Identification and Characterization of a Mucosal Antimicrobial Peptide Expressed by the Chinchilla (Chinchilla lanigera) Airway*

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Cationic antimicrobial peptides (APs) are produced at mucosal surfaces and play a key role as a first line of defense against infection. To understand how APs might impact disease progression in otitis media (OM), our goal was to identify and characterize APs expressed by the epithelium lining the uppermost airway of the chinchilla, the established rodent host for the study of the bacterial-viral pathogenesis in OM. Using a molecular approach, we cloned a cDNA encoding a homolog of human β-defensin 3, designated chinchilla β-defensin-1 (cBD-1), and found by Northern analysis expression of the corresponding mRNA in nasopharyngeal and tongue mucosae as well as skin. By reverse transcription-PCR, cBD-1 mRNA was also detected in RNA isolated from trachea, lung, and Eustachian tube tissues. The predicted mature form of cBD-1, expressed as a recombinant peptide in *Escherichia coli*, demonstrated bactericidal activity against the three primary opportunistic pathogens of OM as well as *Candida albicans*. Continuation of this and other APs will allow us to determine their role in bacterial colonization of the upper airway as well as how viruses might contribute to the pathogenesis of OM by modulating AP expression.

Otitis media (OM) is the most common pediatric infectious disease occurring at least once in ~83% of children by age 3 (1, 2). The most common sequela of OM is conductive hearing loss accompanied by delays in language, behavioral, and cognitive development. *Streptococcus pneumoniae*, nontypeable *Haemophilus influenzae* (NTHI), and *Moraxella catarrhalis* are responsible for the majority of bacterial OM. These bacteria colonize the nasopharynx (NP) soon after birth and are typically carried asymptomatically, thereby constituting members of the normal nasopharyngeal flora. During an upper respiratory tract infection (most often viral), the defensive functions of the Eustachian tube (ET) are compromised, and the load of bacterial pathogens in the NP increases significantly (3, 4), thus facilitating sequential ascension of the ET and invasion of the middle ear cavity. Retrograde ascension of bacteria into the middle ear is ordinarily prevented, in part, by innate host defenses at this site. These defenses include the mucociliary action of the ET epithelium, which moves debris away from the middle ear cavity and into the oropharynx for swallowing, as well as the activity of several antimicrobial components such as surfactants, complement, preexisting secretory IgA, lysozyme, and lactoferrin (2, 5, 6).

One class of antimicrobial peptides (APs), the β-defensins, are of special interest, since they are implicated in defending the mucosal barrier against colonization and/or invasion by pathogens. β-defensins are small (3–5 kDa) cysteine-rich, highly cationic peptides produced by various epithelia with broad spectrum activity against bacteria, fungi, and some viruses in vitro (7). Of the four human β-defensins (hBD) that have been characterized to date, only expression of hBD-1 and hBD-2 have been investigated in the upper airway. hBD-1 is constitutively expressed in normal and inflamed nasal and middle ear mucosae, whereas hBD-2 is expressed only in inflamed tissues (8, 9). Additionally, inducible hBD-2 expression was demonstrated in both a human middle ear epithelial cell line and in primary nasal epithelial cells in response to proinflammatory cytokines or bacterial components, a common feature of hBD-2 expression in multiple in vitro systems (10). The detection of these defenses in nasal and middle ear mucosae suggests a role for these peptides in maintaining the health of the uppermost airway and thus perhaps preventing OM.

Experimental OM is currently modeled in the chinchilla for several reasons (1, 11). The chinchilla ET is semipatulous, similar to the “floppy” ETs of a human child. The animals possess a large accessible cephalid bulla, thereby permitting serial recovery of middle ear fluids without compromising the integrity of the tympanic membrane. Chinchillas do not develop OM naturally, yet they can be colonized and/or infected with many of the human viral and bacterial pathogens associated with OM (1). In order to dissect the role APs may play during the pathogenesis of OM, we sought to identify and characterize those peptides expressed in the uppermost airway of this chinchilla host. This work describes cloning of a cDNA encoding a chinchilla β-defensin, expression of the transcript in the uppermost airway, and the antimicrobial activity of the defensin in vitro.
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EXPERIMENTAL PROCEDURES

