Isolation and characterization of Human β-Defensin-3, a novel human inducible peptide antibiotic

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Running Title: A novel human β-defensin
Summary

The growing public health problem of infections caused by multiresistant Gram-positive bacteria - in particular *Staphylococcus aureus* (*S. aureus*) - prompted us to screen human epithelia for endogenous *S. aureus*-killing factors. A novel 5-kilodalton, nonhemolytic antimicrobial peptide (human ß-defensin-3, hBD-3) was isolated from human lesional psoriatic scales and cloned from keratinocytes. hBD-3 demonstrated a salt-insensitive broad spectrum of potent antimicrobial activity against many potential pathogenic microbes including multi-resistant *S. aureus* and Vancomycin-resistant *Enterococcus faecium*. Ultrastructural analyses of hBD-3-treated *S. aureus* revealed signs of cell wall perforation. Recombinant hBD-3 (expressed as a His-Tag-fusion protein in *E. coli*) as well as chemically synthesized hBD-3 were indistinguishable from naturally-occurring peptide with respect to their antimicrobial activity and biochemical properties. Investigation of different tissues revealed skin and tonsils to be major hBD-3 mRNA expressing tissues. Molecular cloning and biochemical analyses of antimicrobial peptides in cell culture supernatants revealed keratinocytes and airway epithelial cells as cellular sources of hBD-3. Tumor necrosis factor alpha and contact with bacteria were found to induce hBD-3 mRNA expression. hBD-3 therefore might be important in the innate epithelial defense of infections by various microorganisms seen in skin and lung, such as cystic fibrosis.

Introduction

Epithelia of macroorganisms represent the first barrier against invading microorganisms. However, despite constant exposure to these microbial threats invasive infections and pathological disorders are rather rare and usually locally limited. Previous studies have demonstrated that plants and invertebrates produce a set of antimicrobial proteins, that are highly effective at killing a wide variety of microorganisms.
(1). Although vertebrate epithelia are a rich source of antimicrobial proteins (2), it is a very recent observation, that human epithelia mount an innate chemical defense system by secreting antimicrobial peptides (3).

The small (3-5 kDa), cationic defensins represent an important peptide family among antimicrobial peptides. Two subfamilies, the alpha-defensins and beta-defensins that are distinguished on the basis of the connectivity of their six cysteine residues, and more recently the cyclic theta-defensin from macaque leukocytes (4), have been identified in vertebrates (3).

In humans two α-defensins, HD-5 and HD-6, are produced by epithelial granulocytes of the small intestine (5, 6).

The first β-defensin has been isolated from bovine tongue (7). Subsequently 13 novel β-defensins were purified from bovine neutrophils (8) and the three-dimensional structure including the disulfide array of one of these β-defensins has been determined (9).

The first isolated human β-defensin hBD-1 (human beta-defensin-1) was purified from hemofiltrates (10) and was later found in urine as a Gram-negative bacteria killing antibiotic (11). mRNA of this antimicrobial peptide is constitutively expressed in various epithelia (10-14).

The second human β-defensin, hBD-2, was discovered in extracts of lesional scales from patients suffering from psoriasis, a noninfectious proinflammatory and hyperproliferative skin disease (15, 16). hBD-2 is expressed in inflamed skin and lung and is induced in epithelial cells upon treatment with TNF-α (15, 17), IL-1β (17, 18) and contact with mucoid forms of Pseudomonas aeruginosa bacteria (17).

Both human β-defensins show microbicidal activity predominantly against Gram-negative bacteria like E.coli and P. aeruginosa. However, they demonstrate only low, if any, microbicidal activity against Gram-positive bacteria such as Staphylococcus aureus (3, 15, 19), a bacterium that causes infections ranging from skin abscesses to life-threatening conditions such as endocarditis and toxic shock (20).
Recent investigations revealed that α-defensins also have the ability to attract T cells (21). Very recent investigations indicate that human β-defensins attract immature dendritic cells and memory T cells via its chemokine receptor CCR6 (22) providing a link between innate epithelial defense and adaptive immunity.

