"AN ALIPHATIC ESSENTIAL AMINO ACID INFLUENCES THE EXPRESSION OF HOST DEFENSE PEPTIDES IN COLONIC EPITHELIAL CELLS: IN VITRO FINDINGS AND POTENTIAL CLINICAL IMPLICATIONS IN CROHN'S DISEASE"

by

KATE OSEI-BOADI

B.S., University of Ghana, 2005
M.S., Kansas State University, 2009

AN ABSTRACT OF A DISSERTATION

submitted in partial fulfillment of the requirements for the degree

DOCTOR OF PHILOSOPHY

Department of Human Nutrition
College of Human Ecology

KANSAS STATE UNIVERSITY
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2014

Abstract

Background and Objective: Crohn's disease (CD) patients express low levels of host defense peptides (HDPs) especially β-defensins, which may compromise intestinal barrier function. Antibiotic treatment for bacterial infections in CD is limited and rarely curative, making it necessary to find alternative therapeutic approaches. We therefore investigated to what extent an essential amino acid; L-isoleucine (L-ILE) might induce the expression of human β-defensins (HBDs) in colonic epithelial cells as an alternative approach to help patients with CD. Antimicrobial activity of HBD2 was also assessed against four bacterial isolates which can cause secondary infections in CD.

Methods: HTB-37 Caco-2 cells were stimulated with L-ILE at a concentration of 0 - 500µg/ml for 6 hours. Total RNA was extracted using RNeasy Micro Kit (QIAGEN). Reverse transcription was carried out with Superscript ®III First-Strand Synthesis System. The cDNA was amplified using specific primers for HBD1-3. Antimicrobial activity of HBD2 was determined using the broth dilution assay.

Results: HBD1 was constitutively expressed under all conditions. HBD2 was expressed in HTB-37 cells after stimulation with L-ILE. Below 25µg/ml L- ILE stimulation, no expression of HBD2 was observed. HBD2 exhibited antimicrobial activity against bacterial isolates tested, with minimal inhibitory concentration (MIC) of 32, 64 and 128 µg/ml for both strains of *E. coli*, *S. aureus* and *P. aeruginosa* respectively.

Conclusions: Our results indicate that L-ILE stimulation of HTB-37 Caco-2 cells can induce HBD2 expression. Data collected from our *in vitro* studies might have major implications for modifying the intestinal microbiota towards a healthier state in CD patients. Promoting the expression of HBD2 by colonic cells may lead to a lower rate of infection in these patients. Future *in vivo* studies are warranted to determine the potential clinical use of intra colonic administration of L-ILE in CD patients. The observed antimicrobial activity of HBD2 against bacterial isolates provides evidence that it is a crucial component of mucosal epithelial defense against infections which can complicate disease symptoms in CD.

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Dedication

I dedicate this work to my dad Mark Osei-Boadi. Hands down I would not be here if it was not for your love and unending support. You ensured that I had the best of education and all the good things that life could offer. I cannot wait to be in a position to extend the same love and care to you. You are the true definition of a real dad. Every time I have thought of giving up, your belief in my abilities kept me going. I love you so much and thank God for placing you in the capacity as my dad.

Preface

This manuscript was written in a format intended to be submitted for publication. Chapter 1 is a general introduction which outlines the rationale and the hypothesis tested in this research. Chapter 2 is an investigation of the effect of amino acid stimulation on expression of epithelial host defense peptides and the clinical implications for Crohn's disease patients. Chapter 3 is an investigation of the activity of human beta-defensin 2 against bacteria relevant to infections in Crohn's disease patients. Chapter 5 is a reflection on all the findings in the manuscript.

Chapter 1 - General Introduction

Inflammatory bowel disease

Ulcerative colitis (UC) and Crohn's disease (CD) are two of the main inflammatory bowel diseases (IBD). In addition to UC and CD there are other forms of inflammatory conditions that affect the gastrointestinal tract (GI) that have also been studied in detail. Our study will focus on the two main types that are characterized by chronic immune-mediated, unregulated inflammation of the intestinal mucosa of the GI tract. Although there are distinctions between the functional changes in UC and CD, there are also similarities and commonalities that sometimes make it difficult to differentiate between the two.

They are both considered to be chronically idiopathic conditions and present with symptoms characterized by unregulated inflammation, but the main difference lies in the location of the GI tract affected. Whereas CD can affect any anatomic location of the gut, UC is more restrictive in nature and affects mostly the colon, and in rare cases, the rectum [1] (Figure 1.1). The causes of IBD, even though not very well understood, are considered to be a combination of several factors and could range from environmental factors like cigarette smoking and unsanitary living conditions, microbial infections, diet or genetic susceptibility, to ethnic background [2]. Both CD and UC are usually diagnosed in younger populations, especially during the late pubertal stage of growth or during the initial stages of adulthood, although UC occurs a few years later than the former [3]. When it comes to the treatment of IBD, factors such as disease location and the severity of the disease determine the course of treatment. The goal is to treat acute cases and focus on maintaining remission in patients. Most of the treatment options include the use of salicylates, corticosteroids, immunosuppressants and immunomudulators, although other emerging therapeutic options are being considered [4].

UC, as well as CD, continue to be important health concerns not only within the US but also all over the world. Developed parts of the world such as North America and Northern Europe have very high incidences of IBD [3]. The number of Americans afflicted with IBD fall in the millions. A two year cross-sectional study using claims data from about 12 million Americans conducted by Kappelman *et al.* indicated that approximately 1.2 million Americans are living with IBD with a prevalence of CD and UL at 241.3 and 263 for adults (> 20 years of

age) and 57.8 and 33.8 per 100,000 cases in pediatrics (< 20 years) respectively [5]. The low prevalence of IBD in pediatrics may be explained by the peak age of onset of IBD which is between 15 to 30 years old, with only approximately 10% of cases occurring under 18 years [2]. With the high prevalence of IBD in the United States the significant economic burden on the country cannot be ignored as this places a constraint on healthcare resources. A US study to investigate the cost of treatment for CD and UC estimated the mean yearly cost of CD and UC to be $8,265 and $5,066 respectively using data from insurance claims from 33 states between 2003 and 2004 [6]. Compared to adults (> 20 years of age), cost was slightly higher in children (< 20 years). A similar study using data from a 2006 kids inpatient database consisting of patients under 20 years determined the mean cost per person per year for CD and UC to be slightly higher at $10,176 and $11,836 respectively [7]. In the next paragraphs the clinical effects of UC and CD are discussed.

Ulcerative colitis

According to the Crohn's and Colitis Foundation of America, ulcerative colitis is a type of IBD that affects the large intestine, colon and rectum, and is characterized by open sores and ulcers due to uncontrolled inflammation [8]. This ultimately results in clinical symptoms ranging from extra intestinal manifestations like abdominal discomforts to loose bowel, diarrhea, passage of pus and bloody stools. Nutritionally this can result in loss of appetite and weight loss. Colonic and small bowel obstruction hardly occur.

Structurally, UC is characterized by continuous inflammation, muscular thickening, mucin reduction and grandular damage [9]. In the acute phase of disease it is characterized by atypical mucosal pattern due to reduced mucin production, hyperemia and edema which can be detected by radiology and colonoscopy. As disease progresses severe ulcerations appear in the mucosal wall, which changes the architecture of the colonic wall leading to the formation of inflammatory pseudopolyps [10] (Figure 1.3). In the chronic phase of the disease, there is mural involvement manifested by mural thickening and luminal narrowing [10]. Clinical diagnosis is made using findings from endoscopic, radiological and histological studies.

There are different types of UC ranging from a milder form like ulcerative proctitis which affects only the rectum, proctosigmoiditis (affects rectum and sigmoid colon), left-sided colitis, (starts from rectum and extends into the splenic flexure) to a more complex form pan-ulcerative

colitis which spans the entire colon (Figure 1.2). UC and CD can be distinguished from each other but there are anatomic overlaps when inflammation is targeted in the colon.

In infections complicating UC, the most studied is cytomegalovirus infection [11-12] but this is mostly found in patients who fail to respond to steroid therapy. It should be noted that cytomegalovirus infection also occurs in CD patients.

Crohn's disease

This type of major IBD is a chronic inflammatory condition that can affect any part of the gastrointestinal (GI) tract from the mouth to the anus. However it affects more commonly the ileum and the beginning of the colon. The difference between CD and UC lies in the location of the GI tract affected. In addition, inflammation in CD occurs in patches and is segmented (discontinuous) leaving areas of healthy intestines alternating with inflamed sections and has the appearance of a cobblestone pattern with mucosal swellings and ulcerations as shown in Figure 1.3.

Fat-wrapping, a condition where fat creeps on the bowel wall, has been shown to correlate with transmural inflammation and was detected only in CD when intestinal resections were reviewed for fat-wrapping indicating it to be one of the changes accompanying CD [13] (Figure 1.3). Another pathology that has been shown to correlate to inflammation in CD is bowel wall thickening. A previous study by Rae Lee and his group showed that 95% (55 out of 58) of computed tomography (CT) scans of patients with CD had bowel wall thickening. The prevalent anatomic locations were the terminal ileum (56%), ascending colon (29%), caecum (22%), other small bowel (13%), transverse colon, (9%), descending colon (2%) and appendix (5%) [14]. As disease progresses, other structural features that manifest include lymphoid ulcers and chronic granulomatous lesions [9]. Small bowel and colonic obstruction/complication which are rare in UC are common in CD and manifest themselves as either fistulae, strictures or stenosis and abscesses as part of disease progression [15-18], (Figure 1.4) all of which may require several surgical procedures in the patient's lifetime. Infections may also complicate these conditions. When intestinal stenosis or strictures occur they could be inflammatory which can be resolved with medical therapy or fibrotic in nature which requires surgery in most cases [19-20].

Ulcerations in CD can extend far beyond the inner linings and affect all the layers of the bowel wall, which usually are a distinguishing feature from UC.