Animals—Healthy adult chinchillas (~500–700 g) were purchased from Rauscher’s Chinchilla Ranch (LaRue, OH) and fed chinchilla chow (Cincinnati Lab Supplies, Cincinnati, OH) and water ad libitum. The animals were free of middle ear disease as evidenced by otoscopy and tympanometry. For recovery of normal airway and other tissues, animals were deeply anesthetized with xylazine (2 mg/kg; Fort Dodge Animal Health, Fort Dodge, IA) and ketamine (10 mg/kg, Phoenix Scientific Inc., St. Joseph, MO), exsanguinated by cardiac puncture, and sacrificed with an overdose of sodium pentobarbital (The Butler Co., Columbus, OH). Tissues of interest were then dissected, snap frozen in liquid nitrogen, and stored at –80°C until needed.

Antimicrobial Assays—For microbial assays, E. coli strain ML35, NTHI strains 86-028NP and 1128, M. catarrhalis strain 1857, Streptococcus pneumoniae (serotype 14), and Candida albicans were cultured to midlog phase. NTHI 86-028NP and 1128 were cultured in brain heart infusion (BHI) broth supplemented with 2 μg of NAD/ml (Sigma) and 2 μg of hemin/ml (Sigma) or on chocolate agar. M. catarrhalis 1857 and S. pneumoniae (serotype 14) were cultured in BHI. C. albicans was grown in YPD broth or on YPD agar. Static cultures of NTHI, S. pneumoniae, and E. coli were incubated in 5% CO2 at 37°C, whereas M. catarrhalis and C. albicans broth cultures were incubated at 37°C in air with shaking (200 rpm). E. coli ML35 is a standard strain used for assay microbial activity, since it expresses a mutated form of lipopolysaccharide that renders it highly sensitive to the action of APs (12). E. coli was grown in LB broth or on LB agar. Various concentrations of recombinant cBD-1were incubated for 1 h at 37°C in 5% CO2 with 1 × 10⁴ microorganisms in 100 μl of 10 mM sodium phosphate buffer containing either 1% sBHI for S. pneumoniae, NTHI, and M. catarrhalis or 1% YPD for C. albicans. Bacteria and yeast were serially diluted and plated onto chocolate agar and YPD agar, respectively, and the colony-forming units of surviving microorganisms per ml was determined following overnight incubation at 37°C in 5% CO2. Data from a minimum of three replicate assays per strain are presented as mean percentage of survival ± S.D. relative to concentration of recombinant cBD-1 (r(cBD-1)).

Cloning of cBD-1 cDNA—Total RNA was isolated from chinchilla tongue and trachea as described (13). Single-stranded cDNA was synthesized from these RNA samples using a modified oligo(dT) primer (Table I; Marathon cDNA synthesis primer, Clontech, Palo Alto, CA), according to the supplier’s recommended modification of published methods (13). The resulting cDNA product was used as a template in PCR (30 cycles, 94°C for 30 s, 50°C for 30 s, and 68°C for 2 min) with 0.2 μM each of oligonucleotide primers, MBD-84s, and the Clontech anchor primer, AP1 (Table I). The sequence of primer MBD-84s was derived from common sequences in mouse β-defensins 3 and 4 and rat β-defensin 2 (13). The DNA products were separated by electrophoresis through a 1.2% agarose gel, purified using the QIAquick gel purification kit (Qiagen) and electroeluted into E. coli ElectroMax DH10B (Invitrogen) using a Bio-Rad gene pulser (25 microfarads, 200 ohms, 2.5 V). Transformants were selected on LB agar containing 50 μg of kanamycin/ml and 40 μg of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal)/ml. Plasmid DNA from white colonies was isolated using the QIAprep miniprep kit (Qiagen). Plasmid inserts were sequenced by dye terminator chemistry using an Applied Biosystems Model 3100 Genetic Analyzer by the DNA Sequencing Core Facility of the Columbus Children’s Research Institute. Resulting sequences were searched against the National Center for Biotechnology Information (NCBI) database using the BLASTN algorithm. A plasmid containing the cBD-1 insert was saved as pCBD1.