Whereas skin infections caused by Gram-negative bacteria are rather rare, *S. aureus* is a major cause for skin- and lung infections, in particular in atopic dermatitis (23). The high abundance of hBD-2 in skin (16) might explain its high resistance against Gram-negative bacterial infection. In contrast, the factors that protect skin from *S. aureus*-infection remain speculative. We therefore hypothesized that human skin produces peptide antibiotics directed against *S. aureus* in addition to the Gram-negative bacteria killing hBD-2.

In the present study, we report the discovery of a novel human epithelial broad spectrum and multi-resistant bacteria killing peptide antibiotic, which we termed human beta-defensin-3 (hBD-3), that is inducibly expressed by various human epithelial cells.

**Experimental Procedures**

**Culture of Epithelial Cells.** Foreskin-derived keratinocytes, airway epithelial cells and the A549 lung epithelial cells were prepared and cultured as described previously (17, 24). Supernatants of the cells stimulated for 48 hrs with 10⁹/ml heat-killed (65°C, 45 min) *Ps. aeruginosa* (clinical isolate) in FCS-free medium (bacteria-to-cell ratio: 200:1) were collected for purification of antimicrobial factors. For stimulation and subsequent RNA isolation primary keratinocytes, tracheal and bronchial cells were cultured in 6-well tissue culture plates (9.6 cm²/well, Falcon). Second passage cells were used at 70-80% confluence. After removal of growth medium and twice washing with PBS cells were cultured in KGM-Medium (Clonetics) lacking bovine pituitary extract (BPE) for 24 h and were subsequently stimulated with
recombinant TNF-α (Pepro Tech Inc.) or heat-killed (65°C, 30 min) bacteria in 2 ml of serum free growth medium.

**Purification and characterization of hBD-3.** Pooled lesional psoriatic scales (7 – 50 grams) or heel callus (80 – 120 grams) were extracted with acidic ethanolic citrate buffer as described previously (25). After diafiltration (Amicon filters, cut off: 3 kDa) of extracts (or the supernatants of cultured epithelial cells) against sodium phosphate buffer (10 mM, pH 7.4), material was applied to a *S. aureus* affinity column, which was prepared using a NHS-activated sepharose column (HiTrap, 5 ml; Pharmacia) and 5 ml of a *S. aureus* (clinical isolate) suspension (10⁹ bacteria/ml) by a procedure similar to that previously described for a *Ps. aeruginosa* affinity column (17). Briefly, extracts or cell culture supernatants were applied to the affinity column that had been previously equilibrated with 10 mM phosphate buffer, pH 7.4, and bound peptides were eluted with 0.1 M glycine buffer, pH 3.0, containing 1M NaCl. After equilibration of the column with 10 mM phosphate buffer, pH 7.4, the effluent was applied to the column and bound material was eluted as described above. This step was performed four times to increase the efficacy of the column to bind peptides. The eluates were collected and diafiltered against 0.1% trifluoroacetic acid (TFA), pH 3, for subsequent RP-HPLC.

*S. aureus* affinity column bound material was then purified by preparative wide-pore RP-8-HPLC column (300 x 7 mm, C8 Nucleosil, 250 x 12.6 mm, Macherey and Nagel) that was previously equilibrated with 0.1 % (v/v) TFA in HPLC grade water containing 20 % (v/v) acetonitrile. Proteins were eluted with a gradient of increasing concentrations of acetonitrile containing 0.1 % (v/v) TFA (flow rate: 2 ml/min). Aliquots (10-30 µl) of each fraction were lyophilized, dissolved in 5 µl 0.1 % (v/v) aqueous acetic acid and tested for antimicrobial activity against *S. aureus* or *E. coli* by a radial diffusion plate assay (26).

Fractions containing antimicrobial activity against *S. aureus* were further purified by cation exchange HPLC followed by RP-18-HPLC as described for purification of hBD-2 (17).
Electrophoretic mobility was investigated using SDS-polyacrylamide gels (SDS-PAGE) in the presence of 8M urea and Tricine (27) under nonreducing conditions as described for chemokines (28). Peptides were visualized by silver staining (27).

Protein sequencing was done using a pulsed liquid phase 776 automated protein sequencer (Perkin Elmer Applied Biosystems). Electrospray mass spectrometry (ESI-MS)-analyses were performed in the positive ionisation mode with a QTOF-II Hybrid-mass spectrometer (Micromass).

