Published in final edited form as:

Inflamm Bowel Dis. 2013 April ; 19(5): 942–953. doi:10.1097/MIB.0b013e318280b11a.

Human β -Defensin 3 Peptide is Increased and Redistributed in Crohn's Ileitis

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Abstract

Background—Antimicrobial peptides (AMPs) maintain a sterile environment in intestinal crypts, limiting microbial colonization and invasion. Decreased AMP expression is proposed to increase the risk for inflammatory bowel disease. Expression and function of inducible AMPs, human β -defensin 2 and 3 (hBD-3), remain poorly characterized in healthy and chronically inflamed intestine.

Methods—hBD-2 and hBD-3 peptide concentrations in serum and intestinal biopsies of ulcerative colitis, Crohn's Disease (CD), and healthy subjects were measured by ELISA. hBD-2 and hBD-3 mRNA was quantified by qPCR in biopsies from the terminal ileum (TI) of CD patients and healthy controls. hBD-3 peptide localization in the TI was visualized by confocal microscopy.

Results—Immunoreactive hBD-3 peptide is present in the TI and colon in healthy subjects. In the TI of CD patients, hBD-3, but not hBD-2 peptide, is increased four-fold, whereas hBD-2 peptide is elevated in the serum. hBD-3 mRNA in the CD TI remains unchanged and does not correlate with hBD-3 peptide expression. hBD-3 is localized to Paneth cell granules and the apical surface of the healthy columnar epithelium. In CD, hBD-3 peptide location switches to the basolateral surface of the columnar epithelium and is diffusely distributed within the lamina propria.

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Conclusion—hBD-3 peptide throughout the healthy gastrointestinal tract suggests a role in maintaining balance between host defenses and commensal microbiota. Increased and relocalized secretion of hBD-3 toward the lamina propria in the CD TI indicates possible local immunomodulation during chronic inflammation, while increased serum hBD-2 in CD implicates its systemic antimicrobial and immunomodulatory role.

Keywords

beta-defensins; inflammatory bowel disease; Crohn's Disease; immunomodulation; epithelium; innate immunity

Introduction

Inflammatory bowel disease (IBD) is hypothesized to arise from a loss of immune tolerance toward antigens derived from commensal microbiota. While it is widely accepted that tolerance is maintained by a balance between effector and regulatory T cells in the mucosa¹, recent genetic findings in Crohn's disease (CD) have demonstrated the importance of intestinal epithelial cells (IECs).^{1, 2} In addition to acting as a physical barrier, IECs, specifically goblet and Paneth cells, secrete mucins and antimicrobial peptides (AMPs) respectively, which form a semi-impenetrable barrier lining the luminal surface.^{3, 4} Unlike mucins, which are known to maintain intestinal tolerance, the link between AMPs and immune tolerance is not established.^{4, 5} The most widely studied class of intestinal AMPs is alpha (α) defensins, which are small (3-5 kDa) cationic proteins with broad spectrum activity against gram positive and negative bacteria, fungi, viruses and parasites.⁶ Alpha-defensins, specifically human defensin 5 (HD-5) and HD-6, are produced and stored as pro-proteins. Upon secretion, often induced by commensal organisms,⁷ HD-5 and HD-6 are cleaved by Paneth cell trypsin into their mature active form.^{8, 9} Elimination of Paneth cells in mice results in an accumulation of bacteria within the lamina propria (LP) and mesenteric lymph node (MLN), confirming the importance of AMPs in the intestine.¹⁰ Alterations in AMP expression within the intestine lead to overgrowth of commensal bacteria and colonization by more pathogenic organisms.¹¹ These studies implicate intestinal AMPs as important in maintaining a sterile environment proximal to the apical surface of IECs and limiting the density of commensal bacteria along the intestinal mucosa.

Alpha-defensins are distinguished from the β -defensins, by differences in (i) placement of the six cysteine residues along the protein backbone of the respective sub-family molecules, (ii) disulfide linkages, and (iii) cells that express them.⁶ Epithelial cell-derived human β -defensin 3 (hBD-3) is salt insensitive and has multiple positively charged arginine and lysine residues, yielding the highest net-positive charge (+11) of known human AMPs.¹² The high positive charge may contribute to its broad spectrum antimicrobial activity.⁶ Unlike other β -defensins, hBD-3 binds to bacterial products attenuating inflammatory cytokine responses.¹³ HBD-3 expression is induced by various bacterial-derived pathogen associated molecular patterns (PAMPs). Analysis of the hBD-3 promoter reveals STAT1 and AP-1 consensus sequences, implicating interferons (IFN) and growth factors that activate mitogen activated protein kinases as additional regulators of hBD-3 expression.^{14, 15}

The production of hBD-3 by keratinocytes, airway epithelial cells and lung-derived epithelial cells,⁶ combined with the proposed contribution of AMPs in immune tolerance, influenced recent reports of hBD-3 mRNA expression within crypt epithelial cells of both the small and large intestine.¹⁶ In addition, the finding that hBD-3 is elevated during chronic inflammation in patients with psoriasis led to studies measuring hBD-3 mRNA expression levels in IBD.¹⁶ HBD-3 mRNA was reported to be increased in inflamed regions of the ulcerative colitis (UC) colon compared to healthy controls¹⁶ and is unchanged in the CD

