HUMAN β-DEFENSIN 3 PEPTIDE IS INCREASED AND REDISTRIBUTED IN CROHN’S ILEITIS

by

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Human β-Defensin 3 peptide is increased and redistributed in Crohn’s ileitis

Abstract

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Epithelial cell-derived antimicrobial peptides (AMPs) maintain a sterile environment in intestinal crypts, limiting colonization and invasion of commensal microbiota. Decreases in AMP expression are proposed to increase the risk for onset of inflammatory bowel disease (IBD). The expression and function of the inducible AMP, hBD-3, remain poorly characterized in the healthy and chronically inflamed IBD intestine. We report that immunoreactive hBD-3 peptide is present in the terminal ileum (TI) and entire length of the colon in healthy subjects, and confocal microscopy reveals it is localized to Paneth cell granules and the apical surface of the epithelium. In the TI of CD patients, hBD-3 peptide is selectively increased 3-fold, hBD-3 mRNA is elevated, and the peptide’s location switches to the basolateral surface of the columnar epithelial cell and is diffusely distributed within the lamina propria. These results implicate a critical role for hBD-3 in maintaining mucosal immune tolerance.
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 mucosa [1], 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 somewhat impenetrable barrier lining the luminal surface [3,4]. Unlike mucins, which are well 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 human defensin 6 (HD-6), are first 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), thus 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 allude to a function of intestinal AMPs for maintaining a sterile environment proximal to the apical surface of IECs, limiting the density of commensal bacteria and preventing access of commensal antigens to the underlying resident immune cells.
Alpha-defensins are distinguished from their counterpart, beta-defensins, by a distinct disulfide linkage between six conserved cysteines [6]. Epithelial-derived human beta defensin 3 (hBD-3), one of six isolated human beta-defensins containing the characteristic Cys1-Cys-5, Cys2-Cys-4, Cys3-Cys-6 disulfide linkage, is unique among the beta-defensin family [6,12]. Comprised of a high percentage of the positively charged amino acids arginine and lysine, hBD-3 has the highest net-positive charge (+11) and is salt insensitive [12], compared to other family members. This high positive charge may contribute to its broad spectrum antimicrobial activity [6]. Unlike other beta-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 (MAPK) 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 proposed contribution of AMPs in immune tolerance, led to 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 controls [16], 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 there is also increased expression of hBD-3 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 establish 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 expression by 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-3 mRNA and peptide in the mucosa from control, UC, and CD donors, using both immunofluorescence and ELISA.
MATERIALS AND METHODS

Patients and clinical materials

Biopsies were obtained after informed consent from patients undergoing scheduled colonoscopies at University Hospitals Case Medical Center (UHCMC, Cleveland, OH). Protocols were approved by the Institutional Review Board. Two punch biopsies per site were collected from inflamed regions, as appropriate, 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-3 ELISA

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 H₂O, 0.15 U/mL aprotinin, 20 mM leupeptin, 0.5 mM Na₃VO₄, 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 all tissue is processed. The resulting homogenate was centrifuged at 12,000 rpm for 60 min to remove any remaining debris, and the supernatant transferred to a clean, sterile microcentrifuge tube. Total protein concentration in the cleared extract was measured using a NanoDrop 1000
(ThermoScientific, Wilmington, DE), and defensin concentration was assayed in an optimized two-site hBD-3 ELISA with paired antibodies, using a rabbit anti-hBD3 capture antibody (Ab) (PeproTech, Rocky Hill, NJ), a biotin-conjugated detection Ab (PeproTech) and streptavidin-conjugated HRP secondary Ab (Pierce, Wilmington, DE) [59]. A standard curve was established using serially diluted rhBD-3 (PeproTech). As a control rhBD-2 (PeproTech) and HD-5 (Peptides International, Louisville, KY) were analyzed on each ELISA plate to rule out cross-reactivity. ELISA plates were developed with the fluorogenic reagent ABTS [Roche, Indianapolis, IN], and absorbance measured on a Model 680 microplate reader [Bio-Rad, Hercules, CA], using a wavelength of 455 nm and a subtractive wavelength of 655 nm. Results are reported as amount of hBD-3 peptide/total amount of protein in the homogenate. IL-22 was measured with a sandwich ELISA (PeproTech) and 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 UHCMC pathology lab. 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 was washed with 1X PBS and then blocked with 10% donkey serum (Jackson ImmunoResearch Lab, West Grove, PA) overnight in a 4°C cold room. After the overnight blocking, the tissue was washed with
1X PBS to ensure removal of excess donkey serum. On any given slide, one tissue section was incubated for 1 h at room temperature (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 1X 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 1X PBS. A 1:250 dilution of the nuclear stain DRAQ-5 in 1X PBS was applied to all tissue sections and incubated in the dark for 3 min. Finally, tissue sections were washed with 1X PBS and imaged using a Zeiss 510 laser scanning microscope available through the Case Comprehensive Cancer Center imaging core.