**Antimicrobial/hemolytic assay.** Test organisms were incubated with hBD-3 in 100 µl 10 mM sodium phosphate buffer (pH 7.4) containing 1% (v/v) trypticase soy broth (TSB). To investigate the salt sensitivity of hBD-3, 50 µg hBD-3 was incubated with 1 x 10⁵ colony-forming units (CFU) of *S. aureus* (ATCC 6538) in 100 µl 10 mM sodium phosphate buffer (pH 7.4) and NaCl for 3 hours at 37°C. The antibiotic activity of hBD-3 was analyzed by plating serial dilutions of the incubation mixture and determination of the CFU the following day. The limit of detection (1 colony per plate) was equal to 1x10² CFU per milliliter.

For analysis of hemolytic activity up to 500 µg hBD-3 were incubated with 1x10⁹/ml human erythrocytes at 37°C for 3 hrs either in 10 mM sodium phosphate buffer (pH 7.4) containing 0.34 M sucrose or only in phosphate buffered saline. Following incubation samples were centrifuged at 10000 x g for 10 min and hemolysis was determined by measuring the A₄₅₀ of the supernatants using 0.1% Triton X-100 for 100% hemolysis.

**Transmission electron microscopy of bacteria.** Approximately 10⁸ CFU of *S. aureus* cells (ATCC 6538) were treated with hBD-3 (500 µg/ml) in 100 µl sodium phosphate buffer (pH 7.4) containing 1% (v/v) trypticase soy broth for various lengths of time (30 min – 180 min) at 37°C. The bacteria were then centrifuged (5000 x g, 5 min), immersed in cold (4°C) 5% phosphate-buffered glutaraldehyde (pH 7.8) for 2 h, repeatedly rinsed in cold phosphate buffer (pH 7.8) and postfixed in 1% OsO₄ in 0.34 M sucrose for 1 h.
buffer, and postfixed for a further 2h in 4% phosphate-buffered osmic acid. The sample was dehydrated in acetone and finally embedded in Araldit (Araldit Cy212, Sigma), as described previously (29). Bacteria were examined with an EM 910 electron microscope (Zeiss).

**Cloning of hBD-3 cDNA from Airway Epithelial Cells and Keratinocytes.** Total RNA obtained from primary human foreskin-derived keratinocytes and tracheal epithelial cells was reverse transcribed using standard reagents (Gibco-BRL). A 3′-RACE strategy (30) was used to amplify a hBD-3 specific sequence from the cDNA. Two degenerate primers (5′-GGIATHATHAAYACIYTICARAA-3′ and 5′-CCTAARGARGARCARATHGG-3′) were designed based on hBD-3 amino acid sequence data and used as sense primers for 3′-RACE. The amplified products were subcloned and sequenced. Isolation of the full length cDNA was achieved using a 5′-RACE system for rapid amplification of cDNA ends (Gibco-BRL) according to the manufacturer’s protocol.

**Analysis of hBD-3 gene expression.** Real-time RT-PCR analyses were performed in a fluorescence temperature cycler (LightCycler, Roche Molecular Biochemicals) according to the manufacturer’s instructions. This technique continuously monitors the cycle-by-cycle accumulation of fluorescently labeled PCR product. Briefly, total RNA from cultured epithelial cells was isolated using TRIzol reagent (Gibco-BRL) and 2 µg total RNA was reverse transcribed using standard reagents (Gibco-BRL). The cDNA corresponding to 50 ng RNA served as a template in a 20 µl reaction containing 4 mM MgCl2, 0.5 µM of each primer and 1 x LightCycler-FastStart DNA Master SYBR Green I mix (Roche Molecular Biochemicals). Samples were loaded into capillary tubes and incubated in the fluorescence thermocycler (LightCycler) for an initial denaturing at 95°C for 10 min followed by 45 cycles, each cycle consisting of 95°C for 15 s, 60°C for 5 s and 72°C for 10 s. SYBR Green I fluorescence was detected at 86°C at the end of each cycle to monitor the amount of PCR product formed.
during that cycle. At the end of each run melting curve profiles were produced (cooling the sample to 65°C for 15 s and then heating slowly at 0.2°C/s up to 95°C with continuous measurement of fluorescence) to confirm amplification of specific transcripts. The sequences of the hBD-3 specific intron spanning primers were 5’-AGCCTAGCAGCTATGAGGATC-3’ (forward primer) and 5’-CTTCGGCAGCATTTTGCGCCA-3’ (reverse primer). Amplification using these primers resulted in a 206-bp fragment. The sequences of the β-actin primers were 5’-CTCCTTAATGTCACGCAGGATTTC-3’ (forward primer) and 5’-GTGGGGCGCCCAGGCACCA-3’ (reverse primer) and amplification using these primers resulted in a 520-bp fragment. Cycle-to-cycle fluorescence emission readings were monitored and analyzed using LightCycler Software (Roche Molecular Biochemicals). The software first normalizes each sample by detecting the background fluorescence present in the initial cycles. Then, a fluorescence threshold at 5% of full scale was set and the software determines the cycle number at which each sample reached this threshold. This threshold fluorescence cycle number correlates inversely to the log of the initial template concentration. Relative hBD-3 transcript levels were corrected by normalization based on the β-actin transcript levels. The specificity of the amplification products was further verified by subjecting the amplification products to electrophoresis on a 2% agarose gel. The fragments were visualized by ethidium bromide staining and the specificity of hBD-3 encoding PCR products was verified by sequencing.