terminal ileum (TI) and colon. Yet, steroid treatment of patients with CD ileitis and colitis results in reduced hBD-3 mRNA expression in the TI and colon compared to CD patients not receiving steroid treatment.¹⁷ Despite the lack of comparison of hBD-3 mRNA expression to control patients in this study, these results suggest that hBD-3 expression is also increased in CD. Thus, the actual expression of hBD-3 in IBD is still uncertain. In addition, the well-established over-expression of specific cytokines that contribute to the severity of each form of IBD suggests that hBD-3 expression should be elevated in CD and depressed in UC. For example, mouse and human studies identify IL-13 as the dominant cytokine within the UC mucosa,¹⁸ yet *in vitro* studies indicate that IL-13 inhibits the induction of hBD-3.¹⁴ On the other hand, mouse models of CD as well as human studies report elevated levels of IFN- γ , IL-17, and IL-22 within the CD mucosa,^{1, 19} yet *in vitro* these cytokines are potent inducers of hBD-3 and hBD-2 expression, respectively, in epithelial cells of various origins, including intestinal epithelial cells.^{20, 21} Based on these inconsistencies, in this report we re-examined the expression of both hBD-2 and hBD-3 mRNA and peptide in the mucosa from control, UC, and CD donors, using both immunofluorescence and ELISA. In the TI of CD patients, hBD-3, but not hBD-2, peptide is selectively elevated, whereas the mRNA levels of both defensins remain unchanged. Localization of hBD-3, as determined by confocal microscopy, occurs within Paneth cell granules as well as along the apical surface of columnar epithelial cells in the TI of healthy control subjects, yet the orientation of hBD-3 switches to the basolateral surface in the TI of CD patients, accompanied by accumulation of hBD-3 in the lamina propria. In contrast, hBD-2, but not hBD-3, is selectively elevated in the serum of CD patients. Our results suggest distinct immunomodulatory roles for hBD-3 and hBD-2 in modulating the CD inflammatory processes, due to selective overexpression of each peptide in different locations.

Materials and Methods

Patients and clinical materials

Biopsies and blood were obtained after informed consent from patients undergoing scheduled colonoscopies at University Hospitals Case Medical Center (Cleveland, OH). Protocols were approved by the Institutional Review Board. Two punch biopsies per site were collected from inflamed regions of the terminal ileum (TI), ascending colon (AC), transverse colon (TC), and descending colon (DC) from CD and UC patients. As a control, two punch biopsies were obtained from the same regions of subjects with no family history of IBD. Biopsies were either fixed in 4% formaldehyde or stored at -80°C until use.

HBD ELISAs

Serum was separated by centrifuging blood in SST tubes (Becton Dickinson, Franklin, NJ) and stored at -80°C until use. Frozen biopsies were placed individually into a siliconized microcentrifuge tube (Fisher Scientific, Pittsburgh, PA) containing a sterile, endotoxin-free metallic bead and Triton X-100 lysis buffer (0.5% Triton X-100, 40 mM Tris-HCl pH 7.4, 120 mM NaCl, 0.3% SDS, deionized H_2O , 0.15 U/mL aprotinin, 20 mM leupeptin, 0.5 mM Na_3VO_4 , 2 mM EDTA, 10 mM NaF, 10 mM NaPPi, and 2 mM PMSF; Sigma-Aldrich, St. Louis, MO). The tissue was homogenized using a bead-beater (Retsch, Newtown, PA) for 3 min at a frequency of 30 Hz/s to ensure complete homogenization of all the tissue. The resulting homogenate was centrifuged at 12,000 rpm for 60 min to remove any remaining debris, and the supernatant was transferred to a clean, sterile microcentrifuge tube. Total protein concentration in the cleared extract was measured using a NanoDrop 1000 (ThermoScientific, Wilmington, DE). HBD-3 concentration was initially determined using an optimized two-site ELISA with paired antibodies.²² Briefly, 96-well immunoplates (R&D Systems, City, MN) were coated with 50 μl anti-hBD-3 antibodies (PeproTech,

Rocky Hill, NJ) diluted to 1 µg/ml overnight at 4°C, followed by blocking with 1% bovine serum albumin (BSA) in phosphate buffered saline (PBS). The tissue homogenates and recombinant hBD-3 standard were incubated at room temperature (RT) for 1 h. The wells were washed 3 times with PBS containing 0.1% Tween 20 and incubated at RT for 30 min with 50 µl of the biotinylated secondary antibody (Peprotech) diluted to 0.2 µg/ml. Each plate was washed 3 times and filled with 50 µl/well streptavidin-peroxidase (Roche Diagnostics, Branchburg, NJ; 1:10 000 in PBS containing 0.1% Tween 20). Each plate was incubated at RT for an additional 30 min, washed 3 times as described above, and incubated with 2,2-azino-bis-3-ethylbenzthiazoline-6-sulfonic acid (Roche Diagnostics) in the dark at RT for 20 min. Absorbance was measured at 415 nm with a microplate reader (Model 680, Bio-Rad, Hercules, CA). When available, hBD-2 and hBD-3 concentrations were measured in a commercial ELISA kit (PreproTech) according to the manufacturer's instructions, and absorbance recorded on a VersaMax plate reader (Molecular Devices, Sunnyvale, CA) using a wavelength of 415 nm. As a control HD-5 (Peptides International, Louisville, KY) was analyzed on each ELISA plate to rule out cross-reactivity. Results are reported as amount of hBD-2 or hBD-3 peptide/total amount of protein in the homogenate. IL-22 was measured with a sandwich ELISA (PeproTech), used according to the manufacturer's instructions.

Confocal microscopy

After fixation in 4% formaldehyde, biopsies were embedded in paraffin using a clinical automated processor in the University Hospitals Case Medical Center pathology laboratory. Blocks were serially sectioned 5-7 µm thick and mounted 3 to a slide. Sections were deparaffinized by rendering the slides through a series of 5 min incubations in xylene, 100% ethanol (ETOH), 95% ETOH, and then 70% ETOH (Sigma-Aldrich). Following the removal of paraffin, tissue sections were boiled for 20 min in a citrate buffer solution (DAKO, Carpinteria, CA) in order to optimize antigen retrieval. After cooling, the tissue sections were washed with PBS and then blocked with 10% donkey serum (Jackson ImmunoResearch Lab, West Grove, PA) in a 4°C cold room, overnight. The tissue sections were washed with PBS to ensure removal of excess donkey serum. On any given slide, one tissue section was incubated for 1 h at RT with a rabbit anti-hBD-3 antibody (Novus Biologicals, Littleton, CO) diluted 1:100 in 5% donkey serum, another tissue section was incubated with rabbit IgG isotype (Zymed, Carlsbad, CA) diluted 1:500 in 5% donkey serum, and the remaining tissue section was incubated with 5% donkey serum. The tissue sections were then washed with PBS and incubated for 1 h at RT with a Cy-2-conjugated donkey anti-rabbit secondary Ab (Jackson ImmunoResearch Lab) diluted 1:100 in 5% donkey serum. Following incubation with the secondary Ab, tissue sections were washed with PBS. A 1:250 dilution of the nuclear stain DRAQ-5 in PBS was applied to all tissue sections and incubated in the dark for 3 min. Finally, tissue sections were washed with PBS and imaged using a Zeiss 510 laser scanning microscope available through the Case Comprehensive Cancer Center imaging core.