**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 1X Tris Buffer Solution (TBS) containing 1% Tween-20 (TBST; Fisher Scientific) at room temperature for 60 min and then probed overnight at 4°C under gentle agitation with the primary rabbit anti-hBD-3 Ab used for confocal microscopic studies, diluted 1:1000 into 5% milk in 1X TBST. The membrane was washed with 1X TBST for 30 min with gentle
agitation and subsequently probed for 60 min at room temperature with an anti-rabbit HRP-conjugated 2° Ab diluted 1:1000 in 1X TBST. Following incubation with the 2° Ab, the membrane was washed with 1X TBST for 30 min and developed using Supersignal West Pico substrate (ThermoScientific) and chemiluminescence was detected on BioMax MR film (Kodak, Rochester, NY). To ensure specificity for a correctly folded peptide, 20 ng/well and 40 ng/well of hBD-3 and HD-5 were fractionated on a 16% Tricine-SDS polyacrylamide gel and transferred to a nitrocellulose membrane as described above. Prior to incubating the nitrocellulose membrane with the primary rabbit anti-hBD-3 Ab, the Ab was pre-incubated for 60 min at 4°C with a two fold molar excess of synthetic HD-5 peptide (Peptides International) in 5% milk in 1X 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 previously 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 and Company, Franklin Lakes, NJ). 70% ETOH (Sigma-Aldrich) diluted in RNase free water.
(Gibco, Carlsbad, CA) was added, and extracts mixed by vortex. RNA was extracted using the PureLink RNA micro kit following manufacturer’s protocol, and its concentration quantified in a NanoDrop 2000 (ThermoScientific).

**qRT-PCR**

To analyze hBD-3 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, 1X first-strand buffer, 0.01 mM DTT and 40 units/µL RNaseOUT (Invitrogen) were added to the reaction mixture and incubated for 2 min at 42°C. Following this pre-incubation, 200 units of SuperScript II RT (Invitrogen) was added and incubated for 50 min at 42°C. 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 (Gibco), 1X SYBR green supermix (Bio-Rad), and 500 nM forward and reverse hBD-3 primers (Invitrogen). The forward hBD-3 primer was

\[ATCTTCTGTGCTTTGGGCTCTCCTCCTGTGTTT] \]

and the reverse primer used was

\[AGCAGGCGATCTGTTCTCCTCCCT\]. For normalization purposes, triplicate identical reactions were set-up on the same 96 well plate with 500 nM forward and reverse eef1α primers (Invitrogen). The forward primer for eef1α was

\[CTTTGCGCCGCATCTGTTCTCCT\]

and the reverse primer was

\[CCGTTCCTCCACCAGACCTG\]. 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 ΔΔCt method, normalizing to eef1α.
Statistical Analysis

Mean hBD-3 concentration was plotted with standard error of the mean (SEM). Significance of the differences between each patient group was determined by either an ANOVA or two-sided T-test, where appropriate [Excel 2007, Microsoft, Redmond, WA]. P-values less than 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, 22, 23]. 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, 24], 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) 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 first normalized for total protein content, and then hBD-3 peptide concentrations determined by ELISA [25]. HBD-3 protein 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 = 0.129 for
all conditions; n=4, 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 probability that hBD-3 may also contribute to either the establishment or maintenance of mucosal immune tolerance.

![Bar chart showing hBD3 concentrations in protein extracts of homogenized biopsies obtained from the terminal ileum (TI), ascending colon (AC), transverse colon (TC) and descending colon (DC) from healthy control (HC) volunteers. The signal was developed with ABTS, and absorbance recorded at 490 nm and a subtractive wavelength of 655 nm. All conditions are not significant; p = 0.129 n=4, as calculated with the ANOVA test.]