Crohn's disease is also characterized by clinical symptoms such as abdominal pains, diarrhea, loose bowels and bloody stools. Ultimately, CD patients present with malnutrition and weight loss. Diagnosis is usually by physical examinations and history and supported with radiological, endoscopic, histological and laboratory studies due to a lack of a definitive diagnostic test [21].

The different types of CD depend on which area of the GI tract is affected and the symptoms involved. They include ileocolitis which affects the distal part of the ileum and colon, ileitis (affects ileum), gastroduodenal Crohn's disease (affects the duodenum and stomach), jejunoileitis (affects the jujenum) and Crohn's granulomatous colitis which is restricted to the colon (Figure 1.5). The impact of infections in Crohn's disease due to an impaired bowel wall will be discussed in subsequent paragraphs.

Pathophysiology of the colon in IBD

The large intestine comprises the caecum, colon, rectum and the anus and is approximately 1.5m in total length. The prominent part of the large intestine, the colon, shares many similarities with the small intestines and is lined with mucosal epithelial cells such as goblet mucus cells, absorptive cells, Paneth cells, crypt cells, caveolated cells and M-cells.

The mucosal epithelial layer of the colon plays a major role in the pathophysiology of inflammatory bowel disease and is key to the mucosal immune system where it functions to separate the internal milieu of the host from the external environment. In CD, the mucosal epithelial wall of the colon becomes thickened and the many changes which occur which have been already discussed above may predispose patients to infection due to a weak barrier function. The epithelial wall of the colon in UC patients is thin and has ulcers, which do not extend beyond the inner lining of the bowel wall. This distortion of the architecture of the bowel wall of the colon in IBD may have implications for its immune function. The normal intestinal epithelia just like skin epithelial cells express host defense peptides (HDPs) as part of the innate immune system which functions as part of the barrier mechanism to protect the host from infections. However in both types of IBD these peptides are differentially regulated and this may

be due to the differential distortion of the epithelial lining and integrity of the bowel wall in both diseases.

Epithelial expression of host defense peptides (HDPs) in colonocytes in ulcerative colitis and Crohn's disease

The innate immune system is an important part of the human host defense system and serves as the first line of attack against invading pathogens. Antimicrobial peptides also known as host defense peptides (HDPs) which are highly conserved play an important role at the intestinal mucosal surface and are an indispensable part of the innate immune system. The two main sub-groups of antimicrobial peptides are the defensins and cathelicidins. Cathelicidins are characterized by a conserved N terminal region (cathelin domain) and a variable C terminal which differentiates one cathelicidin from the other [22-23]. Defensins, on the other hand, are composed of two main types, α and β, and the difference between the two lies in the arrangement of the disulfide bridges [24]. The α-defensins are expressed by neutrophils and paneth cells of the small intestines [25-26]. The main types are human neutrophil peptides (HNP) 1-4 and human defensin (HD) 5 and 6. Beta defensins are mainly epithelial cell derived and are well studied, playing a major role in immune defense through their potent direct antimicrobial activity against invading pathogens. They include HBD1-4.

For the purpose of our study we will focus on HBD 1-3 because those are the HDPs expressed by epithelial cells of the colon which is the target of our intervention. HBD4 was not included because it is concentrated in the testis and gastric antrum with low amounts expressed by the uterus, thyroid gland, lung and kidney [27]. Whereas HBD1 is constitutively expressed in epithelial cells, HBD2 and HBD3 are induced by various stimuli such as bacteria, cytokines, nutrients and under inflammatory conditions [28-33].

The role of HDPs in IBD is a topic of research interest and there is increasing evidence to suggest that altered innate defense mechanisms may play a role in IBD [34]**.** For the scope of our work I will focus on β-defensin. It should, however, be noted that other defensins such as α-defensins HD-5 and HD-6 are also induced in the mucosa of IBD patients [35-36]. When it comes to β-defensins they are differentially expressed in UC and CD patients. The evidence supports a decreased expression of beta-defensins (HBD 2 and 3) in CD whereas there is an up-

regulation in UC patients [34, 37-39]. In an attempt to determine the expression of β-defensins in the colon of CD and UC patients, the prevalence of HBD2 transcripts was found to be significantly lower in CD compared to UC colonic biopsies even though both samples were positive for HBD2 [40]. In a follow-up study by the same author and his group, using specimens from inflamed and non-inflamed mucosa from patients with CD and UC, they found an increased induction of HBD2 and HBD3 in UC but this up-regulation was not seen in CD colon mucosal samples [41]. Langhorst *et al*., [39] in their investigations also showed the expression of HBD2 in colonic epithelial enterocytes of UC patients using immunohistochemical methods. Zilbauer and his group observed a similar trend in their investigation to analyze β-defensin expression in large bowel biopsies from children with IBD. In their investigations, HBD2 and HBD3 were induced in both CD and UC biopsies, however HBD2 induction was significantly lower in CD than UC biopsies [42]. Similarly colonic epithelial cells from a mouse model of UC showed a significant up regulation of mBD-3 an orthologue of HBD2 compared to healthy controls [38]. This same trend was mirrored by Fahlgren and his co-workers who demonstrated an increased induction of HBD3 in colonic epithelial cells from UC patients which was absent in epithelial cells from CD patients when compared to healthy controls [43].

The potential clinical relevance of the differential expression of HDPs (increased expression in UC versus a low expression in CD) as far as infection rates will be discussed next.

Infection in ulcerative colitis and Crohn's disease

Inflammatory bowel disease affects the distal part of the ileum and the colon in most patients, and it is the colon that contains the highest amount of intestinal bacteria. An increasing mass of data implicates a dis-regulated mucosal immune response to resident microbial flora in the pathogenesis of IBD, especially in the case of CD. The evidence points to the fact that the epithelial mucosa in CD is more susceptible to infection from commensal and pathogenic microorganisms [44-45].

The epithelial cells of the gastrointestinal tract produce a variety of HDPs such as β-defensins to lyse microorganisms which may invade the host. A defect in expression of β-defensins in CD may be a mechanism through which this antimicrobial function is affected. Nuding and his group comparing the antimicrobial activity of colonic mucosal extracts from CD

and UC patients showed that the antimicrobial activity in CD cationic extracts compared to UC and controls cationic extracts was significantly lower for microorganisms such as *Escherichia coli*, *Enterococcus faecalis*, *Bacteroides vulgatus* and *Staphylococcus aureus* [46]. *Escherichia coli* which are found in the normal flora in the epithelium as well as during an infection have been studied extensively in IBD patients. *Escherichia coli* strains have been recovered from the intestinal mucosa of both chronic and early lesion biopsies of CD patients [47]. Additionally it has been shown that *E. coli* colonizes the differentiated intestinal cells of patients with CD and may interfere with barrier function [44]. A defective barrier function can lead to exposure to luminal bacteria and their pathogenic components. *Escherichia coli* has also been implicated and associated with bacterial translocation and complications due to infection following surgical procedures in CD [48]. A study by Darfeuille-Michaud and group to assess adhesive invasive *E. coli* (AIEC) in the colon and ileum of controls, CD and UC patients revealed that *E. coli* was specifically associated with ileal mucosa in CD patients as evidenced by 21.7% and 36.4% AIEC in chronic and early ileal mucosa lesions and 3.7% in colonic specimens [45]. Interestingly no AIEC was recovered in colonic specimens of UC patients. A clinical study which measured bacterial concentrations in ileum, ascending and sigmoid colon biopsies from asymptomatic controls and IBD patients found significantly higher concentrations of bacteria in CD than UC when compared to controls [49].

Other microorganisms such as *Yersinia enterocolitica* has also been detected in CD patients causing moderate to severe infections and in some cases toxic colonic dilatations and antibiotic treatment could not resolve all cases [50]. In the same study, *Salmonella typhimurium* infection was detected even though it was not specific to CD patients. Methicillin resistant *Staphylococcus aureus*, although not commonly found in colonic mucosal biopsies, was recently detected in a patient with proctosigmoiditis (CD) [51]. *Clostridium difficile* and cytomegalovirus have also been documented in stool samples of patients with IBD (both CD and UC) and are therefore pathogens that should not be ignored when diagnosing infections in IBD patients [4] [52-54]. *Pseudomonas aeruginosa*, an opportunistic pathogen, is one that is very prevalent in nosocomial infections and one that frequently infects CD patients in hospitals.

The fact that most of the infections were associated with CD than UC, may imply that, since UC patients express increased levels of β-defensins, they may be more effective in clearing infection than CD patients. Conversely, the colonization of the intestinal epithelium of CD

patients with bacteria may be explained by the reduced epithelial HDP expression in these patients. This differential expression of HDPs in CD and UC could be due to varying degree of inflammation and the resulting damage to the epithelium in both diseases. In the case of CD the cobble-stoning and ulcerations may affect the epithelium in a way that may manifest itself as the observed decrease in HBD2 expression which ultimately predisposes to microbial infection. Other writers attribute it to an impairment in NF-κB binding to the defensin promoter or a mutation in NOD-2 [55]. We are proposing that a dis-regulated HBD expression in CD is one of the mechanisms by which CD patients become more prone to secondary infections than UC patients.

Currently, the antibiotic therapy being used for CD has low success rates. Also there are many drug resistant Gram positive pathogens like methicillin-resistant *Staphylococcus aureus*, vancomycin-resistant *Enterococcus spp*., *Streptococcus pneumonia* and Gram negative drug resistant pathogens like *Escherichia coli*, *Pseudomonas aeruginosa* and *Klebsiella pneumoniae* which pose problems in immune-compromised individuals like CD patients. This necessitates the discovery of novel therapeutic options for managing infections. Amino acids like isoleucine are generally regarded as safe and have recently been shown to induce HDPs expression in varying cell lines [56-58]. We therefore set out to investigate whether nutrient ingredients like amino acids can induce the expression of HDPs (β-defensins) in the colonic environment using colonic epithelial cells as a model

Rationale for study

1. Bacterial Infection in CD is due to decreased epithelial host defense peptide expression.
2. The current antibiotic therapies for CD are limited with low rates of success and can create antibiotic resistant bacterial populations.
3. There is an urgent need for an alternative and effective therapy for CD patients with secondary bacterial infection.