Immunohistochemistry

To generate frozen tissue blocks, terminal ileal biopsies from healthy control or CD patients were fixed in 4% paraformaldehyde for 30 min and then embedded into OCT freezing media. Biopsies were serially sectioned 5-7 µm thick and mounted 2 to a slide. Frozen sections were incubated in -20°C chilled acetone for 20 min and then boiled for 20 min in citrate buffer solution (DAKO). Sections were blocked with 10% goat serum for 60 min at room temperature, followed by incubation at RT for 60 min with either rabbit anti-hBD-3 antibody (1:1000; Novus Biologicals) or rabbit IgG isotype antibody (1:1000; Invitrogen) diluted in 10% goat serum. Sections were washed in 1× PBS and then incubated for 60 min at RT with a goat anti-rabbit HRP-conjugated Ab diluted 1:2000 in 10% goat serum. Sections were washed in 1× PBS, incubated with 10% diaminobenzidine (DAB) in hydrogen

peroxide (Thermoscientific) for 10 min at RT. Finally, the sections were washed in 1× PBS, mounted (Vectashield, Vector Laboratories, Burlingame, CA), and imaged using an Olympus BX60 microscope.

Gel electrophoresis and immunoblot

To ensure specificity of the primary hBD-3 Ab used for confocal microscopic analysis, 20 ng/well of hBD-3 and HD-5 were fractionated on a 16% Tricine-SDS polyacrylamide gel. Protein was electrotransferred onto a nitrocellulose membrane (Invitrogen, Carlsbad, CA), which was blocked with 5% non-fat dry milk (Nestle, Glendale, CA) in 1× Tris Buffer Solution containing 1% Tween-20 (TBST; Fisher Scientific) at RT for 60 min and then probed with the primary rabbit anti-hBD-3 Ab (diluted 1:1000 into 5% milk in TBST) used for confocal microscopic studies at 4°C under gentle agitation overnight. The membrane was washed with TBST with gentle agitation for 30 min and subsequently probed at RT with an anti-rabbit HRP-conjugated 2° Ab (diluted 1:1000 in TBST) for 60 min. Following incubation with the 2° Ab, the membrane was washed with TBST for 30 min, developed using Supersignal West Pico substrate (ThermoScientific) and chemiluminescence, and detected on BioMax MR film (Kodak, Rochester, NY). In addition, to ensure specificity for correctly folded hBD-3 peptide, prior to adding the primary rabbit anti-hBD-3 Ab to the nitrocellulose membrane described above, the Ab was pre-incubated for 60 min at 4°C with a two fold molar excess of synthetic HD-5 peptide in 5% milk in TBST. The membrane was then probed with the solution containing rabbit anti-hBD-3 primary Ab and synthetic HD-5 peptide overnight at 4°C. The remaining steps were carried out as described above and chemiluminescence was detected on film.

RNA Extraction

Frozen biopsies were placed into a siliconized microcentrifuge tube (Fisher Scientific) containing an endotoxin and RNase free metallic bead and lysis buffer from the PureLink RNA micro kit (Invitrogen) containing 1% beta-mercaptoethanol (Sigma-Aldrich). Biopsies were homogenized for 3 min at 30Hz/s using a bead beater (Retsch). The resultant extract was removed from debris by centrifugation for 2 min at 12,000 rpm. The cleared extract was transferred to a new RNase free microcentrifuge tube (Corning CoStar, Corning, NY) and passed through an 18 gauge needle (Becton Dickinson). 70% ETOH (Sigma-Aldrich) diluted in RNase free water (Invitrogen) was added, and extracts mixed by vortex. RNA was extracted using the PureLink RNA micro kit following manufacturer's protocol, and its concentration quantified with a NanoDrop 1000 (ThermoScientific).

qRT-PCR

To analyze hBD-3, hBD-2, and HD-5 mRNA expression in intestinal biopsies, 1 µg of RNA was incubated with 25 µg/mL Oligo(dT)₁₂₋₁₈ and 10 mM (each) dNTP mix at 65°C for 5 min. Subsequently, 1× first-strand buffer, 0.01 mM DTT, and 40 units/µL RNaseOUT (Invitrogen) were added to the reaction mixture and incubated at 42°C for 2 min. Following this pre-incubation, 200 units of SuperScript II RT (Invitrogen) was added and incubated at 42°C for 50 min. Final extension occurred at 70°C for 10 min. Each qPCR reaction was done in triplicate in a 96 well plate, containing 5 µL 1:3 diluted cDNA in RNase free water (Invitrogen), 1× SYBR green supermix (Bio-Rad), and 500 nM forward and reverse primers (Invitrogen). The forward and reverse hBD-3 primers were *ATCTTCTGTTTGCTTTGCTCTTCCTGTTTT* and *AGCACTTGCCGATCTGTTCCCTCCTT*, respectively. The forward and reverse primers for hBD-2 were *ATCAGCCATGAGGGTCTTGT* and *GAGACCACAGGTGCCAATTT*, respectively. The forward and reverse primers for HD5 were *GCCATCCTTGCTGCCATTC* and *AGATTTACACACCCCGGAGA*, respectively. For normalization purposes, identical triplicate reactions were established on the same 96 well plate with 500 nM forward and

reverse eef1 α primers: *CTTTGGGTCGCTTTGCTGTT* and *CCGTTCTTCCACCACTGATT*, respectively. qPCR was performed on an iCycler (Bio-Rad) using the following reaction conditions: 95°C for 5 min followed by 40 cycles of 95°C for 30 sec and 54°C for 30 sec. Fold change in hBD-3 mRNA was calculated using the $\Delta\Delta C_t$ method, normalizing to eef1 α .