**Figure 1.** Expression pattern for hBD-3 peptide in the healthy lower gastrointestinal tract. HBD-3 concentrations in protein extracts of homogenized biopsies obtained from the terminal ileum (TI), ascending colon (AC), transverse colon (TC) and descending colon (DC) from healthy control (HC) volunteers were measured by ELISA. The signal was developed with ABTS, and absorbance recorded at 490 nm and a subtractive wavelength of 655 nm. All conditions are not significant; p = 0.129 n=4, as calculated with the ANOVA test.

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

Antimicrobial peptides in the intestine, specifically the α-defensins HD-5 and HD-6 and more recently the Reg family protein RegIIIγ, localize to granules within Paneth cells [10,24]. Consistent with these observations, hBD-3 mRNA has also been reported within IECs residing near the base of the crypt, as well as those near the surface of the lumen.
To contrast the location of hBD-3 mRNA with the sites of peptide accumulation, we identified the expression pattern for hBD-3 protein in the terminal ileum (TI) of the small bowel, a location where high amounts of immunoreactive hBD-3 peptide were found. TI biopsies from healthy donors embedded in paraffin were serially sectioned, mounted onto microscope slides, and labeled with a rabbit anti-human hBD-3 primary antibody coupled to a fluorescent donkey anti-rabbit Cy-2-conjugated secondary antibody. Images were obtained on a confocal microscope. HBD-3 peptide is seen to map to two distinct locations within the healthy terminal ileum. Comparable to the localization of HD-5 and HD-6 [24], hBD-3 protein appears to be stored in Paneth cell granules residing at the base of the intestinal crypt (Figure 2, top panels; long arrows). In examining the villus, hBD-3 protein also seems to colocalize with 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 2, bottom panels; short arrows). These results suggest that hBD-3 contributes to maintaining sterility within intestinal crypts in the small intestine and confirms the importance of Paneth cells in intestinal defense.
Figure 2. Immunofluorescent hBD-3 peptide is detected in granules within the Paneth cells of the terminal ileum. Paraffin embedded terminal ileal biopsies from healthy control volunteers were sectioned, stained with isotype-matched control antibody (data not shown) or rabbit anti-hBD3 Ab, and detected with Cy2-labeled donkey anti-rabbit antibody. Nuclei were identified with DAPI counterstain in the right panels. Images were recorded by confocal microscopy using a 20x objective. **Upper Panels.** HBD-3 localization within Paneth cell granules (long arrows). **Lower Panels.** HBD-3 localization along the apical surface of the villus epithelium (short arrows).

The presence of multiple AMPs and additional immunoregulatory proteins in the storage granules of Paneth cells raises an extra note of caution. To ensure that there is no cross-reactivity between the immunological probes used to detect hBD-3 with other structurally or functionally 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 protein 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 this membrane. The results show that the primary antibody used for confocal microscopic studies only recognizes denatured hBD-3, and not denatured HD-5 (Figure 3A). Recognizing that epitopes of biologically active peptides within paraffin embedded tissue may remain in their native conformation, we also investigated whether properly folded, biologically active 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 protein, the hBD-3 Ab was still able to exclusively recognize hBD-3 protein, confirming the specificity of the antibody reagent for hBD-3 protein (Figure 3B).