Working hypothesis

1. Aliphatic essential amino acids can effectively induce the expression of HDPs (β-defensins) in colonic epithelial cells
2. Increased concentrations of β-defensins in *situ* (colonic epithelial surface) will significantly reduce the colonization of pathologic bacteria

3. By exposing epithelial colonocytes to select amino acids patients with CD may be able to produce endogenous antibiotics (HDPs- β defensins) to ameliorate the clinical signs of bacterial infection in the colon

To test our hypothesis, an *in vitro* study using HTB-37 Caco-2 cells was carried out. Confluent HTB-37 Caco-2 cells were stimulated with different concentrations of (L-isoleucine) L-ILE and expression of specific human β-defensins assessed using RT-PCR in study 1 which is described in detail in chapter 2. A second study was carried out to test the antimicrobial activity of human β-defensin 2 (HBD2) against selected bacterial isolates which are prevalent in CD as outlined in chapter 3. In the first study we were able to induce expression of HBD2 with L-ILE stimulation. The follow-up antimicrobial studies showed that HBD2 was potent against bacteria prevalent in secondary infections in CD.

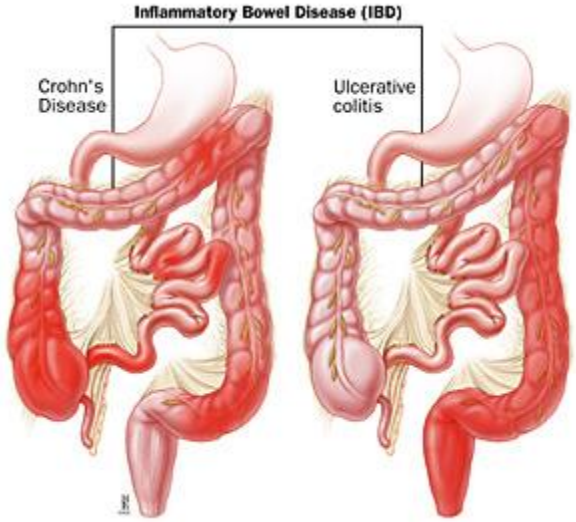

Figure 1.1 Inflammatory bowel disease

Image shows the anatomic locations of the gastrointestinal tract affected in Crohn's disease and Ulcerative colitis as indicated by reddening of the areas.

(From: https://gi.jhsps.org/GDL_Disease.aspx?CurrentUDV=31&GDL_Disease_ID=291F2209-F8A9-4011-8094-11EC9BF3100E&GDL_DC_ID=D03119D7-57A3-4890-A717-CF1E7426C8BA)

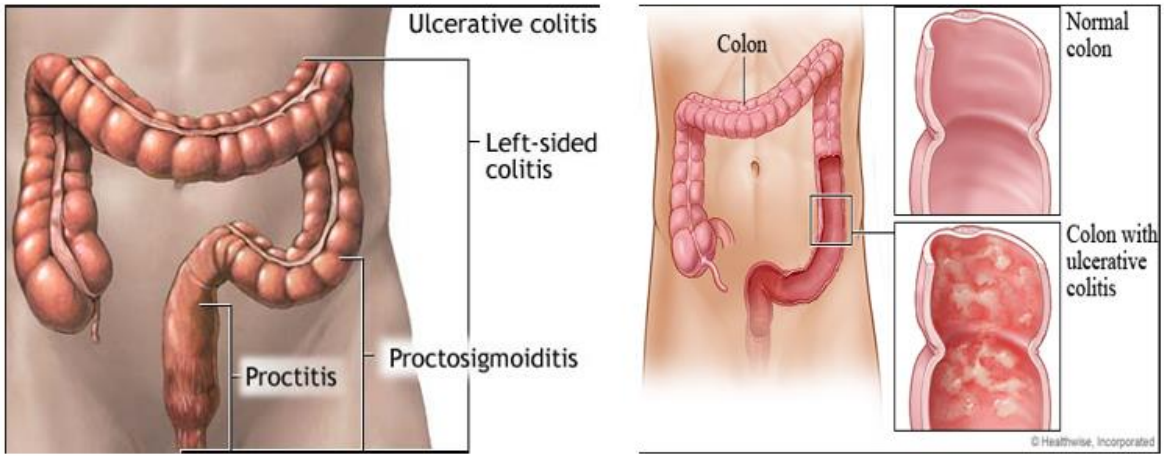

Figure 1.2 Types of Ulcerative colitis showing the different anatomic locations of the large intestines affected

(From: https://www.serovera.com/ulcerative-colitis/)

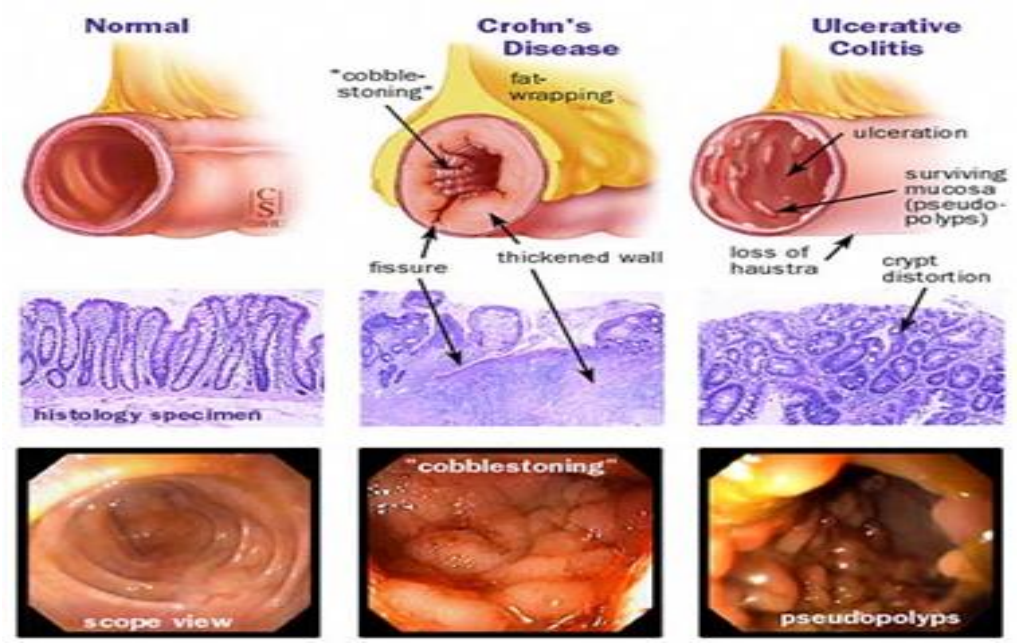

Figure 1.3 Appearance of normal colon, Crohn's disease and ulcerative colitis

Gross (top), histological (center) and endoscopic (bottom) images.

(From https://gi.jhsps.org/GDL_Disease.aspx?CurrentUDV=31&GDL_Disease_ID=291F2209-F8A9-4011-8094-11EC9BF3100E&GDL_DC_ID=D03119D7-57A3-4890-A717-CF1E7426C8BA)

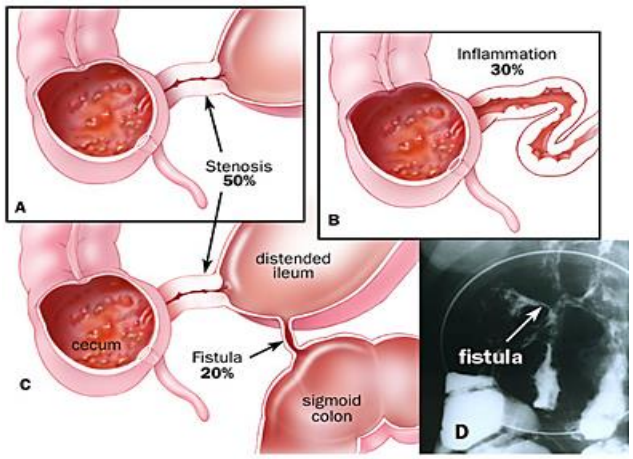

Figure 1.4 Crohn's disease showing stenosis, inflammation and fistula formation during disease progression

The radiographic image of a fistula is shown in D.

(From: https://gi.jhsps.org/GDL_Disease.aspx?CurrentUDV=31&GDL_Disease_ID=291F2209-F8A9-4011-8094-11EC9BF3100E&GDL_DC_ID=D03119D7-57A3-4890-A717-CF1E7426C8BA)

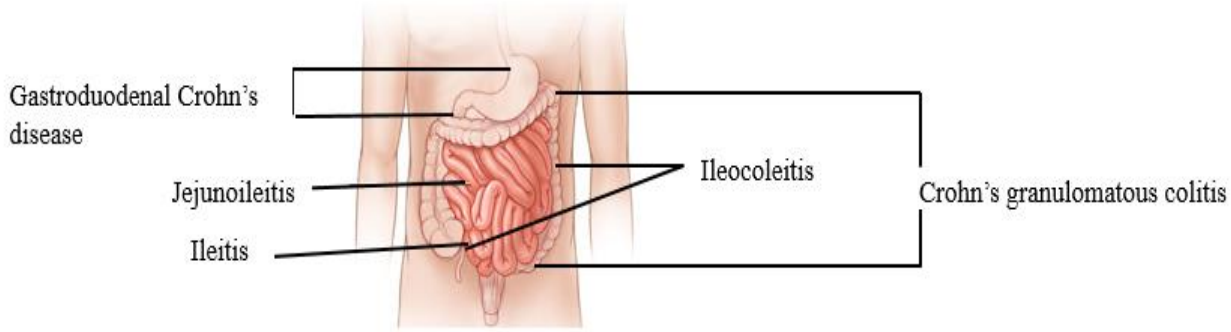

Figure 1.5 Different types of Crohn's disease showing the anatomic locations of the gut affected

Image was adapted from WebMD medical reference from Healthwise with some modifications. (From: http://www.webmd.com/digestive-disorders/small-intestine)

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Chapter 2 - Select Amino Acid Induced Expression of β-defensins

Abstract

Background and objective: Crohn's disease (CD) patients' under-express inducible β-defensins which may affect their intestinal barrier function. In an attempt to come up with alternative ways to treat secondary infections in CD patients we investigated to what extent isoleucine (L-ILE) at different concentrations could stimulate β-defensin expression in a human intestinal (colon) cell line.

