to identify lower respiratory pathogens in infants with cystic fibrosis. *Pediatr. Pulmonol.* 21:267–275.
8. Smith, J.J., S.M. Travis, E.P. Greenberg, and M.J. Welsh. 1996. Cystic fibrosis airway epithelia fail to kill bacteria because of abnormal airway surface fluid. *Cell*. 85:229–236.
9. Gilljam, H., A. Ellin, and B. Strandvik. 1989. Increased bronchial chloride concentration in cystic fibrosis. *Scand. J. Clin. Lab. Invest.* 49:121–124.
10. Joris, L., I. Dab, and P.M. Quinton. 1993. Elemental composition of human airway surface fluid in healthy and diseased airways. *Am. Rev. Respir. Dis.* 148:1633–1637.
11. Goldman, M.J., G.M. Anderson, E.D. Stolzenberg, U.P. Kari, M. Zasloff, J.M. Wilson. 1997. Human β -defensin-1 is a salt-sensitive antibiotic in lung that is inactivated in cystic fibrosis. *Cell*. 88:553–560.
12. Bensch, K.W., M. Raida, H.-J. Mägert, P. Schulz-Knappe, and W.-G. Forssmann. 1995. hBD-1: a novel β -defensin from human plasma. *FEBS Lett.* 368:331–335.
13. Huttner, K.M., M.E. Selsted, and A.J. Ouellette. 1994. Structure and diversity of the murine cryptdin gene family. *Genomics*. 19:448–453.
14. Jespersen, T., M. Duch, and F.S. Pedersen. 1997. Efficient non-PCR-mediated overlap extension of PCR fragments by exonuclease “end polishing.” *Biotechniques*. 23:48–52.
15. O’Reilly, D.R., L.K. Miller, and V.A. Luckow, editors. 1994. Baculovirus expression vectors. Oxford University Press, New York.
16. Valore, E.V., C.H. Park, A.J. Quayle, K.R. Wiles, P.B. McCray, and T. Ganz. 1998. Human β -defensin-1: an antimicrobial peptide of urogenital tissues. *J. Clin. Invest.* 101:1633–1642.
17. Saiman L., F. Mahar, W.W. Niu, H.C. Neu, K.J. Shaw, G. Miller, and A. Prince. 1996. Antibiotic susceptibility of multiple resistant *Pseudomonas aeruginosa* isolated from patients with cystic fibrosis, including candidates for transplantation. *Clin. Infect. Dis.* 23:532–537.
18. Harder, J., J. Bartels, E. Christophers, and J.-M. Schröder. 1997. A peptide antibiotic from human skin. *Nature*. 387:861.
19. Liu, L., C. Heng, C. Zhao, and T. Ganz. 1996. Human α - and β -defensins evolved from a common mammalian ancestor peptide. *J. Invest. Med.* 44:294A. (Abstr.)
20. Thompson, A.B., T. Bohling, F. Payvandi, and S.I. Rennard. 1990. Lower respiratory tract lactoferrin and lysozyme arise primarily in the airways and are elevated in association with chronic bronchitis. *J. Lab. Clin. Med.* 115:148–158.
21. Knowles, M.R., J.M. Robinson, R.E. Wood, C.A. Pue, W.M. Mentz, G.C. Wager, J.T., Gatzky, and R.C. Boucher. 1997. Ion composition of airway surface liquid of patients with cystic fibrosis as compared with normal and disease-control subjects. *J. Clin. Invest.* 100:2588–2595.