For determination of hBD-3 mRNA in different tissues total RNA was isolated from human skin, larynx, pharynx, polyp, tonsil and tongue using the TRIzol reagent (Gibco-BRL). All other RNAs were obtained from Clontech (Palo Alto, CA). Real-time RT-PCR was carried out as described above.
Expression of recombinant hBD-3 in *E. coli*

The cDNA encoding the 45 amino acids containing natural form of hBD-3 was cloned into the expression vector pET-30c (Novagen), which contains an N-terminal His-Tag sequence allowing purification of the fusion-protein by the use of a nickel-affinity column.

A 200 ml culture of transformed *E. coli* (strain BL21, Novagen) was grown to an optical density of 0.6 and expression was induced by adding 1 mM IPTG. Expression was carried out for 4 hrs and bacteria were harvested by centrifugation at 6000 x g for 5 min and lysed by sonication. Extracts were purified with a nickel-affinity column (Novagen) according to the manufacturer’s protocol (Novagen, pET System Manual, 7th Ed.). Bound material was digested with Enterokinase (Invitrogen) and the released 45 amino acids containing form of hBD-3 was further purified by micro reversed phase (RP-18) HPLC eluting with a retention time at 25 min identical to that of natural hBD-3. Tricine-SDS-PAGE revealed a single band migrating as natural hBD-3. The identity of recombinant hBD-3 was confirmed by N-terminal amino acid sequencing and by ESI-MS analyses.

Synthetic hBD-3

hBD-3 was chemically synthesized by JERINI BIO TOOLS GMBH, Berlin, Germany, according to the amino acid sequence deduced from the cDNA sequence. The material eluted in a single peak upon RP-HPLC with a retention time at 25 min identical to that of natural hBD-3. ESI-MS analyses revealed a mass of 5154.7 Da.

Results

**Isolation of a novel human peptide antibiotic: hBD-3.** To address the question whether human skin produces *S. aureus* killing proteins, we analyzed lesional scale extracts of patients
with psoriasis and extracts of healthy person’s heel callus for *S. aureus* killing activity. Initial experiments revealed high *S. aureus* killing activity in crude psoriatic scale extracts as well as heel stratum corneum extracts. In order to enrich and purify staphylocidal activity from psoriatic scale extracts, in which more staphylocidal activity was observed than in heel callus extracts, a *S. aureus*-affinity column was used. Protein(s) with microbicidal activity directed against *S. aureus* were found to bind to the column. Bound proteins were then separated by preparative reversed-phase-C₈ high performance liquid chromatography (HPLC) and HPLC-fractions were analyzed for staphylocidal activity (Fig. 1A). The most prominent staphylocidal activity containing HPLC-fraction was further purified using micro cation exchange HPLC. Staphylocidal activity eluted at high salt concentration from this column indicating a highly basic antimicrobial peptide (Fig. 1B, arrow). Final purification of this antibiotic peptide was achieved by reversed phase C₂/C₁₈ HPLC (Fig.1C). Tricine-SDS-urea-polyacrylamide gel electrophoretic analyses revealed a single line migrating like a 9 kDa polypeptide (Fig. 1C, inset). NH₂-terminal amino acid sequence analyses gave the sequence shown in Fig. 1D (EMBL/Genbank database, accession number P 81534), which indicated a new human antimicrobial peptide. Using degenerated primers the complementary DNA (cDNA) was isolated from primary keratinocytes. The cDNA (EMBL/Genbank database, accession number AJ237673) encodes a 67 amino acid precursor and the predicted 45 amino acids containing mature peptide shows similarity to vertebrate epithelial β-defensins, in particular bovine “enteric β-defensin”, EBD (Fig. 2). Since this novel antimicrobial peptide is the third isolated human β-defensin it was termed human β-defensin-3 (hBD-3).