Methods: HTB-37 Caco-2 cells were stimulated with 0-500µg/ml L-ILE for 6 hours and gene expression of human β-defensins 1-3 assessed using reverse transcription PCR.

Results: HTB-37 cells expressed HBD1 constitutively whereas HBD2 was induced with L-ILE stimulation. Below 25µg/ml L-ILE stimulation, no consistent expression of HBD2 was observed.

Conclusions: L-ILE was able to induce expression of inducible HBD2. L-ILE could potentially be used as a safe and effective alternative immune-modulator in CD patients who suffer from secondary infections.

Introduction

The role of individual amino acids on both the innate and adaptive system has been studied [1-2]. Alanine is a known stimulator of lymphocyte proliferation, arginine a regulator of cytokine production and mediator of autoimmune diseases, whereas lysine has antiviral properties with leucine and tyrosine regulating immune responses [2]. The beneficial effect of amino acid supplementation in humans has been shown in long distance athletes where branched chain amino acid supplementation enhanced peripheral blood mononuclear cells and cytokine production [3].

There is evidence to suggest that amino acid stimulation can induce β-defensin expression both in studies using cell lines and in animal models. Fehlbaum *et al*., demonstrated that when Madin-Darby bovine kidney epithelial cells are stimulated with L-isoleucine (L-ILE) they induced β-defensin expression with maximal activity at amino acid concentrations of 3.12 -

12.5 µg ml [4]. A study by Sherman and others, using human colon cells, HCT 16 showed that HBD1 was up-regulated upon stimulation with amino acids such as arginine and isoleucine (maximal induction at 100-250 µg/ml isoleucine and 250µ/ml for arginine) using quantitative PCR [5]. In another study using a mouse model of pulmonary tuberculosis, mice were infected with different strains of *Mycobacterium tuberculosis* and treated intratracheally with 250µg of L-ILE after 60 days of infection [6]. Treatment with this amino acid induced an increase in β-defensin expression in these mice. The same research group showed that peak HBD2 gene expression could be detected at 25 µg/ml isoleucine stimulation in respiratory alveolar cells A549 (human type 2 alveolar pneumocytes) and this was time and dose dependent. Mao *et al*., showed that isoleucine can induce β-defensin proteins and mRNA (pBD1, 2 and 3) expression in porcine intestinal epithelial cells (IPEC-J2 cell line) with induction peaking at 25-50µg/ml isoleucine [7].

Amino acids together with other substances such as cytokines have also stimulated β-defensin expression. Co-incubation of isoleucine and IL-1α in human colonic epithelial Caco-2 cells led to an isoleucine enhanced IL-1α induction of HBD2 even though isoleucine alone did not stimulate this induction [8]. All these studies suggest the involvement of amino acids in β-defensin expression in different epithelial cells even though peak expression seemed to occur at different concentrations.

Therefore, we sought to investigate further the effect of amino acids on the expression of specific β-defensins (HBD1, HBD2 and HBD3) in Caco-2 (ATCC® HTB-37™) using isoleucine, arginine and glutamine. Findings from this study have implications for IBD, specifically CD, considering the diminished expression of β-defensins in these patients. The rationale of this study was to find an alternative therapy that is safe and effective for CD patients and does not involve the use of commercially available antibiotics. We approached this by using Caco-2 cells, a well-studied system that has been universally recognized as a valid *in-vitro* system to study intestinal epithelial cells. Prior to confluence in their undifferentiated state, Caco-2 cells express low levels of protein markers of both colonocytes and enterocytes, express markers for both intestinal cell types just after confluence, and finally shift towards a predominantly enterocytic phenotype following long term culture post confluence [9]. This makes timing very important in determining the phenotype of cells and hence interpreting results from Caco-2 cell studies.

Our expectation was to induce the expression of HBD2 and HBD3 in amino acid stimulated Caco-2 cells, which may potentially have clinical implications to future therapies for CD bacterial infection.

Materials and methods

Cell line

Human colonic epithelial Caco-2 cells ATCC HTB-37 were purchased from ATCC (American type culture collection). Cells were routinely grown in T25 flask using complete growth media made up of 80% EMEM (Eagle's Minimum Essential Medium cat # 30-2003) and 20% FBS (fetal bovine serum ATCC cat # 30-2020) and kept in an incubator at 5% CO_2 and 95% atmospheric air. The media was changed every 2 to 3 days and cells allowed to grow for 10 to 13 days until they reached 80% confluence. Dulbecco's PBS (1X) solution was used to rinse cells before each media change. Thus prepared, cells were now ready to be used for stimulation experiments. Images of confluent cells (> 80%) were taken using a digital camera attached to a Nikon Eclipse TS100 microscope. The experimental procedure used in all assays is outlined in Figure 2.1

Amino acid and E. coli stimulation of caco-2 cells

Caco-2 cells were grown to 80% confluence and trypsinized with trypsin-EDTA. Cell count was performed after staining with trypan blue and observing under the microscope. Cells were cultured again in complete growth media in T25 flask at a concentration of 1x 10^4 cells per flask. The cells were allowed to differentiate and then stimulated with various concentrations of L-ILE (500, 200, 100, 50, 25, 10, 5, 1, 0.5, 0.05 µg/ml) and incubated at 37^0C at 5% CO_2 for 6 hours. A control of Caco-2 cells with no amino acid stimulation and Caco-2 cells stimulated with *E. coli* pellets was also incubated under the same conditions.

Escherichia coli stimulated Caco-2 cells were used as a positive control because it is well-established that bacterial components induce β-defensin expression in epithelial cells [10]. Briefly one colony of *E. coli* was transferred into Mueller Hinton II broth and kept in an incubator at 37°C with shaking to grow to log phase. The *E. coli* bacterial suspension was heat inactivated in a water bath at 65°C for 1 hour. Inactivated bacteria was centrifuged at 4000g for 10min to separate pellet from the supernatant. Both bacterial pellet and supernatant were

suspended in fetal bovine serum-free culture media (EMEM). The *E coli* pellet suspension was adjusted to $3x10^8$ cells/ml and used for stimulation experiments. After stimulation experiments HTB-37 cells were washed again in 1X PBS and confluent cells harvested in RNA lysis buffer then kept at -80°C until ready to be used for RNA extraction. Excess Caco-2 cells were cryopreserved in a nitrogen tank suspended in complete growth media containing 5% cell culture tested DMSO (V/V). Other amino acids like glutamine and arginine at same concentrations were also used for stimulation experiments but because yield of total RNA was poor we reported on only L-ILE stimulated cells.

RNA extraction and quantification

After incubation of Caco-2 cells with L-ILE total RNA was isolated by using RNeasy Micro Kit (QIAGEN) according to the manufacturer's instructions. The final total RNA was eluted in 14µL of RNase-free water. Total RNA was put in 2 µL aliquots and kept at -80 to be used for cDNA synthesis. RNA yield and quality was measured using the Agilent 2100 Bioanalyzer. The rRNA ratio (28S/18S) of the total RNA ranged between 1. 4 to 2.2 with the RNA integrity number (RIN) from 7 to 10. The RIN is a measure of the integrity of RNA as RNA could be degraded by ubiquitous RNase enzymes during the extraction procedure.

Reverse transcription of total RNA from Caco-2 cells

A total of 0.5-1µg of total RNA from each of the unstimulated, *E. coli*-stimulated and L-ILE stimulated HTB-37 Caco-2 cells from above was reverse transcribed using Superscript ®III First- Strand Synthesis System for RT-PCR (Invitrogen, life technologies). Briefly 1µL each of 50µM oligo(dt), 10mM dNTP mix was added to 0.5-1 µg equivalent of total RNA and the volume adjusted to 10µL using DEPC-treated water. The mixture was homogenized and incubated at 65^0C for 5 min then placed on ice for 1 min. A total of 10µL of a cDNA synthesis mix made up of 2µL of 10X RT buffer, 4µL of 25mM $MgCl_2$, 2µL of 0.1 M DTT, 1 µL of 40U/µL RNaseOUT and 1µL of 200U/ µL Superscript III RT was added to the RNA/ oligo(dt)/ dNTP mix and centrifuged under the following conditions: 50 min at 50^0C and terminated at 85^0C for 5 min. Reaction was chilled on ice, centrifuged briefly and 1µL RNase H added and incubated for 20 min at 37^0C. The cDNA was put in aliquots of 2µL and stored at -20^0C to be used for PCR.

Polymerase Chain reaction (PCR)

A total volume of 2µL of synthesized cDNA was used for the polymerase chain reaction. The cDNA was amplified using specific primers for HBD1, HB2 and HBD3, which are the epithelial associated β-defensins, along with the control housekeeping gene β-actin. Primers were purchased from Eurofins mwg operon Inc, USA. The amplification conditions for HBD1 and 2 were a hot start of 96°C for 5 min, 35 cycles of denaturation at 94 ^{0}C for 1 min, annealing at 66^{0}C for 1 min and extension at 72^{0}C for 1.5 min. This was followed by an extra extension at 72^{0}C for 3 min and cooling at 4^{0}C for 5min. For HBD3 the amplification conditions were 30 cycles of denaturation at 95^{0}C for 45sec, annealing at 60^{0}C for 45sec and elongation at 72 for 1 min after a hot start at 95^{0}C for 1 min. The conditions for the house keeping gene β-actin were 35 cycles of denaturation at 94^{0}C, annealing at 64^{0}C and extension at 72^{0}C for 30 sec. The PCR conditions used are indicated in the articles referenced in Table 2.1. It should be noted that different primers and primer conditions were tried before finally settling on the above conditions since they gave the best results. The PCR products were resolved on a 2% agarose gel to determine the molecular weight of products. The sequences of the forward and reverse strands of the primers used and the expected amplicon sizes of the resulting PCR products in base pairs (bp) are as shown in Table 2.1 with the references for the primers [11-14].