Statistical Analysis

The measures of hBD-2 and hBD-3 peptide and mRNA in tissue or serum were summarized by mean, median (range), standard error of the mean (SEM), and by bar plot. The difference of each measure between two groups of patients with different disease status was examined by T-test after checking normality. For those violating normality, a Kruskal-Wallis test, the nonparametric version of the T-test, was used instead. The difference of hBD-3 peptide among the four locations was examined by analysis of variance (ANOVA) followed by Tukey's pair-wise comparison procedure. The association between two continuous measures was estimated using Pearson correlation coefficient. All tests were two-sided and p-values 0.05 were considered statistically significant.

Results

HBD-3 peptide is expressed in the healthy lower gastrointestinal tract

Multiple inconsistencies exist in the literature regarding the expression profile for hBD-3 mRNA in the healthy and IBD GI tract.^{16, 23, 24} In addition, drawing a parallel with the intestinal epithelial cell-derived α -defensin HD-5, which is packaged in Paneth cell granules before it is secreted,^{7, 25} it is likely that a major discordance between mRNA expression and protein translation or secretion of hBD-3 may exist in the human intestine. Therefore, to clarify this uncertainty, we chose to analyze hBD-3 protein expression in various sections of the small and large intestine of healthy control (HC) subjects. Biopsies were obtained from the terminal ileum (TI), ascending colon (AC), transverse colon (TC), and descending colon (DC) of individuals with no family history of IBD undergoing routine, surveillance colonoscopies. After homogenization, a tissue extract was normalized for total protein content, and then hBD-3 peptide concentrations were determined by ELISA.²² HBD-3 peptide is expressed throughout the entire lower gastrointestinal tract, with maximal expression occurring in the ascending colon, although this elevation did not reach statistical significance in our study population ($p = 0.362$ by Kruskal-Wallis test; Figure 1). The production of hBD-3 peptide in the lower gastrointestinal tract reveals the presence of additional antimicrobial defense mechanisms within the gut, and raises the possibility that hBD-3 may also contribute to either the establishment or maintenance of a balance between host defenses and commensal microbiota.

HBD-3 peptide expression is selectively increased in the Crohn's disease terminal ileum

Crohn's disease is primarily a result of an overt inflammatory response driven by a loss of tolerance to commensal microbiota and their derived antigens.²⁶ Intestinal epithelial-derived AMPs are emerging as important mediators of mucosal homeostasis, limiting interactions of commensal bacteria with the epithelium and underlying immune cells, respectively, thus acting to minimize inflammatory responses.^{10, 27} We therefore propose that changes in AMP expression may alter the homeostatic environment within the intestine, leading to chronic inflammation. Previous reports reveal that mRNA expression of the alpha defensins HD-5 and HD-6 is decreased in the TI of CD patients, and that this decrease is attributed to a frameshift mutation in the NOD2/CARD15 gene encoding a truncated dysfunctional NOD2 receptor.²⁸ However, no reports have yet to link hBD-3 expression with NOD2 activation, and one study shows little to no change in hBD-3 mRNA expression in CD patients.¹⁶ Other reports, however, indicate that a successful anti-inflammatory regimen of corticosteroid

therapy can reduce hBD-3 mRNA in CD patients.¹⁷ Interestingly, in UC patients, hBD-3 mRNA is reported to be increased in the large intestine.¹⁶ In an attempt to clarify these inconsistencies and to focus more specifically on the biological relevance of hBD-3, the peptide was initially measured in intestinal biopsies collected from the terminal ileum (TI), ascending colon (AC), transverse colon (TC), and descending colon (DC) of patients with active CD and UC. Compared to healthy controls, hBD-3 peptide is increased more than 2-fold in the TI of CD patients ($p = 0.08$; $n = 4$ for HC, $n = 14$ for CD, $n = 5$ for UC), whereas no detectable changes in hBD-3 peptide amounts were observed throughout the CD large intestine (Figure 1). In contrast to previous publications that reported hBD-3 mRNA levels, no observable increase in hBD-3 peptide was detected throughout the entire large intestine of UC patients (Figure 1). To further investigate and substantiate our preliminary finding of higher hBD-3 peptide in the TI of CD patients, hBD-3 peptide was measured in additional healthy and CD TI biopsies. In the CD TI, hBD-3 peptide was significantly elevated greater than four-fold compared to healthy controls (Figure 2A; $n = 23$ for HC and $n = 29$ for CD; $p = 0.047$). In contrast, hBD-2 peptide was not increased in the TI of the same patients, ($n = 23$ for HC and $n = 29$ for CD; $p = 0.577$) (Figure 2B). IL-22 expression is reported to be increased in the CD intestine;²¹ thus we measured IL-22 concentrations by ELISA in the biopsy extracts used in Fig. 2A to confirm the presence of active disease. Compared to concentrations in the healthy control TI, we detected a significant increase in IL-22 in the CD TI (Figure 2C; $n = 20$ for HC, $n = 29$ for CD; $p = 0.026$).

HBD-3 and hBD-2 mRNA expression remain unchanged in the CD TI

To address discrepancies between the reported decrease in hBD-3 mRNA expression levels in CD ileitis and the increase in hBD-3 peptide described here, we compared hBD-3 mRNA expression levels in healthy controls and CD TI biopsies. We found no significant changes in hBD-3 mRNA in the CD TI ($n = 13$ for HC and $n = 12$ for CD; $p = 0.221$) by qPCR (Figure 3A). To further analyze differences between β -defensin peptide and mRNA expression in the intestine, we measured hBD-2 mRNA expression and found a slight elevation, although not significant, in the CD TI compared to the healthy control TI (Figure 3B; $n = 12$ for HC and $n = 12$ for CD; $p = 0.068$). As a control, we measured HD-5 mRNA expression in our biopsies and found no significant change between CD TI and healthy control TI (Figure 3C; $n = 12$ for HC and $n = 9$ for CD; $p = 0.694$). These results suggest discordance between hBD-3 mRNA and peptide levels, as well as with previously published reports.