Figure 3. HBD-3 antibody is not cross-reactive with denatured or native HD-5. A. HBD-3 and HD-5 (both at 20 ng/well) were fractionated by gel electrophoresis on a 16% Tricine-SDS-polyacrylamide gel, transferred to a nitrocellulose membrane, and probed with a rabbit anti-hBD-3 Ab, followed by incubation with a goat anti-rabbit-HRP Ab. Signals were developed using Supersignal West-Pico and chemiluminescence was recorded on film. B. HBD-3 and HD-5, at 20 and 40 ng/well, were fractionated as described in panel A, and the resulting membrane was probed with rabbit anti-hBD-3 Ab, after the antibody had been pre-incubated for 60 min at 4°C with a two-fold molar excess of soluble HD-5. The chemiluminescence was recorded on film.
**HBD-3 peptide and mRNA expression are 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 antimicrobial peptides are emerging as important mediators of mucosal homeostasis, limiting interactions of commensal bacteria and antigens with the epithelial and underlying immune cells, respectively, thus acting to prevent 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 demonstrate that hBD-3 mRNA expression is decreased in the TI of CD patients, attributed to a frameshift mutation in the NOD2/CARD15 gene encoding a truncated dysfunctional NOD2 receptor [28], while other reports indicate that a successful anti-inflammatory regimen of corticosteroid therapy can reduce hBD-3 mRNA in CD patients [17]. Yet 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 biologically relevant hBD-3 peptide itself, hBD-3 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 (HC), hBD-3 peptide is selectively increased greater than 2-fold in the TI of CD patients (p = 0.08; n=4 (HC), n=11 (CD), n=5 (UC)), whereas no detectable changes in hBD-3 peptide amounts were observed throughout the large intestine (Figure 4A). In contrast to previous publications, no observable increase in hBD-3 peptide was detected throughout the entire large intestine of UC patients (Figure
To further investigate and substantiate elevated hBD-3 peptide in the TI of CD patients, hBD-3 peptide was measured in additional HC and CD TI biopsies. In additional clinical samples, elevated hBD-3 peptide is still observed in the CD TI and is increased greater than 3-fold compared to HC subjects (Figure 4B; p = 0.059; n=9 (HC) and n=16(CD)). IL-22 expression is reported to be increased within the CD intestine [20]. Thus, we measured IL-22 concentrations by ELISA in the biopsy protein extracts used in Fig. 4A to confirm the presence of active disease. Compared to IL-22 concentrations in the healthy control terminal ileum, we detect an increase in IL-22 in the CD TI (Figure 4C; p = 0.08; n=4 (HC), n=11 (CD)). 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 HC TI and CD TI biopsies. We found a 2-fold increase in hBD-3 mRNA in the CD TI (n=2 (HC) and 4 (CD)) by qPCR (Figure 4D). These results indicate that the inflammatory response in CD, rather than that in UC, induces hBD-3 protein expression, and may suggest discordance between hBD-3 mRNA expression and protein production. Furthermore, the selective over-expression of hBD-3 in CD possibly implicates additional roles for hBD-3 in regulating the inflammatory response.
**Figure 4.** HBD-3 peptide expression is selectively increased in the terminal ileum of CD patients. A. HBD-3 concentrations in tissue extracts of homogenized biopsies obtained from the terminal ileum (TI), ascending colon (AC), transverse colon (TC) and descending colon (DC) from healthy control subjects (HC), Crohn’s Disease patients (CD) and Ulcerative Colitis patients (UC) were quantified by ELISA. The signal was developed with ABTS, and absorbance recorded at 490 nm and a subtractive wavelength of 655 nm (*, p = 0.08, using a two-sided T-test; HC: n=4; CD: n=14; UC: n=5). B. HBD-3 concentrations were measured in additional tissue extracts of homogenized biopsies taken from the TI of HC volunteers and CD patients as described in panel A (p = 0.059, using a two-sided T-test; HC: n=9; CD: n=19). C. IL-22 concentrations were measured in extracts from panel A and quantified by ELISA (p = 0.08 using a two-sided T-test; HC: n=4; CD: n=14). D. HBD-3 mRNA was measured in tissue extracts of homogenized biopsies obtained from the TI of HC volunteers and CD patients by qPCR. Transcripts were normalized to eef1α and fold induction was calculated using the ∆∆Ct method.