Results

HTB-37 cells

HTB-37 Caco-2 cells were cultured in complete growth media and the media changed every 48-72 hours until cells became confluent as shown below in Figure 2.2. At confluence the cells form a monolayer of cells and are attached to each other.

Expression of β-defensins in HTB-37 cells

Human beta-defensins 1, 2 and 3 were quantified using RT-PCR after stimulating HTB-37 Caco-2 cells with L-ILE (1µg/ml - 500µg/ml). This was done in two sets, at higher (25µg/ml - 500µg/ml) and lower (1µg/ml- 50 µg/ml) concentrations of L-ILE. Figure 2.3 (a-d) depict the gene expression levels of the HBDs and housekeeping gene, β-actin, under higher concentrations of L-ILE treatment conditions Constitutively expressed HBD1 amplicon was seen under all treatment conditions (both control untreated Caco-2 cells and L-ILE stimulated cells) as shown

in Figure 2.3b. HBD2 on the other hand was expressed only under L-ILE and *E coli* stimulated conditions as shown in Figure 2.3c. The 206bp amplicon for HBD3 was not expressed under L-ILE concentrations tested in this study (Figure 2.3d). Bands seen in some of the lanes in Figure 2.3d are unspecific products that were not the expected HBD3 target bands.

To test whether this same trend of β-defensin expression will be observed at lower concentrations (1, 5, 10, 25, and 50 µg/ml) of L-ILE stimulation, HTB-37 Caco-2 cells were treated with these concentrations of L-ILE for 6 hours. The results are as shown in Figure 2.4 a-d. HBD1 was constitutively expressed under all treatment conditions. HBD2 was expressed only when stimulated with 50 µg/ml and 25µg/ml L-ILE as shown in figure 2.4c. The same trend of non-specific bands not related to the expected HBD3 amplicon size was obtained at these lower concentrations of L-ILE stimulation of HTB-37 Caco-2 cells.

Discussion

The expression of defensins in epithelial cells of the intestine is very important for intestinal health and function. In this study HTB-37 Caco-2 cells were used as an *in vitro* model of the intestine (colonocytes). Caco-2 cells originated from colon cancer cells and under the right conditions in culture medium can differentiate into colonocyte and enterocyte phenotypes [7, 15-16]. Induction of β-defensin in epithelial barriers has been suggested to have an important therapeutic benefit. HBD1, although constitutively expressed, has been shown to be up-regulated with amino acid stimulation [5]. L-ILE was able to induce an upregulation of HBD1 in HCT-16 a human colon carcinoma cell line [17]. A similar trend was seen in IPEC-J2 cells for porcine β-defensin 2 (pBD2) an orthologue of HBD1 [7]. These are interesting findings since HBD1 is a constitutively expressed β-defensin. We however could not evaluate that since we did qualitative measures of defensin expression.

To the best of our knowledge this is the first study to confirm a direct effect of amino acid induced expression of HBD2 in Caco-2 cells. It must however be noted that this was not the first study to investigate the effect of amino acids on host defense peptide (HDP) expression in this cell line. Recent studies have shown that amino acids, especially L-ILE, may regulate the expression of β-defensins and actually increase their expression. When it comes to amino acid induced expression of β-defensin 2 the results have varied in different cell lines. Increased expression of HBD2 was observed in human pulmonary epithelial cells after L-ILE induction

and the highest expression occurred at 25µg/ml of the amino acid stimulation [6]. The same group in their *in vivo* study also showed a higher expression of mouse β-defensin3 (mBD3) a homolog of HBD2 after intratracheal instillation of L-ILE (at a concentration of 250µg/100 µL). In another study however, HBD2 expression was only observed in Caco-2 cells when pre-treated with IL-1α before isoleucine stimulation [8]. Isoleucine by itself did not induce HBD2 expression. This is contrary to what we observed as we were able to induce HBD2 expression with amino acid (L-ILE) stimulation. This could be explained by such factors as different culture conditions relating to growth media composition, substrate on which cells were cultured, passage of cell line used and the parent cell line used [16]. They used nanogram amounts of L-ILE to stimulate their cells [8] whereas we used microgram amounts for stimulation which may explain observed differences.

Quantitative experiments will need to be conducted to investigate the actual quantities of β-defensins expressed and if these quantities increase with amino acid stimulation as this is difficult to assess with our qualitative measures.

Our data suggest that amino acid exposure to epithelial surfaces at the colonic level might have important clinical implications for CD. The epithelial cells of the normal mucosa is lined with a repertoire of HDPs which together with other immune components act as protective barrier against invading pathogens and harmful antigens. These HDPs, some of which are constitutive (HBD1) or inducible (HBD2 and HBD3) help to maintain immune health at the epithelial surface and are deployed when there is an infection to ward off the invading pathogen. Human β-defensins expressed by epithelial cells of the intestines are major frontiers in the immune defense mechanisms of the host species. Therefore a lack of or decreased expression of these immune molecules at the epithelial surface may affect an organism's ability to fight infections. In IBD patients, especially those with CD, HBD2 seems to be under expressed which can compromise epithelial barrier function in these patients. Lower expression of epithelial β-defensins has been associated with secondary bacterial infections in CD. Currently, antibiotics have proven to be a therapy with limited success necessitating the discovery of novel alternative therapeutic options to treat infections. The ability of a nutrient ingredient like L-isoleucine to induce β-defensin expression in epithelial cells makes it a potential candidate as a novel natural therapy to combat infections in IBD patients. This is especially true for CD patients who have been observed to have a down-regulated expression of inducible β-defensins as compared to UC

patients [18]. Isoleucine, an essential amino acid, may be a potential candidate as an immune-modulator because it has been shown to have low level of toxicity at pharmacological or dietary levels and therefore generally regarded as safe [19].

In our study we used the human intestinal Caco-2 cells as a model of the colon cells because they have been used over the last couple of years as a model for intestinal barrier function. To date the difficulty in establishing small intestinal and colon cells from normal intestines has made these colon tumor cells indispensable for studying diseases that affect the intestines and for gaining insight into processes and mechanisms involved in digestion and absorption of various nutrients in the intestines.

Boosting β-defensin expression in CD patients may enhance barrier function and thus limit bacterial invasion which aggravates disease symptoms and clinical signs of bacterial infection in the colon. Further studies using animal models for CD will pave the way for use in humans. It may be possible to expose the colon of IBD patients to L-ILE using enemas to enhance colonic HDPs expression as an affordable and non-invasive treatment option in the near future.

Conclusions

Our results show that L-ILE alone can induce HBD2 expression in HTB-37 Caco-2 cells. New positive controls for HBD3 will have to be tested to verify the expression of HBD3. Because we used HTB-37 Caco-2 cells as a model for colonocytes it may suggest that exposing epithelial colonocytes from patients with CD to select amino acids may enhance production of endogenous antibiotics (HDPs) to possibly ameliorate the clinical signs of bacterial infection in the colon. L-ILE therefore might be used as a safe, inexpensive and effective immune-modulator in patients with CD. However a first step should involve the use of animal models to test this hypothesis before transferring it into humans.

Table 2.1 Sequences of β-defensin primers used for PCR

Primer	Sequence	Weight bp	Source
HBD1_F	CTCTGTCAGCTCAGCCTC	272	[11]
HBD1_R	CTTGCAGCACTTGGCCTTCCC		
HBD2_F	CCAGCCATCAGCCATGAGGGT	254	[11-12]
HBD2_R	GGAGCCCTTTCTGAATCCGCA		
HBD3_F	AGCCTAGCAGCTATGAGGATC	206	[13]
HBD3_R	CTTCGGCAGCATTTTCGGCCA		
βActin_F	TGTGCCCATCTACGAGGGGTATGC	450	[14]
βActin_R	GGTACATGGTGGTGCCGCCAGACA		