Southern Blot Analysis—Chromosomal DNA was isolated from chinchilla liver using the PureGene DNA isolation kit (Genta Systems, Minneapolis, MN). As a source of human DNA, A549 lung carcinoma

| Name       | Primer sequence                                                                 |
|------------|--------------------------------------------------------------------------------|
| Oligo(DT)  | 5′-CTAATACGACTCACTATAGGGCTGAGGCTTGGC-3′VN-3′                                      |
| m84s       | 5′-GCTTCGATCGATGAGCCCATTT-3′                                                     |
| AP1        | 5′-CCATCTGAATACCCGACTCTAGG-3′                                                    |
| EBDA       | 5′-CTATTTCTCTG-3′                                                               |
| JAD1       | 5′-TATTCTTCGTTCGGACATTTTTC-3′                                                   |
| JAD2       | 5′-CGGGAAGCCTTTATTTCTTGTGCAGCCCAG-3′                                             |

RT-PCR—Total RNA was isolated as described above from chinchilla tongue, nasopharyngeal mucosa, trachea, lung, ET mucosa, and skin. Contaminating DNA was degraded using the DNA-free DNase treatment and removal kit (Ambion, Austin, TX). The Advantage RT-for-PCR kit (Clontech) was employed to generate first strand cDNA from total RNA using random hexamers. Two hundred fifty ng of ET mucosa cDNA was used in 10 μl reactions containing 0.2 μM each of primers cBD-A and cBD-B (Table I). A set of reactions conducted without RT served as a control for DNA contamination. The amplified products were analyzed by agarose gel electrophoresis and then transferred to Hybond N+ in 10× SSC using a Bio-Rad vacuum blotter and probed with cBD-1 cDNA that had been directly labeled with horseradish peroxidase using the Enhance Chemiluminescence labeling and detection kit (Amersham Biosciences). The blot was washed with 0.5× SSC and 0.4% SDS (once for 10 min and twice for 5 min, 50°C), 2× SSC, incubated for 1 min in the ECL substrate, and exposed to x-ray film.

Preparation and Purification of Recombinant cBD-1—A 171-bp fragment encoding the predicted mature cBD-1 was amplified by PCR from the pCBD1 clone using Pfu Turbo DNA polymerase (Strategene, La Jolla, CA) and 0.2 μM each of primers JAD1 and JAD2 (Table I). JAD1 encodes an enterokinase cleavage site positioned directly upstream of the first codon in the cBD-1 cDNA. The fragment was gel-purified, digested with Kpn1 and BamHI, and ligated into similarly digested pET30a (Novagen, Madison, WI) for expression of cBD-1. The ligation mixture was desalted and electroeluted into E. coli ElectroMax DH10B as described above. Positive clones were identified by gel electrophoresis following digestion with Kpn1 and BamHI, and the insert in selected clones was verified by sequencing. A plasmid with the correct sequence was saved as pCBD101.

Recombinant cBD-1 was expressed as an N-terminal His-tagged fusion peptide in E. coli BL21(DE3) (Novagen) transformed with the pCBD101 construct. Bacteria were grown in three 1-liter cultures to an A600 ~ 0.5–0.8 and induced with 1 mM isopropyl-1-thio-β-D-galactopyrano
Chemical Instrumentation Center at The Ohio State University (Co-trometry was performed using a Micromass Q-Tof II by the Campus tested for microbicidal activity as described above. Protein sequencing paraffin. Serial sections (10 in PBS (pH 7.4) containing 2% paraformaldehyde and embedded in (Sp6/T7) (Roche Applied Science). Dissected ET was fixed for2ha t4

EcoRI or PstI, served as template for making digoxigenin-labeled sense pSPT18 (Roche Applied Science). The plasmid clone, linearized with overhangs were filled in with Klenow and cloned into SmaI-digested

cBD-1 cDNA was released from pCBD1 with HindIII, and the cDNA and deduced amino acid sequence of cBD-1 and comparison with other β-defensins. A, conceptual translation of the cBD-1 DNA sequence amplified by RT-PCR from chinchilla trachea and tongue RNA. The defensin-specific primer (positions 1–22) and the anchor primer are underlined. The first 13 nucleotides of the cBD-1 coding sequence are derived from the defensin-specific primer. B, multiple sequence alignment of cBD-1, bBD-3, the putative murine β-defensin 14 (mBD-14) (15), and a bovine β-defensin (bBD; accession number BM480498) (16). Identical and similar amino acids are shaded in black and gray, respectively. The arrow indicates the first amino acid in the mature native form of hBD-3 and recombinant cBD-1.