HBD-3 peptide expression correlates with IL-22 protein, but not with hBD-3 mRNA expression in the TI of CD patients

Due to the elevated expression of hBD-3 peptide and IL-22 protein, used as an indicator of active inflammation, in the CD TI, we determined if the expression pattern of both molecules correlated. By Pearson coefficient analysis, elevated levels of IL-22 correlated with elevated levels of hBD-3, but not hBD-2, peptide in the CD TI (Figure 4A; $n = 29$ for IL-22 and hBD-3; $r = 0.76$; $p = 0.007$ and Figure S1A; $n = 29$ for IL-22 and hBD-2 $r = -0.124$ and $p = 0.554$), suggesting the inflammatory response in CD regulates hBD-3 peptide expression in the TI. To relate our findings on β -defensin peptide to earlier reports on β -defensin mRNA, Pearson analysis demonstrates a lack of correlation between hBD-3 and hBD-2 peptide with their respective mRNA expression profiles in both healthy control and CD TI (Figure 4B; $n = 12$ for hBD-3 peptide and mRNA: $r = -0.171$; $p = 0.594$ and S1B; $n = 12$ for hBD-2 peptide and RNA: $r = -0.081$; $p = 0.802$). Furthermore, there is no correlation between hBD-2 and hBD-3 peptide expression in the CD TI (Figure S1C; $n = 29$ for both hBD-2 and hBD-3 peptide; $r = -0.091$; $p = 0.640$). These results indicate that within the healthy control and CD TI, β -defensin peptide expression is discordant with its mRNA expression. This suggests that β -defensin mRNA transcription and translation is tightly regulated and possibly cyclic. Overall, the selective expression of hBD-3 peptide in the CD

TI, possibly driven by an active inflammatory process, suggests distinct roles of hBD-3 and hBD-2 in the healthy control and CD mucosa.

HBD-3 peptide localizes to the luminal surface of the intestinal epithelium and within Paneth cell granules in the healthy small intestine

Antimicrobial agents in the intestine, specifically lysozyme, the α -defensins HD-5 and HD-6, and more recently the Reg family lectin RegIII γ , localize to granules within intestinal crypt Paneth cells.^{2, 10, 25} HBD-3 mRNA has also been reported in intestinal epithelial cells residing near the base of the crypt, as well as those near the surface of the lumen.¹⁶ To confirm these earlier observations we isolated RNA specifically from Paneth cells and columnar epithelial cells by laser capture microscopy. RNA specific for various antimicrobial peptides was reverse transcribed, and then AMP-specific cDNA was pre-amplified prior to detection by qPCR. We were successful in detecting HD-5 in the Paneth cell samples and RegIII γ in the columnar epithelial cells, as previously reported, with a Ct value of 25. HBD-3 and LL37 mRNA were not detected by 40 cycles in either the Paneth cell or columnar epithelial cell samples, which is the limit of sensitivity using this optimized amplification system (data not shown). To place these Ct values in perspective, when we measured HD-5 and hBD-3 mRNA from bulk ileal biopsy tissue (Fig. 3), with yields of RNA greater than 5 μ g / sample, the Ct value for HD-5 was 25, while the Ct value for hBD-3 was >35. Thus the expression ratio between HD-5 and hBD-3 mRNA was maintained using laser capture microdissection, but hBD-3 mRNA expression is below the limits of sensitivity.

In contrast, when we compared the location of hBD-3 mRNA with the sites of peptide accumulation, we identified the expression pattern for hBD-3 peptide in the TI of the small bowel, a location where high amounts of immunoreactive hBD-3 peptide were found. Paraffin embedded TI biopsies from healthy donors were serially sectioned and imaged by confocal microscopy using a rabbit anti-human hBD-3 primary antibody and a fluorescent donkey anti-rabbit secondary antibody (Figure 5A). HBD-3 peptide maps to two distinct locations within the healthy control terminal ileum. Surprisingly, hBD-3 localizes to Paneth cell granules residing at the base of the intestinal crypt (Figure 5A, top panels; long arrows). HBD-3 also colocalizes to the reported site of hBD-3 mRNA expression,¹⁶ as intense fluorescence is observed along the apical surface of columnar epithelial cells in close contact with the lumen (Figure 5A, bottom panels; short arrows). Immunofluorescent staining for hBD-3 in an additional donor is shown in Supplemental Figure S2. To address the possibility of autofluorescence influencing these findings, apical surface localization of hBD-3 in the healthy control TI was confirmed by immunohistochemistry (Figure 5B). These results suggest that hBD-3 contributes, along with other intestinal AMPs, in maintaining sterility within intestinal crypts in the small intestine and reaffirms the importance of Paneth cells in intestinal defense.

To ensure that there is no cross-reactivity between the immunological probes used to detect hBD-3 with other structurally similar AMPs found in the Paneth cell, we undertook studies to determine the specificity of these results. The primary rabbit anti-hBD-3 antibody used for confocal microscopic imaging was tested for cross reactivity against HD-5 via immunoblot. Equal amounts of HD-5 and hBD-3 peptide were fractionated by Tricine-SDS-PAGE, using a 16% acrylamide gel and subsequently transferred onto a nitrocellulose membrane. Primary rabbit anti-hBD-3 antibody was used to probe the membrane. The results showed that the primary antibody used for confocal microscopic studies recognized only denatured hBD-3, and not denatured HD-5 (Figure S3A). Moreover, since epitopes of biologically active peptides within paraffin embedded tissue remain in their native conformation, we also investigated whether non-denatured HD-5 could compete with hBD-3 for binding to this rabbit antibody. Prior to incubation with the membrane, the hBD-3

antibody was pre-incubated for 1 h with a two fold molar excess of HD-5 peptide. Despite incubation with HD-5 peptide, the hBD-3 Ab was still able to exclusively recognize hBD-3 peptide, confirming the specificity of the antibody reagent for hBD-3 peptide (Figure S3B).