**HBD-3 protein 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; the apical surface samples the luminal contents and secretes a variety of extrinsic factors, such as mucus, 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. To assign a functional role for hBD-3 in the healthy and inflamed intestine, the location of hBD-3 protein in the terminal ileum of the CD patient was analyzed by confocal microscopy (Figure 5). As described earlier in the non-IBD, healthy control terminal ileum, hBD-3 peptide (shown in green) distribution is limited to the apical surface of the columnar epithelia (red arrow; top right panel pair). Surprisingly, in the CD TI, hBD-3 protein relocates to the basolateral surface (red arrow; bottom left panel pair) of the intestinal epithelium in actively inflamed CD tissue, and also accumulates within the lamina propria. In a histologically defined inactive CD region of the TI, the hBD-3 peptide
remains redistributed to the basolateral surface of the epithelium, but its accumulation in the lamina propria is not observed (red arrow, bottom right panel pair). 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 within the lamina propria may indicate an immunomodulatory role for hBD-3 within the TI of CD patients.
Figure 5. HBD-3 protein relocates to the basolateral surface of the columnar epithelium and accumulates in the lamina propria in the CD TI. Paraffin embedded terminal ileal biopsies from healthy control volunteers, patients with active and inactive CD were sectioned, stained with isotype-matched control antibody (isotype) or rabbit anti-hBD3 Ab, and detected with Cy2-labeled donkey anti-rabbit antibody. Nuclei were identified with DAPI counterstain shown in right hand panels of each pair. Images were recorded by confocal microscopy using a 20x objective. A. Isotype control primary antibody B. Control tissue, displaying the accumulation of hBD-3 along the apical surface of the columnar epithelial cells in the villi. C. Actively inflamed Crohn’s disease tissue, in which hBD-3 is redistributed along the basolateral surface of the epithelium, with elevated expression in the adjoining lamina propria. D. Tissue from a CD patient, in whom inflammation of the TI was inactive. While staining for hBD-3 in the lamina propria is reduced, possibly due to the diminished inflammatory cell infiltration, hBD-3 accumulates along the basolateral surface of the epithelium in inactive disease. Arrows indicate the apical or basolateral location of hBD-3 signal.
**Discussion**

In this study, we demonstrated that the peptide expression level and anatomical distribution of hBD-3 are distinct in the healthy and chronically inflamed IBD intestine. HBD-3 peptide is expressed in the healthy lower gastrointestinal tract, localized to Paneth cell granules and along the apical surface of the epithelium in the terminal ileum. In contrast to previous studies of hBD-3 mRNA expression [16], we show elevated hBD-3 peptide and mRNA in the terminal ileum of Crohn’s ileitis patients. Unlike in the healthy terminal ileum, hBD-3 peptide localization in the CD terminal ileum redistributes to the basolateral surface of the epithelium and also accumulates in the lamina propria, only in active disease. The apical orientation of hBD-3, its presence in Paneth cell granules, and its broad spectrum antimicrobial activity suggest that in the healthy mucosa hBD-3 is secreted into the lumen and functions to keep the crypt sterile, thus contributing an important role in maintaining mucosal tolerance within the intestine.

The intestinal mucosa is unique compared to other mucosal sites, such that immunocompetent cells do not mount an overt inflammatory response in the presence of continual stimulation from the commensal microbiota and derived antigens that are separated by an epithelial barrier only a single cell layer thick [2]. This phenomenon is defined as immune tolerance and is critical for proper function of the gastrointestinal tract [2]. It is well established that tolerance is maintained by a delicate balance between various innate and adaptive immune cells [1]. To further understand mechanisms important for establishing and sustaining tolerance, investigators studied inflammatory bowel disease, which is widely believed to represent a pathological loss of tolerance.
Genetic analyses identified multiple genes that increase the susceptibility of IBD onset [2], among which were those important for epithelial cell immunological function, implicating the epithelium as an important mediator of mucosal tolerance [2]. The epithelium, in addition to being a physical barrier, secretes antimicrobial peptides produced by Paneth cells that act as a chemical barrier to provide a sterile crypt environment preventing the attachment and invasion of commensal bacteria, thereby limiting the interaction of commensal antigens with intestinal immune and epithelial cells and preventing an overt, uncontrolled inflammatory response [10,26,27]. This is best evidenced in a mouse model lacking Paneth cells, in which translocation and invasion of commensal bacteria into the mesenteric lymph node is increased [10]. In the human intestine, this concept is confirmed by the storage of the alpha defensins, HD-5 and HD-6, within Paneth cell granules [24]. In addition to alpha defensins, another class of AMPs, beta defensins, is also primarily produced by epithelial cells [6]. Of the six isolated human beta defensins, human beta defensin 3 (hBD-3), possesses the most potent broad spectrum antimicrobial activity, able to kill both gram negative and positive bacteria and multiple viruses and fungi [12]. Thus, it may serve to protect and maintain epithelial homeostasis upon microbial challenge or invasion.