By electrospray mass spectrometry its exact molecular mass was found to be 5154.59 Da, which is 6 Da less than the mass calculated from the deduced hBD-3 amino acid sequence (5161.20 Da), supporting the idea that hBD-3 contains three cysteine bridges and the amino acid sequence shown in Fig. 2.
We were able to isolate 88 µg pure hBD-3 from 7 grams psoriatic scales and 15 µg hBD-3 from 112 grams human skin-derived stratum corneum. The recovery of hBD-3 peptide after three HPLC purification steps was found to be very low. Losses were estimated to be in the range of 80 – 95 % of the quantity originally present.

hBD-3 peptide could be expressed as His-Tag-fusion protein in *E. coli*. Cleavage of the fusion protein, which was found to be weakly active against *E. coli*, with Enterokinase and subsequent RP-HPLC-analysis led to a single peptide giving by ESI-MS a molecular mass of 5154.2 Da, which is exactly the mass calculated for full length hBD-3. Antimicrobial activity against *E. coli* (Fig. 3B) and *Staphylococcus aureus* was found to be equivalent to that seen for natural hBD-3. Synthetic hBD-3 gave a single peak by RP-HPLC at the same retention time as natural hBD-3 and gave a molecular mass of 5154.7 Da upon ESI-MS analyses. Interestingly, synthetic hBD-3 also demonstrated the same antimicrobial activity as natural hBD-3 (Fig. 3B).

**hBD-3 exhibits salt-insensitive broad spectrum antimicrobial activity and no hemolytic activity.** Analyses of the in vitro antimicrobial properties of hBD-3 revealed antimicrobial activity against several potential pathogenic Gram-positive bacteria (*S. aureus, Streptococcus pyogenes*) as well as Gram-negative bacteria (*Pseudomonas aeruginosa, E. coli*) and the yeast *Candida albicans* (Fig. 3A). Furthermore hBD-3 kills multi-resistant *S. aureus* and Vancomycin- resistant *Enterococcus faecium* at similar low concentrations (Fig. 3A). When *S. aureus* was treated at higher cell densities of 8x10⁵/ml we observed slightly higher killing concentrations (hBD-3 concentration necessary to kill 90% bacteria = 4 µg/ml) as we found when 8x10⁴/ml *S. aureus* were used (hBD-3 concentration necessary to kill 90% bacteria = 2.5µg/ml).

*S. aureus* was killed by hBD-3 at low and physiologic salt concentrations (Fig. 3C). Reduced antimicrobial activity was only observed at supraphysiological salt concentrations.
Since several cationic antimicrobial peptides have been reported to exhibit cytotoxic activity against eukaryotic cells, hBD-3 was also assayed for hemolytic activity against human erythrocytes. No significant hemolytic activity (< 0.5 %) was observed using concentrations of hBD-3 up to 500 µg/ml at physiologic salt concentrations. However, significant hemolytic activity was seen at high hBD-3 concentrations in 10 mM sodium phosphate buffer containing 0.34 M sucrose (Fig. 3A).

**Ultrastructure of hBD-3-killed Staphylococcus aureus.** In order to develop an insight into the mechanisms by which *S. aureus* is possibly killed by hBD-3, we examined the morphological changes of *S. aureus* exposed to hBD-3 by transmission electron microscopy. As shown in Fig. 4, *S. aureus* shows signs of perforation of the peripheral cell wall with explosion-like liberation of the plasma membrane within 30 min. After 2 hrs most cells undergo bacteriolysis with different degrees of cellular disintegration.