RESULTS

Cloning of the cDNA for cBD-1—The 5' portions of defensin cDNAs that encodes the prepro-region of these peptides share considerable nucleotide identity (13). Defensin-specific primers were thereby designed from a consensus of these invariant sequences comprising the 5'-untranslated and proximal coding regions in mouse β-defensins 3 and 4 and rat β-defensin 2 cDNAs. These primers were used in an anchored PCR to am-

FIG. 1. A, conceptual translation of the cBD-1 DNA sequence amplified by RT-PCR from chinchilla trachea and tongue RNA. The defensin-specific primer (positions 1–22) and the anchor primer are underlined. The first 13 nucleotides of the cBD-1 coding sequence are derived from the defensin-specific primer. B, multiple sequence alignment of cBD-1, bBD-3, the putative murine β-defensin 14 (mBD-14) (15), and a bovine β-defensin (bBD; accession number BM480498) (16). Identical and similar amino acids are shaded in black and gray, respectively. The arrow indicates the first amino acid in the mature native form of hBD-3 and recombinant cBD-1.
pressed sequence tag library (16), respectively (Fig. 1). cDNAs were used as templates in a PCR with the defensin/H925 primer containing a 31-nucleotide extension at its 5’ end. The tongue and reverse transcribed into cDNA using an oligo(dT) 3’-end. The cBD-1 gene was detected only in chinchilla DNA and is present in a single copy.

BLASTX searches of the NCBI databases revealed that the conceptual translation of 52% (29 of 56) of the cDNA clones (Fig. 1A) from chinchilla trachea and tongue shared a sequence that was 77% identical to the untranslated human defensin 14 (15) and 59% 68% identical to the translated sequence of murine β-defensin 1 (16) and a bovine β-defensin identified from an expressed sequence tag library (16), respectively (Fig. 1B). The 12-kb placental band was also present in the uppermost airway tissues and thus was not a different copy. The conceptual translation of the cBD-1 cDNA was 77% identical to the unprocessed hBD-3 peptide (14) (Fig. 1A). As such, the term “chinchilla β-defensin one” (cBD-1) was coined. The conceptual translation of the cBD-1 cDNA was also found to be 59% and 68% identical to the translated sequence of the murine β-defensin 14 (15) and a bovine β-defensin identified from an expressed sequence tag library (16), respectively (Fig. 1B). The 12-kb placental band was also present in the uppermost airway tissues and thus was not a different copy. The conceptual translation of the cBD-1 cDNA was 77% identical to the unprocessed hBD-3 peptide (14) (Fig. 1A). As such, the term “chinchilla β-defensin one” (cBD-1) was coined.

Prior to blotting and probing with 32P-labeled cBD-1 cDNA, the DNA was blotted, probed with horseradish peroxidase-labeled cBD-1 cDNA, and detected by chemiluminescence. The 175-bp product in each sample hybridized to the cBD-1 probe (Fig. 3B). The PCR products from tongue, lung, and skin were directly sequenced independently, confirming that the RT-PCR products were cBD-1 cDNA (data not shown). The expression data indicated that the cBD-1 transcript was produced throughout the airway of the chinchilla, albeit at a much lower level in trachea, lung, and skin. To confirm and extend these observations, RT-PCR was performed using cBD-1-specific primers and DNase-treated total RNA from the same tissues used for Northern analysis. As shown in Fig. 3B, a prominent 175-bp fragment corresponding to the expected size for cBD-1 was amplified from tongue, nasopharyngeal mucosa, lung, and skin as well as from ET RNA. A less intense 175-bp product was also observed in the reaction using tracheal RNA as a template, whereas a faint product of the same size was amplified from lung RNA. To confirm that the RT-PCR products were indeed cBD-1 cDNA, the DNA was blotted, probed with horseradish peroxidase-labeled cBD-1 cDNA, and detected by chemiluminescence. The 175-bp product in each sample hybridized to the cBD-1 probe (Fig. 3B). The PCR products from tongue, lung, and skin were directly sequenced independently, confirming that the RT-PCR products were cBD-1 cDNA (data not shown). The expression data indicated that the cBD-1 transcript was produced throughout the airway of the chinchilla, albeit at a much lower level in trachea, lung, and ET.