In this study, using intestinal biopsies collected from the terminal ileum, ascending colon, transverse colon and descending colon of healthy control patients, hBD-3 peptide was detected within the terminal ileum and throughout the entire length of the colon. Maximal hBD-3 protein expression occurs within the ascending colon, possibly due to the exponential increase in bacterial load from the terminal ileum to the ascending colon [29]. HBD-3 may prevent the invasion of commensal bacteria into the terminal ileum,
which may not be designed to handle an elevated bacterial load, as increased pattern
recognition receptor (PRR) expression, such as TLR4, is reported to be higher in the TI
compared to the colon [30]. Confocal microscopy reveals hBD-3 protein stored within
Paneth cell granules. Previously identified AMPs with similar structure are also stored in
Paneth cell granules [24]. To rule out hBD-3 antibody cross-reactivity with such AMPs,
a competitive immunoblot demonstrates that the hBD-3 antibody used for confocal
microscopy does not recognize denatured and native epitopes of Paneth cell specific HD-5.
Incubation of HD-5 peptide in molar excess with the primary hBD-3 antibody, prior to
incubation on tissue sections will further prove specificity of the hBD-3 antibody. These
findings strengthen the concept that Paneth cells are critical for maintaining a sterile crypt
environment via storage of antimicrobial peptides. Previously unreported for other
human antimicrobial peptides, hBD-3 also localizes to the apical face of the luminal
intestinal epithelium, suggesting hBD-3 protects the full surface of the epithelium from
interactions with commensal microbiota. This is consistent with the ability of hBD-3,
possibly due to high cationicity, to bind microbial associated molecular patterns,
preventing their ligation to cognate receptors and thus reducing proinflammatory
cytokine induction [13]. Additional confocal images are needed to validate these results.
Detection of constitutive hBD-3 protein expression within the lower gastrointestinal tract
indicates possible redundancy of intestinal epithelial defenses, extending the assertion
that antimicrobial peptides maintain intestinal homeostasis.

Perturbations in antimicrobial peptide expression levels are implicated in disease
pathogenesis in the intestine, particularly in inflammatory bowel disease [23]. 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 for the intracellular PRR NOD2 [23]. Decreased expression of these antimicrobial peptides is hypothesized to cause an increase in 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. Antimicrobial peptide expression is not solely regulated through the NOD2 receptor, as multiple proinflammatory cytokines, such as TNF-\(\alpha\), IL-1\(\beta\), IFN-\(\gamma\), IL-17 and IL-22, all implicated in Crohn’s ileitis pathogenesis, also act to induce antimicrobial peptide expression [20,21]. It remains unknown if the Leu1007fsinsC mutation alters signal transduction initiated by these cytokines resulting in inhibition of antimicrobial peptide expression. Furthermore, studies have yet to implicate regulation of hBD-3 by NOD2 engagement, and protein levels of these antimicrobial peptides have not been measured in Crohn’s Ileitis patients with the Leu1007fsinsC mutation.

In contrast to the hypothesis that Crohn’s ileitis is associated with decreased AMP expression, we report a three-fold increase in hBD-3 peptide within the terminal ileum of these patients. HBD-3 protein levels in all colonic regions of Crohn’s ileitis patients were similar to those detected in the healthy colon. Thus, it seems that hBD-3 protein is selectively increased in the TI of patients with ileitis; however additional patient samples are required to reach statistical significance. To confirm the presence of active disease in our patient samples, we measured IL-22 concentrations in biopsy extracts. In agreement with previously published studies [20], we report elevated levels of IL-22 in ileitis biopsies compared to healthy control. Due to the inconsistency between the increase hBD-3 peptide in Crohn’s ileitis presented in this report and the decrease in hBD-3
mRNA expression described in current literature [16], we measured hBD-3 mRNA in ileal biopsies obtained from healthy controls and Crohn’s ileitis patients. Thus far, we observed approximately a 2-fold increase in hBD-3 mRNA in the terminal ileum of CD patients. With larger numbers of subjects, a significant increase in hBD-3 mRNA in CD patients is likely to be recorded. If no significant hBD-3 mRNA increase is observed following the addition of more subjects, it may be possible that regulation of hBD-3 mRNA expression is cyclic, indicating strict post-transcriptional, regulatory mechanisms control hBD-3 production. It also remains to be determined if patients enrolled in the current study carry the Leu1007fsinsC mutation. Therefore, conclusions cannot be drawn regarding the effect of NOD2 mutations on hBD-3 protein expression. Future experiments are required to determine if a correlation exists between NOD2 polymorphisms and hBD-3 expression levels. Discrepancies between our data of elevated hBD-3 protein and the current hypothesis that a decrease in AMPs contributes to the onset of CD can possibly be explained by 1) lack of NOD2-dependent regulation of hBD-3 expression or 2) severity of disease in different patient populations. Severity of disease has been reported to correlate with decreased AMP expression due to epithelial deterioration resulting in fewer cells capable of producing AMPs [31]. Previous studies implicating a correlation between NOD2 polymorphisms with decreased AMP expression failed to evaluate the histological integrity of the intestinal sections used, thus lessening a direct contribution of NOD2 mutations to decreased AMP expression in active disease [27,28].