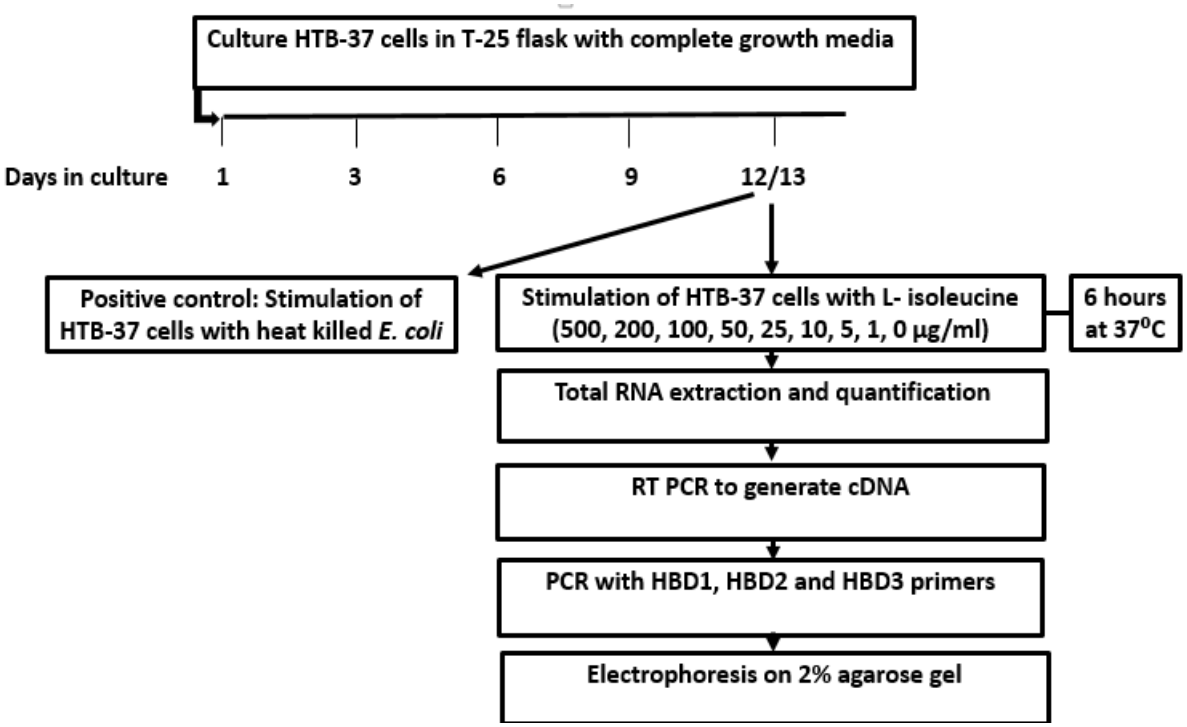

Figure 2.1 Schematic representation of experimental procedure

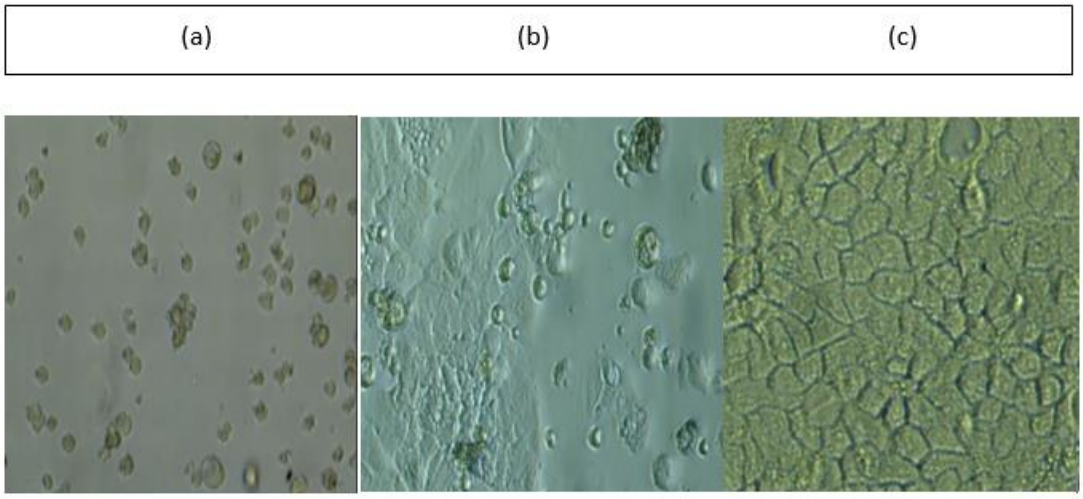

Figure 2.2 HTB-37 cells before confluence and at confluence

Cells are sparsely distributed prior to confluence (a) on day 2 of cell culture and attach to each other forming a monolayer of confluent cells [low density of cells (b) and high density of cells (c)].

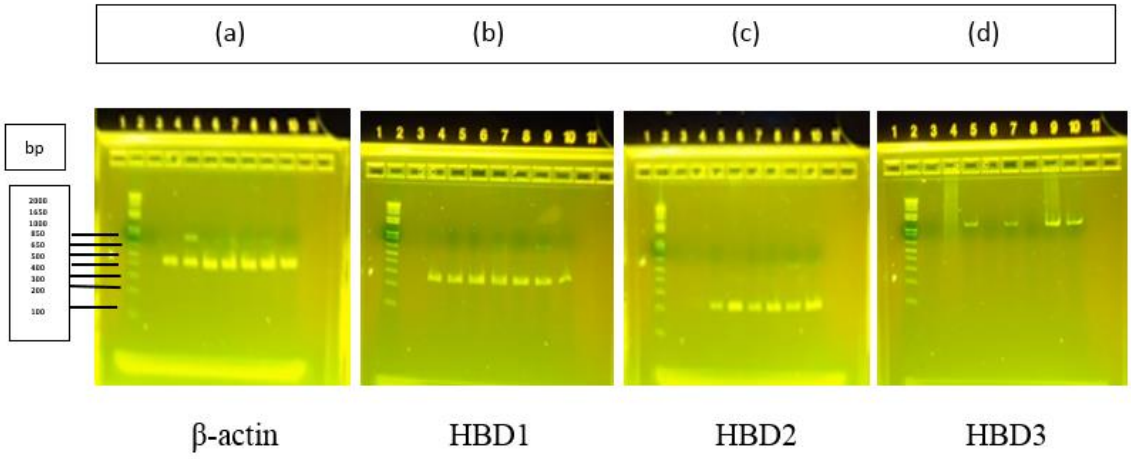

Figure 2.3 Human β-defensin gene expression in HTB-37 Caco-2 cell line after stimulation with different concentrations of L-ILE

Lane 2: E-Gel 1 Kb plus DNA ladder, lane 3: water, lane 4: unstimulated HTB-37 cells, lane 5: 500µg/ml, lane 6: 200µg/ml, lane 7: 100µg/ml, lane 8: 50µg/ml, lane 9: 25µg/ml L-ILE and lane 10: *E. coli* stimulated HTB-37 cells. HBD1 and 2 were detected as a 272 bp and 254 bp bands respectively. The housekeeping gene β-actin was observed as a 450 bp

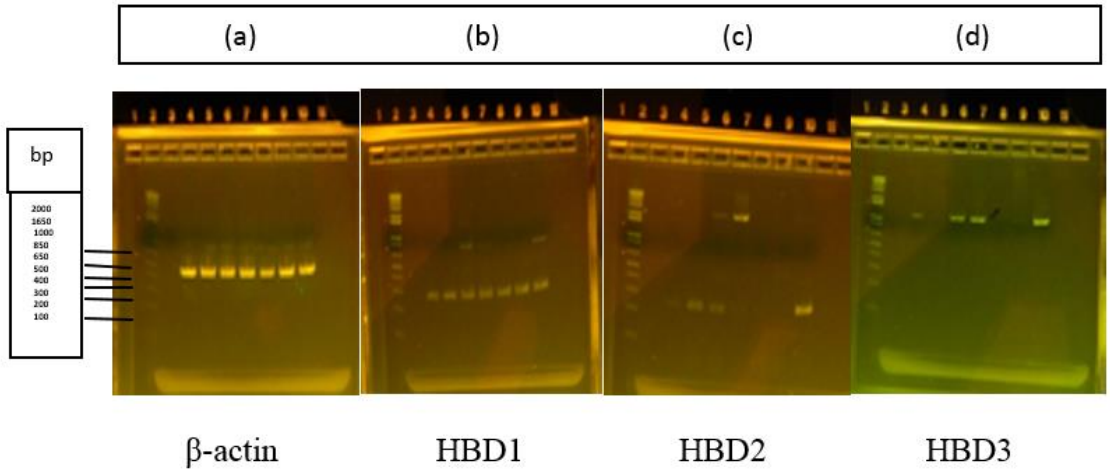

Figure 2.4 Human β-defensin gene expression in HTB-37 Caco-2 cell line after stimulation with lower concentrations of L-ILE

Lane 2: E-Gel 1 Kb plus DNA ladder, lane 3: water, lane 4: unstimulated HTB-37 cells, lane 5: 50µg/ml, lane 6: 25µg/ml, lane 7: 10µg/ml, lane 8: 5µg/ml, lane 9: 1µg/ml L-ILE and lane 10: *E. coli* stimulated HTB-37 cells.

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Chapter 3 - Antimicrobial Activity of Human Epithelial β-defensins against Prevalent Intestinal Bacteria in Crohn's Disease

Abstract

Background and objective: In order to enhance barrier health and fight infections the epithelial lining of the intestine (colon) synthesizes immune molecules such as host defense peptides (HDPs). Decreased expression of HDPs, especially human β-defensins (HBD2), has been observed in CD patients and colon biopsies from these patients have shown the presence of bacterial isolates indicating a break in barrier function. We tested the antimicrobial activities of HBD2 against *E. coli*, *S. aureus* and *P*. aeruginosa, all bacterial isolates that have been observed to infect CD patients.

Methods: The antimicrobial activity of HBD2 was tested against bacterial isolates using the broth dilution assay. The effect of NaCl concentration on peptide activity was also investigated. The minimal inhibitory concentration (MIC) of HBD2 was defined as the lowest concentration of peptide at which bacterial growth was inhibited as measured by lack of visual turbidity.

Results: HBD2 was antimicrobial against tested microorganisms. The MIC for HBD2 as determined by the broth dilution assay for both strains of *E. coli* was 32 μg/ml, 64μg/ml for *S. aureus* and 128 μg/ml for *P. aeruginosa*. At high NaCl concentration (150mM) this activity was inhibited.

Conclusions: Here we report that HBD2 can inhibit growth of bacterial isolates prevalent in CD. It was effective in killing both Gram positive and Gram negative bacteria at low sodium concentrations. Our findings are consistent with the function of HBD2 as an important component of the innate immune system with antimicrobial property. HBD2 serves as a first line of defense, protecting mucosal surfaces from pathogens. HBD2 therefore has potential for use as a source of natural antibiotic for treatment of infections in diseases such as Crohn's disease (CD).

Introduction

Host defense peptides (HDPs) are ubiquitously found in both animal and plant species and have been widely studied as important players in immune function. Defensins, which are a type of HDPs, are evolutionarily conserved and are antimicrobial against a wide range of microorganisms thus providing protection against infections [1-3]. Several β-defensins have been characterized and extensively studied in humans [1, 4-6]. They are cationic in nature and are made up of approximately 36 to 50 amino acids with a characteristic 6 cysteine-residue which gives rise to 3 disulfide linkages, a feature that is used to classify defensins into α or β defensins (Figure 3.1 and 3.2). Human beta defensin 1 (HBD1) is constitutively expressed in epithelial cells whereas HBD2 and HBD3 are expressed upon induction [7-9]. All three beta-defensins are characterized by helixes at the N terminal end and beta strands, all of which contribute to their structure and function [10-12], (Figure 3.2).