**Analysis of hBD-3 Gene Expression.** To investigate the tissue distribution of hBD-3 mRNA expression we analyzed mRNA obtained from various body sites by real-time RT-PCR. Low or no hBD-3 mRNA-expression was seen in most of the analyzed organs including the respiratory tract, gastrointestinal tract and genito-urinary tract, whereas strong expression was detected in skin and tonsils (Fig. 5A).

To investigate the cellular origin of hBD-3 we first analyzed cultured primary keratinocytes as well as respiratory tract epithelial cells for hBD-3 mRNA expression. As shown in Fig. 5B, primary keratinocytes express hBD-3 mRNA at a low level. Similarly we found hBD-3 mRNA-expression at a low level in primary tracheal (Fig. 5C), nasal and bronchial airway epithelial cells.

We next assessed whether inflammatory stimuli upregulate the expression of the hBD-3 gene in epithelial cells. TNF-α induced hBD-3 gene expression in primary keratinocytes (Fig. 5B)
as well as in primary tracheal epithelial cells (Fig. 5C) at physiologically relevant concentrations. Also the contact of keratinocytes or primary tracheal epithelial cells with heat-inactivated Gram-negative and Gram-positive bacteria like *Ps. aeruginosa* and *S. aureus*, respectively, induced hBD-3 mRNA (Fig. 5B-C).

**hBD-3 peptide is produced by keratinocytes and lung epithelial cells.**

We then investigated, whether epithelial cells produce hBD-3 peptide. Biochemical analyses of culture supernatants of primary keratinocytes as well as A549 lung epithelial cells previously pretreated with *Ps. aeruginosa* led to the isolation of a peptide antibiotic showing identical biochemical properties including the N-terminal sequence as seen for the skin-derived hBD-3 (data not shown). We were able to purify approximately 10 µg hBD-3 from the supernatants of both, 10⁹ primary keratinocytes and 10⁹ A549 cells indicating that skin keratinocytes as well as epithelial cells of the respiratory tract represent cellular sources for hBD-3.

**Discussion**

It has been previously demonstrated that the epithelia of plants (31), insects (32), amphibians (33), and several mammals (34) are protected from bacterial infection by a chemical defense shield. The recent isolation of the human epithelial peptide antibiotics human beta-defensin-1 (hBD-1) (10) and human beta-defensin-2 (hBD-2) (15) and the demonstration of their expression in major epithelia like skin (14, 16), respiratory tract (17, 18, 35-37), urogenital tract (11) and gut (38) confirms the hypothesis that human epithelia are similarly protected. Although in human secretions such as tears secretory phospholipase A2 may represent one of the most potent Gram-positive bacteria killing factors (39), no systematic analyses have been
performed to elucidate why human healthy skin is protected from *S. aureus* infection. Our previous observation, that hBD-2 is not bactericidal towards *S. aureus* (15, 17) prompted us to investigate human skin extracts for *S. aureus* killing factor(s). These analyses have led to the purification of a novel peptide antibiotic, which we identified as human beta-defensin 3 (hBD-3).

A very recent data bank search indicated that upon sequencing of human chromosome 8 bacterial artificial chromosomes (BACs) the hBD-3 gene was identified 15000 bp distant from the hBD-2 gene (GenBank accesssion AF189745) further supporting the idea that all human ß-defensins are clustered on chromosome 8 (40).

Although originally purified as a *S. aureus* killing peptide antibiotic, our data clearly show, that hBD-3 is a broad-spectrum peptide antibiotic that kills at low micromolar concentrations many other potential pathogenic microbes including *P. aeruginosa*, *Streptococcus pyogenes*, multi-resistant *S. aureus*, Vancomycin-resistant *Enterococcus faecium* and the yeast *Candida albicans*. The human ß-defensins 1 and 2 are less potent peptide antibiotics and predominantly active against Gram-negative bacteria and yeasts (15, 17, 18).