In order to localize expression of cBD-1 mRNA to a specific cell type within the mucosae of the uppermost airway, we performed in situ hybridization on tissue sections of the ET. As shown in Fig. 4, cBD-1 mRNA was detected in the ciliated cell type within the mucosae of the uppermost airway.
columnar epithelia in the proximal and middle portions of the ET when sections were hybridized to an antisense cBD-1 RNA probe. No signal was detected when serial sections were exposed to the sense cBD-1 RNA probe.

Purification and Antimicrobial Activity of (r)cBD-1—(r)cBD-1 was expressed in E. coli as a His tag fusion peptide and purified. Lane 1, uninduced control; lane 2, E. coli induced for 2.5 h at 37 °C with 1 mM isopropyl-1-thio-β-D-galactopyranoside. His-tagged cBD-1 is the 10-kDa band. Lane 3, batch purification of His-tagged cBD-1 using Ni²⁺-NTA resin. Lane 4, enterokinase treatment of His-tagged cBD-1 cleaved the His tag from cBD-1. cBD-1 and the His tag are 5 and 4.8 kDa, respectively. Lane 5, cBD-1 after removal of the His tag using the Ni²⁺-NTA resin.

B, reverse phase HPLC fraction containing recombinant cBD-1 after weak cation exchange chromatography demonstrating the homogeneity of the preparation. The preparation was also homogeneous by acid urea (AU)-PAGE (data not shown).

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FIG. 5. Expression and purification of recombinant cBD-1. A, cBD-1 was expressed as an N-terminal His-tagged fusion peptide in E. coli BL21(DE3) and purified. Lane 1, uninduced control; lane 2, BL21(DE3) induced for 2.5 h at 37 °C with 1 mM isopropyl-1-thio-β-D-galactopyranoside. His-tagged cBD-1 is the 10-kDa band. Lane 3, batch purification of His-tagged cBD-1 using Ni²⁺-NTA resin. Lane 4, enterokinase treatment of His-tagged cBD-1 cleaved the His tag from cBD-1. cBD-1 and the His tag are 5 and 4.8 kDa, respectively. Lane 5, cBD-1 after removal of the His tag using the Ni²⁺-NTA resin.

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Antimicrobial peptides are effector molecules of the innate immune system, implicated in controlling infections by directly clearing pathogens, acting as opsonins, and/or recruiting inflammatory cells to the site of infection (7). In order to begin characterizing the role that APs play during colonization and infection by the bacterial pathogens responsible for OM, we have identified a homolog of human β-defensin 3 in airway tissues of the chinchilla, the primary animal host for studying OM.

Taking advantage of the conservation in the 5′ region of β-defensin genes, we used an anchored PCR strategy to clone the cDNA for cBD-1, a chinchilla homolog of hBD-3, from trachea and tongue RNA. The 77% amino acid identity between cBD-1 and hBD-3 was not expected, considering that on average, defensins share 40% amino acid similarity among species (17). This unexpected degree of identity between cBD-1 and hBD-3 suggests evolutionary pressure to produce this particular β-defensin in skin and the upper respiratory tract. The presence of bovine and murine homologs of cBD-1 was present in about half of the clones sequenced, cBD-1 was microbicidal against all test organisms.

(r)cBD-1, respectively. Similarly, C. albicans was also killed when exposed to (r)cBD-1, albeit within a narrow range of concentrations (12.5–20 μg/ml) (Fig. 6B). The concentrations necessary to kill 50% of microorganisms were comparable with that observed for hBD-3, indicating that cBD-1 has broad spectrum antimicrobial activity.