It has been documented in CD patients that there is under-expression of HDPs, especially β-defensin 2, when compared to patients with ulcerative colitis (UC) [13-15]. Considering the function of HDPs as being antimicrobial, it is not surprising that bacterial isolates have been detected in biopsies from the intestines of CD patients [16-17]. Our previous study showed that L-ILE can induce the expression of HBD2 in epithelial colonic cells (HTB-37 Caco-2 cells) a model of intestinal cells. As a follow up, in this study we tested the antimicrobial activity of HBD2 against methicillin resistant *S. aureus* (MRSA) a Gram positive bacterium known to cause infection, and two strains of *E. coli* and *P. aeruginosa*, Gram negative bacteria some of which have been detected in biopsies of CD patients [16]. *Pseudomonas aeruginosa*, an opportunistic pathogen, can infect CD patients whilst in the hospital. We included MRSA because it was recently detected in mucosal biopsies from a 24 year old CD patient [17] although not a common colon mucosal pathogen.

The broth dilution assay was used to estimate antimicrobial activity as described elsewhere with some modifications [18].

Materials and methods

Bacterial strains

Bacterial strains used for antimicrobial assays were commercial *E. coli* ATCC 1883, an *E. coli* clinical isolate from a CD patient with diarrhea, *P. aeruginosa* ATCC 53923 and methicillin resistant *S. aureus* (MRSA) ATCC 33591. All bacteria were grown on TSA w/ sheep blood and trypticase Soy Broth (TSB) substituted with other media components as described in the antimicrobial assay for HBD2 and K9cath 21N below. Bacteria were grown following recommendation from NCCLS [18] with some modifications.

Synthesis and preparation of HBD2

The sequence of the HBD2 used is as indicated in Table 3.1. The peptide sequence was obtained from the protein database (PDB) of gene bank and sent for synthesis at Peptide 2.0 Inc (Virginia, USA). The purity of all peptides was > 95%. All peptides were dissolved in 0.01% acetic acid to obtain a stock concentration of 1mg/ml and stored at -20^{o}C. The formation of the 3 disulfide bonds was induced by air oxidation. This involved dialysis in a 500 µl dialysis tube (Spectrum Laboratories Inc. Rancho Dominguez, CA) overnight in 1 liter 50mM Tris-HCl (pH 8.0), 1mM EDTA and 1 mM dithiothreitol at 4^{o}C. Another dialysis was carried out in $NaHCO_3$ and other components as described in [19]. A peptide, K9CATH 21N, that was derived from K9CATH peptide, already tested and found to be antimicrobial against bacterial isolates was included as a positive control [20].

Antimicrobial activity

To determine antimicrobial activity a broth dilution method as described in NCCLS methods [18] was adapted with some modifications. All bacteria were grown at a temperature of 37^{0}C on blood agar plate (TSA w/ sheep blood-Remel) overnight. A colony of each strain was transferred into trypticase soy broth (Difco) and grown under aerobic conditions with shaking at 37^{o}C to mid logarithmic phase. The bacterial suspension was centrifuged at 900 x g and pellets washed and suspended in 10mM sodium phosphate buffer which contains [Na+] = 17.83 mM at pH 7.4. For the determination of antimicrobial activity a broth dilution assay was used. Briefly, after suspending bacterial pellets in phosphate buffer and comparing to 0.5 McFarland standard

which is approximately 10^8 CFU/ml, the concentration of bacteria suspension was adjusted to ~ 1x 10^6 CFU/ml after measuring optical density at 600nm.

For antimicrobial assay approximately 10^6 CFU/ml was used. Briefly, 50µL of microbial suspension was added to 25 µl of H_2O and 25µl of 30mM phosphate buffer containing [Na+] = 53.49mM on a 96 well plate. A 50µl HBD2 working stock at different concentrations was added to the wells of the 96 microtiter plate. In a growth control experiment all bacteria were incubated in phosphate buffer and trypticase soy broth with no peptide. A sterile control of assay media with no bacteria or peptide was also included. Plates were incubated at 37°C in a shaking incubator (140 rpm) for 2 hours. At the end of 2 hours an additional 150 µl of trypticase soy broth was added to wells and incubated at 37°C for 24 hours. The same procedure was used to test the control peptide K9CATH 21N. The concentrations of peptides used for assays were 0, 0.5, 1, 2, 4, 8, 16, 32, 64, 128, 256µg/ml. All assays were performed in duplicates. The effect of sodium was tested at low and physiological concentrations (10 mM and 150 mM respectively) by substituting assay media with NaCl and running the antimicrobial assay against bacterial isolates using HBD2. Minimal inhibitory concentration (MIC) was used as a measure of growth inhibition and this was defined as the lowest concentration of the peptide (HBD2 or K9CATH 21N) at which microbial growth was prevented as indicated by lack of turbidity.

Results

Antimicrobial activity of HBD2

To determine the antimicrobial activity of HBD2 a broth dilution assay was carried out at different concentrations of the peptide. As shown in Table 3.2 HBD2 displayed antimicrobial activity against tested bacteria at low sodium concentration, [Na^+] = 10mM. Both strains of *E. coli* ATCC 1883 and the clinical isolate from IBD patient were susceptible to HBD2 (MIC = 32µg/ml). *Pseudomonas aeruginosa* another Gram negative bacteria was also susceptible to HBD2 with a slightly higher MIC of 128µg/ml. The same pattern of activity of HBD2 was observed for *S. aureus* ATCC 33591 with a MIC of 64µg/ml.

We tested a control peptide K9cath 21N against the clinical isolate of *E. coli* and commercial *E. coli*, ATCC 1883 as well as *S. aureus*, ATCC 33591 and the MIC was 64 µg/ml for all three. The control peptide exhibited a similar pattern of antimicrobial activity against bacterial isolates consistent with how similar these HDPs function as antibacterial agents.

K9CATH 21N is a peptide that we have worked with on different occasions in our laboratory and shown to be antimicrobial against both Gram positive and Gram negative bacteria so we tested it as a positive control to ensure the broth dilution assay was working.

The effect of NaCl on antimicrobial activity of HBD2 was investigated. At physiological (high) concentration, [Na^+] = 150mM in the assay medium, the observed antimicrobial activity of HBD2 at a [Na+] = 10mM was reduced recording MICs as high as ≥250μg/ml (about 8 fold increase) as shown in Fig 3.3 This is consistent with other studies investigating the effect of salt on activity of HDPs [19, 21-23,].

Discussion

Human β-defensin 2, an antimicrobial peptide expressed by epithelial cells, has been shown to kill both Gram positive and Gram negative bacteria. Apart from killing bacteria directly, they are also mediators of the adaptive immune system. Microbial pathogens are able to invade the host system and may potentially pose serious clinical consequences. In CD, the intestinal epithelium is compromised by inflammation and ulcerations which may ultimately affect HDP production as part of the first line of defense against invading pathogens. The antimicrobial property of these HDPs makes them indispensable for protecting the mucosal surface of the intestinal tract which is in constant contact with the complex multitude of microbial organisms in the lumen. The ability of HBD2 which is down-regulated in CD, to kill tested microbes in our study shows that HBD2 is important for clearing bacterial infections. These HDPs kill by either creating pores in the bacterial membrane leading to leakage of cytoplasmic content or through electrostatic interactions with anionic membrane of the bacteria [24].

Several factors affect microbial killing by HDPs such as NaCl concentration. Our findings indicated that at low sodium concentration the activity of HBD2 is enhanced as evidenced by the low MIC values recorded (Table 3.2). Many studies have reported a reduced antimicrobial activity of HDPs in high salt concentrations [21]. The sensitivity of *E. coli* to HBD2 was reduced eightfold in the presence of 150mM NaCl [22]. Of the human β-defensins, HBD3 is the one that is considered to be less sensitive to salt concentration and has been shown to be antimicrobial against *S. aureus* and *E. coli* [25]. Here in our study with HBD2, when 150mM [Na] was used in assay media the sensitivity of bacteria to peptide was inhibited (MIC ≥

256). Other studies have also showed an antimicrobial effect of HBD2 on other strains of *E. coli* and *S. aureus* [22-21]. Another study showed a lack of significant activity of HBD2 against bacterial growth in the presence of high NaCl and serum but inhibition of bacterial growth seemed to be enhanced with the addition of carbonate [26]. The ability of HBD2 to kill at low salt concentration *in vitro* is relevant to their activity in the epithelial layer of the human colon where sodium concentration is low as assessed by measurement of fecal sodium content in normal and IBD patients [27].

Importance of variability in different strains of the same microorganism relative to how they respond to the HDPs should not be underestimated when evaluating the antimicrobial activity of a species of bacteria. Jolly and her group investigating the antimicrobial activity of HBD2 and 3 against oral pathogen demonstrated strain specific activity of these peptides against some of the tested pathogens [28]. For our studies, differences in the strains of *E. coli* tested did not change the MIC of HBD2 recorded (32µg/ml for both strains). Host defense peptides from different organisms have been consistently shown to be effective in killing different strains of E. coli [19, 22-23]. The MIC for HBD2 against different strains of *S aureus*, *P. aeruginosa* and *E. coli* was determined elsewhere to be 62µg/ml using the micro-dilution assay [22]. The difference in observed results may be due to differences in culture media, CFU/ml of bacteria used for assay and the duration of bacterial incubation.