We were able to express a recombinant hBD-3 fusion protein in *E. coli*, which to our surprise could be enzymatically cleaved to generate a fully active recombinant version of hBD-3 with biochemical and biological properties indistinguishable from those of the naturally-occurring hBD-3 peptide. Only a few reports describe the expression of antimicrobial peptides in bacteria (41), a fact which reflects the difficulties of expressing bactericidal peptides in a bacterial host cell. In addition, correct folding is a general problem in proteins with a high number of cysteine-bridges when expressed in bacteria. However, our observation that recombinant as well as chemically synthesized hBD-3 are indistinguishable from natural hBD-3 with respect to their antimicrobial activity and biochemical properties, makes it likely that recombinant and synthetic hBD-3 show the same tertiary structure as natural hBD-3, a hypothesis which remains to be proven.
In order to elucidate how *S. aureus* is possibly killed by hBD-3, we examined morphological changes occurring upon hBD-3-treatment of *S. aureus* by transmission electron microscopy. The morphological effects resemble those seen when *S. aureus* is treated with penicillin, an antibiotic that interferes with the crosslinking of the bacterial peptidoglycan cell wall (42). Therefore, mechanisms by which hBD-3 affects *S. aureus* seem to be completely different from those discussed for neutrophil α-defensins where lamellar mesosome-like structures at the cell membrane level were seen in *S. aureus* (43). Striking electron-dense deposits were present in the periplasmic space affixed to the outer membrane when *E. coli* was investigated (44). It has been suggested that antimicrobial peptides bind and insert into the cytoplasmic membrane due to their cationic and amphiphilic characteristics, where they assemble into multimeric pores (45). However, a very recent investigation indicates, that - at least in the case of the octamer-forming hBD-2 - bactericidal activity could also result from electrostatic charge-based mechanisms of membrane permeabilization, rather than a mechanism based on formation of bilayer-spanning pores (46). It remains to be determined whether hBD-3 kills bacteria by a similar mechanism and how hBD-3 affects cell wall perforation in *S. aureus*.

The identification of hBD-3 in normal stratum corneum and the isolation of hBD-3-peptide from culture supernatants revealed skin keratinocytes as a possible cellular source of hBD-3. Expression of hBD-3 in epithelial cells was further confirmed by the detection of hBD-3 mRNA in primary keratinocytes as well as in primary respiratory epithelial cells, where we also isolated the protein from culture supernatants. Whereas low hBD-3 mRNA expression was found in many normal epithelial tissues including that of the respiratory tract and genito-urinary tract, real-time RT-PCR revealed high levels of hBD-3 mRNA-expression in skin and surprisingly, tonsils. It is interesting to speculate, that possibly microbial stimulation is responsible for these findings.
The isolation of 10- to 30-fold higher amounts of hBD-3 from psoriatic lesions, when compared with normal stratum corneum, indicated that hBD-3 is also inducible by inflammatory stimuli. Like hBD-2 (16, 18) and the epithelial bovine β-defensins LAP and TAP (47) and unlike hBD-1 (48), proinflammatory cytokines such as TNF-α induce hBD-3 in primary epithelial cells at physiologically relevant concentrations. Furthermore the contact of epithelial cells with bacteria induces hBD-3 gene expression, a finding that is known for hBD-2 in keratinocytes (15), airway epithelial cells (17) and intestinal epithelium (38). Thus hBD-3 represents the second member of the human beta-defensin family where expression is regulated by inflammatory stimuli at a transcriptional level.

Several reports indicate that inactivation of antimicrobial peptide activity in patients with cystic fibrosis (CF) may contribute to the recurrent airway infections (49). Elevated salt concentrations in the airway surface fluid of patients with CF, a matter that has been controversially discussed (49), inactivate the antimicrobial activity of human β-defensins (13, 18, 35), possibly by inhibiting the binding of positively charged defensins to negatively charged bacterial surfaces. In contrast to both known human β-defensins (18, 35), our findings indicate that bactericidal activity of hBD-3 is not salt-sensitive at physiologic salt concentrations, which makes this β-defensin of particular relevance in CF.

In summary, the discovery of a novel human epithelial broadspectrum antimicrobial peptide confirms the hypothesis that antimicrobial peptides represent an integral part in the innate immunity of human epithelia (as is found in organisms lacking an adaptive immune system (50)) which complements the adaptive cellular immune system and offers an immediate host response against infectious agents.