HBD-3 peptide redistributes to the basolateral surface of the villus epithelium and accumulates in the lamina propria of the CD terminal ileum

The intestinal epithelium is polarized, where the apical surface samples the luminal contents and secretes a variety of extrinsic factors, such as mucins, prostaglandins, and trefoil proteins that protect the lining of the gut wall,² while the basolateral surface interacts with various immune and mesenchymal cells in the underlying lamina propria. By conducting immunofluorescence confocal microscopic analysis, we were able to discern a difference in the distribution of hBD-3 expression in healthy TI compared to active and inactive CD TI (Figure 6A). As previously described, hBD-3 distribution in healthy TI is expressed along the apical surface of the columnar epithelia (short white arrow; top right panel pair). In contrast, in the CD TI, hBD-3 relocates to the basolateral surface (long white arrow; bottom left panel pair) of the intestinal epithelium in actively inflamed CD tissue, and also accumulates within the lamina propria (pink arrows). Immunofluorescent staining for hBD-3 in an additional CD donor is shown in Supplemental Figure S4. This observation was confirmed by immunohistochemistry (Figure 6B). In an inactive region of the CD TI, hBD-3 remains redistributed to the basolateral surface of the epithelium (long white arrow, bottom right panel pair), but its accumulation in the lamina propria is less pronounced. The redistribution of hBD-3 to the basolateral surface of the intestinal epithelium in both active and inactive CD suggests an active process by which the intestinal epithelium senses a shift in the luminal microbial distribution in CD, and responds by redirecting hBD-3 inward, possibly tracing a breach in the epithelial barrier. Furthermore, the accumulation of hBD-3 within the lamina propria in active CD lesions could lead to “cross-talk” between hBD-3 and immune cells. We and others have shown that this peptide has immunomodulatory activity with cells of the adaptive immune system.²⁹⁻³¹

HBD-2, but not hBD-3 is elevated in the serum of CD patients

Patients afflicted by Crohn's disease exhibit extra-intestinal inflammatory conditions over the course of the disease. Such manifestations include rheumatoid arthritis and uveitis.³² Inflammatory mediators, such as IL-22, IFN- γ and TNF- α are increased in the serum, possibly contributing to extra-intestinal inflammation.^{33, 34} A recent study reported elevated hBD-2 in the serum of systemic lupus erythematosus patients, and this increase correlates with disease activity and the levels of anti-dsDNA antibodies.³⁵ We measured hBD-2 and hBD-3 in the serum of healthy control and CD patients, and detected baseline expression of both peptides in healthy controls, with higher expression of hBD-3 than hBD-2 (Figures 7A and 7B). In contrast to what we report in CD TI tissue, hBD-2 (Figure 7B), but not hBD-3 (Figure 7A) is significantly elevated more than three-fold in the serum of CD patients compared to healthy controls (for hBD-2: $n = 14$ for HC, $n = 12$ for CD, $p = 0.01$ by two-tailed T test; for hBD-3: $n = 12$ for HC and CD, $p = 0.453$ by Kruskal-Wallis test). Although there was no significant elevation of hBD-3 in the serum of CD patients, hBD-2 and hBD-3 peptide levels were found to be significantly correlated in the serum of CD patients (Figure S5A; for hBD-3 and hBD-2 peptide $n = 11$, $r = 0.576$ and $p = 0.05$). As a control for ongoing inflammation, patients with CD showed a greater than 10-fold increase in serum C-reactive protein (CRP) compared to healthy controls (Figure 7C; $n = 11$ for HC and CD, $p < 0.0001$), yet there was no correlation between CRP, hBD-3, and hBD-2 peptide levels in the serum of CD patients (Figure S5B; for hBD-3 and CRP: $n = 11$, $r = -0.171$, $p = 0.615$, Figure S5C; for hBD-2 and CRP: $n = 11$, $r = -0.161$, $p = 0.636$). Constitutive expression of hBD-2 and hBD-3 peptide in the serum of healthy controls suggests a systemic immune homeostatic function, possibly protecting against systemic microbial infection. Elevation of hBD-2 in the

serum of CD patients suggests a potential immunomodulatory role for hBD-2 contributing to extra-intestinal disease manifestations.

Discussion

The intestinal mucosa is populated by immunocompetent cells that fail to mount an inflammatory response in the presence of continual stimulation from commensal microbiota.² This immune tolerance is critical for the proper functioning of the GI tract² and is maintained through a delicate balance between innate and adaptive immune cells.¹ Disruption of intestinal immune tolerance is often associated with inflammatory bowel disease. Among the multiple genes that increase the susceptibility to IBD² are those important for epithelial cell immune function. Epithelial cell-derived AMPs constrain the attachment and invasion of commensal bacteria, limiting their interaction with mucosal immune cells and preventing an uncontrolled inflammatory response.^{10, 26, 27} In the intestine, α -defensins, HD-5 and HD-6, are stored within Paneth cell granules,²⁵ and β -defensins are proposed to be produced by epithelial cells, although characterization of intestinal β -defensin peptide expression has not been reported.⁶ We demonstrate in the healthy intestine that the broad spectrum AMP, hBD-3¹² peptide, but not mRNA, is stored within Paneth cell granules and is localized along the apical surface of columnar epithelial cells, suggesting that hBD-3, along with other AMPs, is secreted into the lumen and functions to keep the crypt sterile, thus contributing to mucosal tolerance. In addition, we demonstrate hBD-3 peptide is present in the terminal ileum and throughout the entire length of the colon. Maximal hBD-3 peptide is found in the ascending colon, possibly due to the exponential increase in bacterial load from the proximal to distal colon.³⁶ High hBD-3 expression in the TI, despite lower bacterial load than the colon, is consistent with increased pattern recognition receptor expression, such as TLR4, which is reported to be higher in the TI compared to the colon.³⁷