DISCUSSION

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cBD-1 mRNA was abundantly transcribed in chinchilla NP mucosa, skin, and tongue mucosa as compared with ET, trachea, and lung. This result is reminiscent of findings with other defensins in mice, rats, and cows that are expressed at a high level at mucosal surfaces (19–21). The increased expression level at these portals of entry may reflect a primary defense against infection by pathogenic microbes. Alternatively, since the sites of abundant cBD-1 expression are typically those that are populated with normal flora, its expression may be at least partially regulated by the presence of commensal microorganisms. The commensal microflora is thought to contribute to host defense by stimulating the production of APs (22–24). For example, Krisanaprakornkit et al. (22) demonstrated that the majority of normal, uninflamed gingival tissue samples tested expressed hBD-2 mRNA, which may have been, in part, due to the ability of cell wall extracts from the oral commensal Fusobacterium nucleatum, but not the periodontal pathogen Porphyromonas gingivalis, to up-regulate hBD-2 expression in human gingival epithelial cells. Indeed, virulence factors of certain bacteria have been found to block the ability of eucaryotic cells to produce APs (25).

We have successfully expressed cBD-1 as a recombinant peptide in E. coli. There are several examples of expression and purification of defensins in E. coli; however, the majority of these peptides were isolated under native conditions (14, 26–29). Our attempts to perform a similar purification resulted in very poor recovery of (r)cBD-1 due to the formation of inclusion bodies. Consequently, (r)cBD-1 was purified under denaturing conditions. Such conditions, in combination with the reducing environment of the E. coli cytoplasm (30), would not be expected to promote the disulfide bond formation required for defensin antimicrobial activity. Nonetheless, the recombinant peptide was microbicidal against NTHI, S. pneumoniae, M. catarrhalis, and C. albicans in the low micromolar range. Moreover, the experimental mass of (r)cBD-1 was essentially identical to the theoretical mass of the properly folded peptide. These results indicate that (r)cBD-1 is completely oxidized. The formation of the active (r)cBD-1 may have occurred during subsequent processing of His-tagged peptide. Similar findings have been recently demonstrated for a mouse cathelicidin, cryptdin 4, in which active peptide was purified from E. coli under denaturing conditions without a need to oxidize the peptide (31).

Recombinant cBD-1 had broad spectrum activity against Gram-positive and -negative bacteria and yeast. The concentrations needed to kill the 50% of bacteria and yeast ranged from ~1.25 to 14 μg/ml. These concentrations are within the physiological range for antimicrobial peptides including defensins (32–34). For example, hBD-2 can be detected in nasal secretions from healthy donors at concentrations between 0.3 and 4 μg/ml (32). Furthermore, defensins can act synergistically with other APs such as lysozyme and lactoferrin, two of the most abundant APs in mucosal fluids, reducing the effective antimicrobial concentration in vitro (34, 35). Interestingly, the bacteria that cause OM are themselves normal flora of the pediatric nasopharynx. Because these bacteria are constantly exposed to mucosal fluids, the microbes would be expected to have mechanisms of AP resistance. Indeed, for NTHI, cell wall modifications that reduce AP sensitivity have been reported (36). Data from our laboratory also support this notion in that resistance to cBD-1 is mediated, in part, by the NTHI sap locus (sensitivity to antimicrobial peptides), an operon important for survival of enteric bacteria in the presence of APs (25).

2 K. M. Mason, R. S. Munson, Jr., and L. O. Bakaletz, unpublished data.
In conclusion, we have identified cBD-1, a homolog of human β-defensin 3, expressed by chinchilla airway mucosae. Our results indicated that cBD-1 is expressed throughout the airway, particularly in the nasopharyngeal mucosa and the ciliated columnar epithelium of the ET. This broad spectrum and potent antimicrobial activity; therefore, the native peptide may have a role in maintaining the sterility of the middle ear. The identification of the chinchilla AP cBD-1 in this model host will facilitate a better understanding of the role of innate immunity during colonization and subsequent spread of opportunistic pathogens to the middle ear during experimental, and perhaps natural, OM.

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