Finally, the discovery of this human inducible, epithelial antimicrobial peptide may prove to be a vital advance in dealing with skin and respiratory tract-infection and in the development of novel strategies for antimicrobial therapy, i. e. by artificial stimulation of epithelial peptide antibiotic synthesis – as recently shown with the amino acid 1-Isoleucin (51).
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Footnotes:

The nucleotide and protein sequences reported in this paper have been submitted to the EMBL/GenBank database under accession numbers P81534 and AJ237673.
After we had submitted the protein sequence (P81534) and the cDNA sequence (AJ237673) for hBD-3, sequencing of a chromosome 8 BAC clone (AF189745) revealed the presence of a nucleotide sequence encoding a putative ß-defensin-like protein identical to hBD-3. Thereafter two cDNA sequences were available in the GenBank database, which encode hBD-3 (Accession number AF217245 and AF295370).

**Figure Legends**

**Fig. 1.** Identification and purification of hBD-3. *Staphylococcus aureus*-affinity column-bound proteins of lesional psoriatic scale extracts were separated by RP-8-HPLC (A) and the fraction containing high titer antimicrobial activity (arrow) was purified to homogeneity by micro-cation exchange HPLC (B) followed by analytical C\textsubscript{3}/C\textsubscript{18}-RP-HPLC (C). Tricine-SDS-Urea-PAGE of the resulting peak and silver-staining revealed a single band migrating as a 9 kDa peptide (C, inset). NH\textsubscript{2}-terminal amino acid sequence of 25 residues (single letter code, X = not identified) revealed a novel human antimicrobial peptide (D) (EMBL/Genbank database, accession number P81534).

**Fig. 2.** Peptide sequence of hBD-3. The deduced amino acid sequence (single-letter code) of the native hBD-3 peptide based on the complementary DNA sequence obtained from human keratinocytes and tracheal epithelia cells is shown. For comparison amino acid sequences of the human ß-defensins hBD-1 and hBD-2, bovine epithelial ß-defensins TAP, LAP and EBD, bovine neutrophil ß-defensin BNBD-12 as well as the ß-defensin consensus sequence (including the putative disulfide bridges) are aligned. (The dashes in the ß-defensin sequences represent gaps due to the alignment). The complete cDNA sequence of hBD-3 has been submitted to the EMBL/Genbank database with accession number AJ237673.
**Fig. 3.** Antimicrobial/hemolytic activity of hBD-3. For analysis of antimicrobial activity hBD-3 was incubated for 3 hours at 37\(^{0}\)C in 100 µl 10 mM sodium-phosphate-buffer (pH 7.4) containing 1% trypticase soy broth and the indicated concentrations of hBD-3. To determine the number of colony-forming units (CFU) serial dilutions were plated and colony counts were performed the following day. For analysis of hemolytic activity hBD-3 was incubated at 37\(^{0}\)C for 3 hrs with 1x10\(^{9}\)/ml human erythrocytes in either 10 mM sodium phosphate buffer (pH 7.4) containing 0.34 M sucrose (closed boxes) or only in phosphate buffered saline (opened boxes). Hemolysis was determined by measuring the A\(_{450}\) of the supernatants using 0.1% Triton X-100 for 100% hemolysis.

Panel B shows that natural, recombinant and chemically synthesized hBD-3 exhibit identical antimicrobial activity. All investigations shown in A and C were performed with synthetic hBD-3.

**Fig. 4.** Morphology of hBD-3-treated *Staphylococcus aureus*. Transmission electron micrographs of *Staphylococcus aureus* (10\(^{8}\)/ml) incubated in 10 mM phosphate buffer for 2 hours (A) or treated with synthetic hBD-3 (500 µg/ml) for 30 min (B) or 2 hours (C and D) are shown. Bar = 0.1 µM.

**Fig. 5.** Tissue-expression of hBD-3 mRNA. Low hBD-3 mRNA expression (analyzed by real-time RT-PCR) was detected in many tissues (A). Normal skin and tonsils showed the highest hBD-3 transcript level. (n.d. = not detected). hBD-3 mRNA is expressed in cultivated human primary keratinocytes (B) or primary tracheal epithelial cells (C) and is upregulated by treatment of the cells with heat-inactivated bacteria (10\(^{8}\)/ml) or TNF-\(\alpha\) (10 ng/ml) for 6 hrs. The mucoid clinical isolate of *Pseudomonas aeruginosa* proved to be the strongest inducer of hBD-3. Bars represent the relative hBD-3 transcript levels normalized to ß-actin transcript levels.
Figure 1
Figure 2
Figure 3
Figure 4
Figure 5
Isolation and characterization of Human β-Defensin-3, a novel human inducible peptide antibiotic

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