Crohn's ileitis is associated with a decrease in HD-5, HD-6, and hBD-2 mRNA expression in genetically susceptible individuals carrying the Leu1007fsinsC mutation in the gene encoding intracellular NOD2.²⁴ Decreased AMP expression is hypothesized to increase the invasion of commensal bacteria into the lamina propria, providing continual antigenic stimulation to the underlying immune cells, thus breaking tolerance. However, flaws remain in this hypothesis. AMP expression is not solely regulated through NOD2, as multiple cytokines, such as TNF- α , IL-1 β , IFN- γ , IL-17, and IL-22, all implicated in Crohn's ileitis pathogenesis, also act to induce AMP expression.^{20, 21} In particular, studies have yet to implicate regulation of hBD-3 by NOD2 engagement, and these AMP peptide levels have not been measured in Crohn's ileitis patients with the Leu1007fsinsC mutation. In contrast to the proposal that Crohn's ileitis is associated with a decrease in AMPs, we report a four-fold increase in hBD-3 peptide in the terminal ileum of ileitis patients, whereas hBD-3 mRNA in this patient cohort remains unchanged. Due to proposed diverse genetic etiologies of CD, future studies are needed to measure hBD-3 peptide levels in CD patients harboring different genetic mutations linked to CD susceptibility. Differences in mRNA expression between studies suggest that hBD-3 mRNA synthesis is cyclic, indicating strict post-transcriptional regulation of hBD-3 production and the possibility that this peptide is stored in differentiated epithelial cells. Disparate expression profiles between hBD-3 peptide and mRNA stresses that fundamental conclusions cannot be based solely on hBD-3 mRNA studies, and that hBD-3 peptide measurements are critical. To confirm the presence of active disease,²¹ IL-22 is elevated in our ileitis biopsies compared to healthy control and correlates with hBD-3, but not hBD-2, peptide expression, suggesting that ongoing inflammation in CD drives the expression of hBD-3. This finding is consistent with studies reporting that steroid treatment reduces hBD-3 mRNA expression in the intestine of CD patients¹⁷ and an increase in hBD-3

mRNA in the actively inflamed TI of pediatric CD patients.³⁸ Thus, hBD-3 appears to be selectively increased at the site of inflammation.

While hBD-3 is located along the apical surface of the epithelium in the healthy control TI, hBD-3 localization in the CD TI redistributes primarily to the basolateral surface of the epithelium and accumulates in the lamina propria only in active disease. These findings suggest that the intestinal epithelium senses a change in the distribution of “danger signals.” We propose that the mucosa of patients with Crohn's disease, whose defective epithelial barrier function facilitates translocation of commensal bacteria,^{39, 40} responds by actively redirecting AMP to restore protection. HBD-3 remaining localized to the basolateral surface in an inactive region of CD may suggest epithelial “memory,” allowing for a quick response if disease activity were to reinitiate, or a permanent change in epithelial cell physiology.

The biologic function of the apical and basolateral surfaces on intestinal epithelial cells is distinct due to differential protein distribution.⁴¹ Key regulators of epithelial cell polarization are Rab proteins, small GTPases that mediate intracellular protein trafficking.⁴² IL-6 induction of Rab5, for instance, redirects proteins to early endocytic compartments, whereas IL-12 induction of Rab7 redirects proteins to lysosomes.⁴³ Thus, cytokines implicated in inflammatory diseases may regulate spatial distribution of immune effector proteins in the epithelium. We propose that CD is not only influenced by the release of specific immune effector molecules, but is also affected by changes in the spatial distribution of these effectors.

Patients with CD exhibit impaired epithelial barrier function,^{40, 44} which results in translocation of bacterial products such as LPS and peptidoglycans into the blood stream.³² In addition to killing microbes, AMPs bind to and neutralize molecules such as LPS and peptidoglycan.¹³ This motivated us to measure hBD-2 and hBD-3 in the serum. In healthy controls, circulating hBD-2 and hBD-3 were detected, with hBD-3 higher than hBD-2. However, in the serum of CD patients, hBD-2, but not hBD-3, is elevated. Although the source of hBD-2 in serum is not known, monocytes⁴⁵ and neutrophils⁴⁶ are identified to express hBD-2 mRNA and peptide. Furthermore, the amino acid isoleucine and bovine serum albumin (BSA) stimulate the secretion of hBD-2 from colonic epithelial cells.⁴⁷ It is possible that serum proteins and nutrient metabolism enhance systemic antimicrobial defenses and establish a first response during infection, limiting the dissemination of microbes.

The detection of hBD-2 and hBD-3 in the healthy intestine suggests that, similar to previously characterized α -defensins, β -defensins also contribute to maintaining mucosal tolerance. The redistribution of hBD3 from the apical to basolateral membrane in CD TI, and its presence in the lamina propria of involved CD, indicate that this peptide responds to a microbial challenge by interacting with resident and infiltrating immune cells. The increase in hBD-3 expression in the TI of ileitis patients suggests that hBD-3 may compensate for reduced levels of the other intestinal AMPs.²⁴ The versatility of hBD-3 as an AMP and immunomodulatory peptide, coupled to its unique expression profile, denotes a dual function for this peptide in maintaining homeostasis at sites of confrontation between microorganisms and host.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank Dr A. Hise for the use of the Bead Beater lysis instrumentation, Ms. Charlotte Chung for sectioning frozen biopsies, Dr. P. Leahy for his analysis of gene expression using Laser Capture Microscopy, and Drs. B. Jiang and R. Jurevic for teaching us the theory and practice behind real time PCR. Dr. Z. Feng provided us with recombinant hBD-3, and Drs. J. Katz, R. Wong, P. Gholam, and A. Chak were instrumental in obtaining intestinal biopsies. This work was supported by grants from the National Institute of Health (2R01 DK-54213 and 1R21 AI-53188 to A.D.L.; R01 DE-018276 and P01 DE-019759 to A.W.), the Cytometry & Imaging Microscopy Core Facility of the Case Comprehensive Cancer Center (P30 CA-43703), the Case Western Reserve University Skin Diseases Research Center (pilot funding from 5P30 AR-039750 to J.P.M. and A.D.L.), the Crohn's and Colitis Foundation of America (to R.M.V.).

Grant Support: This work was supported by grants from the National Institute of Health (2R01 DK-54213 and 1R21 AI-53188 to A.D.L.; R01 DE-018276 and P01 DE-019759 to A.W.), the Cytometry & Imaging Microscopy Core Facility of the Case Comprehensive Cancer Center (P30 CA-43703), the Case Western Reserve University Skin Diseases Research Center (pilot funding from 5P30 AR-039750 to J.P.M. and A.D.L.), the Crohn's and Colitis Foundation of America (to R.M.V.).