In addition to an increase in hBD-3 protein levels, we also describe a change in hBD-3 localization in the terminal ileum of Crohn’s ileitis patients. In active CD, hBD-3 is
redistributed to the basolateral surface of the epithelium and accumulates within the lamina propria. HBD-3 remains localized to the basolateral surface in the terminal ileum of inactive ileitis, however, the accumulation of hBD-3 within the lamina propria is lost. These findings suggest that the intestinal epithelium senses a change in the distribution of “danger signals.” We propose that since Crohn’s disease patients, with active and inactive disease, who have defective epithelial barrier function allowing for possible translocation of commensal bacteria [32,33], respond by actively redirecting defense proteins such as hBD-3 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 at the same location, or a permanent change in epithelial cell physiology.

Intestinal epithelial cells are polarized with an apical and basolateral membrane. The phenotypic and biologic function of each membrane is distinct due to differential protein distribution [34]. Key regulators of intestinal epithelial cell polarization are Rab proteins, which are small intracellular GTPases that regulate intracellular protein trafficking [35]. Distinct Rab GTPases associate with proteins as they exit the trans-Golgi network (TGN) and shuttle proteins to either the apical or basolateral membrane. Up to 70 different Rab proteins have been identified [35]. Depending on which Rab protein associates with a protein exiting the TGN, the protein is either shuttled to the apical, basolateral or both membranes [35]. For example, Rab18 transports proteins to both the basolateral and apical membrane, whereas Rab3 and Rab14 shuttle proteins solely to the apical membrane [34,36,37]. Currently, it remains unknown if inflammatory diseases alter the repertoire of Rab proteins within epithelial cells, thus changing the spatial distribution of
proteins. It is reported, however, IL-6 and IL-12 regulate Rab5 and Rab7 expression, respectively [38]. IL-6 induction of Rab5 redirects proteins to early endocytic compartments, whereas IL-12 induction of Rab7 redirects proteins to lysosomes [38]. Thus, cytokines implicated in inflammatory diseases may regulate spatial distribution of immune effector proteins in the epithelium. Therefore, we propose that CD is not only mediated by the release by specific immune effector molecules, but is also affected by changes in the spatial distribution of these effectors. The shift in hBD-3 spatial distribution in intestinal epithelial cells as a consequence of the CD disease process warrants further investigation into the mechanisms of hBD-3 polarization. Its accumulation within the lamina propria in active CD suggests hBD-3 may have an additional role in maintaining tolerance. In addition to having antimicrobial properties, hBD-3 modulates various immunological responses [39]. Future studies merit investigating the immunomodulatory contributions of hBD-3 in the healthy and IBD intestine.

The results presented here are the first to characterize the expression pattern and localization of hBD-3 peptide in the healthy and IBD intestine. The detection of hBD-3 in the healthy intestine suggests that, similar to previously characterized intestinal AMPs, hBD-3 also contributes to maintaining mucosal tolerance. The selective increase in hBD-3 expression in the terminal ileum of ileitis patients, where other AMPs are reported to be decreased [23], suggests that intestinal AMPs have distinct functions in health and disease. This premise is further supported by the redistribution of hBD-3 from the apical surface of the epithelium in the healthy TI to the basolateral surface of the epithelium in the CD TI. The versatility of hBD-3 as an AMP and immunomodulatory peptide,
coupled to its unique expression profile, indicate a dual function for this peptide in the healthy non-inflamed and inflamed intestine.
References