The control K9CATH 21N peptide had antimicrobial activity against all three bacteria tested showing that the assay condition was optimal for that peptide and that both defensins and cathelicidins may function using similar mechanisms for direct microbial killing. Bacteria killing by these HDPs may be a very effective means through which the host protects itself from infections. The study of HDPs specifically HBD2 therefore may provide insight into designing alternative antibiotic therapy for secondary bacterial infections in diseases such as CD. The ability of HBD2 to effectively kill tested clinical and bacterial isolates which have been implicated in CD patients as causing infection has very significant clinical relevance in the quest for finding alternative therapy for CD patients

Conclusions

HBD2 was antimicrobial against all microorganisms tested in this study and is an important inducible component of the innate immune system. The ability of HBD2 to kill *E coli,* a clinical

isolate from an IBD patient, as well as *P aeruginosa* and *S. aureus*, all of which are opportunistic microorganisms that affect CD patients in the hospital, makes it an indispensable natural antibiotic. The ability to induce its production naturally in epithelial cells in the colon may have a protective function against infections. Using nutrient ingredients like isoleucine to induce the expression of HBD2 (from our first study) is therefore a step in the right direction in the quest to find alternative therapies for dealing with secondary infections in CD.

Table 3.1 Peptide sequences for β-defensins used for multiple sequence alignment

Name	Sequence	Gene bank accession number	Protein database ID (PDB ID)
HBD1	DHYNCVSSGGQCLYSACPIFTKIQGTCYRGKA KCCK	AAC51728	1KJ5
HBD2	GIGDPVTCLKSGAICHPVFCPRRYKQIGTCGLP GTKCCKKP	AAC69554	1E4Q
HBD3	GIINTLQKYYCRVRGGRCAVLSCLPKEEQIGKC STRGR KCCRRKK	AAG02237	1KJ6

C 1

Table 3.2 Antimicrobial activity of HBD2 against bacterial isolates

Bacteria	Classification	Source	Clinical relevance	MIC (µg/ml)[b]
E coli clinical isolate	Gram negative	Clinical isolate from CD patient	Clinical isolate was obtained from CD patients with chronic diarrhea	32
E. coli ATCC 1883	Gram negative	Bovine feces	Diarrhea causing and can infect CD patients while in hospital	32
P. aeruginosa ATCC 53923	Gram negative	Soil	Opportunistic bacteria that can infect CD patients while in hospital. Can also cause Gastrointestinal infection, urinary tract infections, skin and soft tissue infection, pneumonia, sepsis	128
S aureus ATCC 33591	Gram positive	Nosocomial or community associated	Hospital acquired infections, sepsis, bacteremia, skin. Infections, abscesses, bone and joint infections. Has been reported in patients with CD	64

[b] the MIC was determined by a broth dilution method

Figure 3.1 Multiple sequence alignment of HBD1, 2 and 3 using clustal W

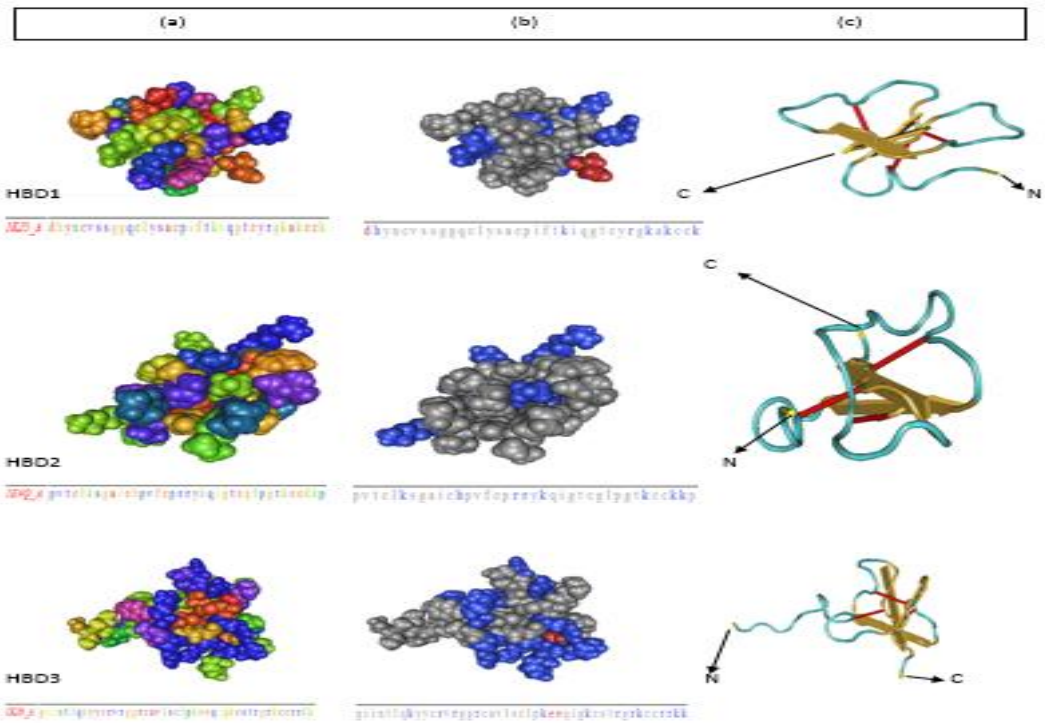

Figure 3.2 Three dimensional structures of human beta-defensins (HBDs)

(a) On the top left depicts the fill space diagram for the peptides based on residues in each peptide as indicated by different colors. (b) The middle column depicts a fill space diagram based on charge of side chain, red is positive, blue negative and grey neutral. (c)The rightmost column shows the worm diagram for the peptides with the three disulfide bonds shown in red, the N and C terminal shown in yellow. These diagram was generated with the program Cn3D 4.3

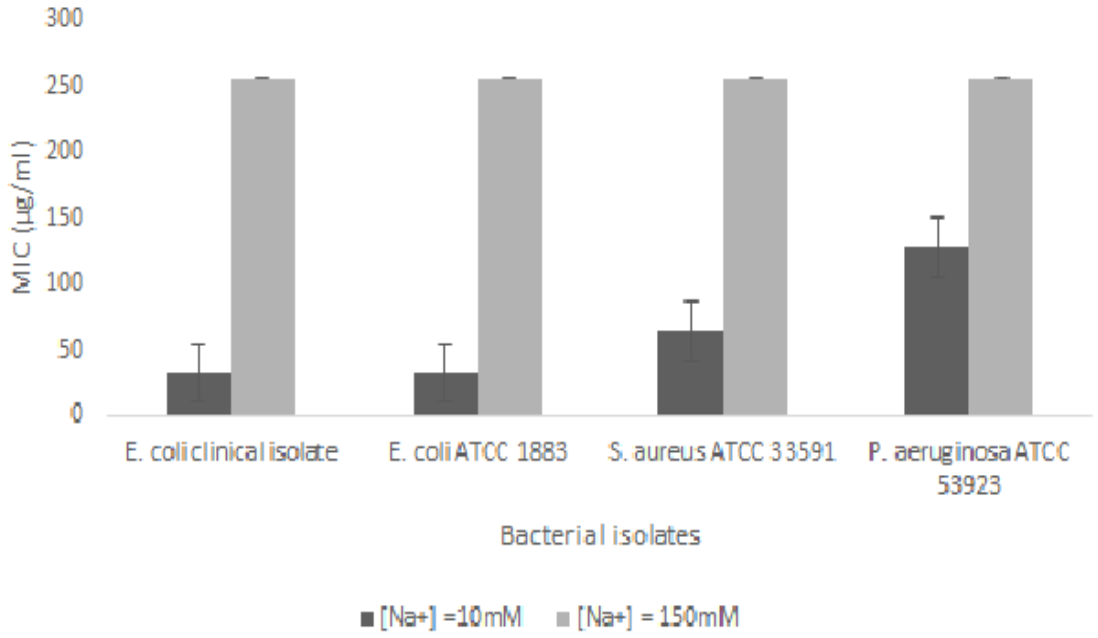

Figure 3.3 Effect of [Na+] on antimicrobial activity of HBD2

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Chapter 4 - Reflections

My knowledge of inflammatory bowel disease has widened as I have researched this topic throughout the course of my graduate studies. The extent of inflammation in both UC and CD, determines disease severity as this influences the accompanying disease symptoms. HBDs are down-regulated in CD in comparison to UC and this may be explained by differences in the extent of damage to the bowel wall. Due to the transmural (affecting all layers of the bowel) nature of inflammation in CD as opposed to the mucosal and submucosal manifestation in UC my theory is that this might predispose CD patients to more infection due to the all-inclusive damage to the epithelial wall of the bowel in CD. The characteristic cobblestone appearance of the epithelial wall of the bowel may also play a role in affecting the production of HDPs. Knowing that β-defensins are epithelial derived it is not surprising that damage to the bowel epithelial wall will cause an attenuation in the production of these peptides. Conversely, the deep ulcerations which are common in UC in my opinion should make them more susceptible to infection than CD; however, the opposite is true from literature which indicates that other mechanisms may explain the observed trend of expression of HDPs in these inflammatory conditions.

As part of an effective treatment strategy there is the need to screen patients for infection prior to the start of any therapy. With problems with antibiotic resistance a nutrient ingredient like L-isoleucine could be a possible candidate for potential treatment, especially since it is generally considered safe and we have shown it to induce HBD2 expression directly in a human cell line. Also being a nutrient it has the potential to repair damaged epithelial bowel wall in CD patients to enhance HDPs production which will ultimately enhance barrier function. L-isoleucine may also have implications for microbiota health in the colon. Animal models of CD will however need to be used to gain more substantial evidence before it can be applied in humans.

As far as the antimicrobial activity of HBDs the assay condition used plays a major role. High NaCl tends to decrease activity of peptides. Proper folding of the disulfide peptide (HBD2) is very crucial to peptide activity and this seems to occur at low salt concentrations which is representative of the salt levels at the epithelial surface of the colon.

As with any research I had limitations such as issues with time and finances which did not allow assessment of quantitative measures (qRT-PCR). Also amplicons will have to be sequenced to confirm the identity of the expressed HBD2. A new positive control for assessing HBD3 expression will have to be investigated to eliminate any pitfalls due to assay conditions. This will confirm that the lack of its expression under the conditions investigated in this study was not due to a lack of optimal assay condition for HBD3 expression.