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Abbreviations

alpha defensin	α-defensin
AC	ascending colon
AMP	Antimicrobial peptides
beta defensin	β-defensin
BSA	bovine serum albumin
CRP	c-reactive protein
CD	Crohn's disease
DAB	diaminobenzidine
DC	descending colon
ELISA	enzyme linked immunosorbent assay
ETOH	ethanol
HC	healthy controls
hBD	human β-defensin
HD	human defensin
IBD	inflammatory bowel disease

IFN	interferon
IL	interleukin
LP	lamina propria
LPS	lipopolysaccharide
NOD	nucleotide-binding oligomerization domain
PRR	pattern recognition receptor
PAMP	pathogen associated molecular pattern
PBS	phosphate buffered solution
qPCR	quantitative polymerase chain reaction
SDS	sodium dodecyl sulfate
TI	terminal ileum
TC	transcending colon
TBST	tris-buffered solution with tween-20
TNF	tumor necrosis factor
UC	ulcerative colitis

Figure 1. hBD-3 peptide is expressed throughout the lower gastrointestinal tract

Intestinal biopsies, obtained after written informed consent from healthy control (HC), Crohn's disease (CD) and ulcerative colitis (UC) patients, were collected from the terminal ileum (TI), ascending colon (AC), transverse colon (TC) and descending colon (DC) and homogenized in lysis buffer. hBD-3 peptide concentrations were determined by ELISA, and the results were normalized to total protein in the extracts. No significant differences were found between hBD-3 peptide levels in the four locations samples ($n = 4$ for all locations; $p = 0.362$ by Kruskal-Wallis test).

Figure 2. HBD-3, but not hBD-2, peptide is elevated in the terminal ileum of Crohn's disease patients

Intestinal biopsies, obtained from healthy control (HC) and CD patients, were homogenized as described in the legend to Figure 1. **(A)** hBD-3 (n = 23 for healthy control and n = 29 for CD patients; p = 0.047) and **(B)** hBD-2 (n = 23 for healthy control and n = 29 for CD patients; p = 0.577) peptide were measured by ELISA, and the results normalized to total protein. As a measure of ongoing inflammation, **(C)** IL-22 (n = 23 for healthy control and n = 29 for CD patients; p = 0.026) was measured in the extracts by ELISA, and results were similarly normalized. Significance was calculated by a two-sided T-test.

Figure 3. Defensin mRNA remains unchanged in the terminal ileum of Crohn's disease patients
Total RNA was isolated from intestinal biopsies of healthy control (HC) subjects and CD patients using a micro RNA extraction kit (Invitrogen). cDNA, derived from 1 μ g RNA after reverse transcription, was diluted 1:5 and served as a template to measure the fold increase in (A) hBD-3 (n = 13 for HC and 12 for CD; p = 0.221), (B) hBD-2 (n = 12 for HC and n = 12 for CD; p = 0.068), and (C) HD-5 (n = 12 for HC and n = 9 for CD; p = 0.694), as an internal control, mRNA by real time-PCR. Ct values were normalized to the housekeeping gene eef1 α , and fold changes were calculated using the $\Delta\Delta$ Ct method. Changes in mRNA expression in the CD TI did not reach significance for any defensin studied as tested by two-sided T-tests.

Figure 4. HBD-3 peptide correlates with IL-22 secretion in the terminal ileum of CD patients Pearson coefficient analyses were performed to determine the correlation (**A**) between hBD-3 peptide and IL-22 expression levels in the CD TI ($n = 29$; $r = 0.76$; $p = 0.007$), as well as the correlation between (**B**) hBD-3 peptide with corresponding mRNA in the same CD patient biopsies ($n = 12$; $r = -0.171$; $p = 0.594$).

Figure 5. HBD-3 localizes to Paneth cells and the apical surface of columnar cells in the non-IBD terminal ileum

(A) TI intestinal biopsies were collected from healthy control patients after written informed consent and embedded in paraffin. Paraffinized biopsies were serially sectioned and mounted to microscope slides. Sections were deparaffinized, and following rehydration, were boiled in citrate buffer to enhance antigen retrieval. To detect hBD-3 (green), sections were blocked and either incubated with a rabbit anti-hBD-3 antibody or a rabbit IgG isotype control antibody for 1 h at RT. Sections were then incubated with a cy-2 conjugated donkey anti-rabbit antibody followed by DAPI nuclear counter stain (blue). HBD-3 localization was observed using a Zeiss laser-scanning confocal microscope; 488 nm and 640 nm excitation were used to detect hBD-3 and DAPI, respectively. (B) TI biopsies were collected from healthy control patients, fixed in 4% paraformaldehyde, embedded in OCT, frozen and serially sectioned. After blocking, sections were incubated either with a rabbit anti-hBD-3 antibody or a rabbit IgG isotype antibody for 1 h at RT. Tissue sections were then incubated with an HRP-conjugated goat anti-rabbit antibody followed by 10% DAB diluted in hydrogen peroxide. Long white arrows depict hBD-3 localization in Paneth cells and short white arrows depict hBD-3 localization along the apical surface of columnar intestinal epithelial cells. Results are representative of three patients.

Figure 6. HBD-3 localization switches to the basolateral surface of columnar epithelial cells and lamina propria in the CD terminal ileum

(A) TI intestinal biopsies were collected from control subjects, and active and inactive CD patients and embedded in paraffin. Paraffinized biopsies were sectioned and stained for hBD-3 (green) and DAPI (blue), as described in the legend to Fig. 5A. Corresponding serial sections of active and inactive CD tissue were stained with hematoxylin and eosin. (B) TI intestinal biopsies were collected from CD patients with active disease, fixed, embedded, frozen, serially sectioned, and stained, as described in the legend to Figure 5B. Short white arrows show apical localization of hBD-3 in the control TI, whereas long white arrows depict basolateral hBD-3 localization in the TI of CD patients. Diffuse lamina propria staining for hBD-3 is only observed in the active CD samples (pink arrows). Results are representative of three donors for each disease status.

Figure 7. HBD-2, but not hBD-3, peptides are elevated in the serum of CD patients

Serum was collected from control and CD subjects. (A) hBD-3 (n = 12 for healthy control and CD patients; p = 0.453 by Kruskal-Wallis test), (B) hBD-2 (n = 14 for healthy control and n = 12 for CD patients; p = 0.01), and (C) CRP (n=11 for healthy control and n = 11 for CD patients; p<0.0001), as a control inflammatory mediator, levels were measured by ELISA and statistical analyses performed using two-tailed T test.