Systemic levels of human β-defensin 1 are elevated in patients with cirrhosis

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Abstract

Background Bacterial translocation (BT) commonly occurs in cirrhosis. Reliable biomarkers for BT are currently lacking. Human beta defensin-1 (hBD-1) is a member of the family of natural antimicrobial peptides produced by epithelial cells and participates in the mucosal defensive mechanisms that prevent BT. The aim of the present study was to examine the local and systemic expression of hBD-1 in patients with cirrhosis.

Methods Plasma concentrations of hBD-1 and of soluble CD14 (sCD14) proteins were measured by ELISA in patients with chronic viral hepatitis, cirrhosis, and healthy controls. Relative mRNA expression of various natural antimicrobial peptides was determined by real-time PCR in biopsies from the terminal ileum and colon.

Results We found significant upregulation of hBD-1 and sCD14 in the peripheral blood of patients with cirrhosis compared to patients with chronic viral hepatitis and healthy controls. The etiology of cirrhosis did not affect the concentration of either protein. The levels of hBD-1 protein correlated significantly with the levels of sCD14 in blood collected from hepatic veins of cirrhotic patients. In contrast, no significant differences were observed in the intestinal mucosal mRNA expression of the Paneth cell specific defensin A5 or hBD-1 between patients with cirrhosis and healthy controls.

Conclusions hBD-1 is upregulated in patients with cirrhosis and highly correlates with the lipopolysaccharide-induced protein sCD14. hBD-1 may serve as a biomarker of BT in patients with cirrhosis.

Keywords Cirrhosis, bacterial translocation, human beta defensin-1, soluble CD14, natural antimicrobial peptides, biomarkers

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Introduction

Bacterial translocation (BT) is a major component of the pathophysiology of cirrhosis [1,2]. It is directly or indirectly involved in the pathogenesis of spontaneous bacterial peritonitis, hepatic encephalopathy, and hepatorenal syndrome [3]. All of these cirrhosis-related complications carry high morbidity and mortality; hence, BT may be considered as an adverse outcome and bad prognostic factor in the natural history of cirrhosis [4]. Therefore, recognition of BT in the individual patient may allow for timely intervention and improvement of the outcome [5]. It follows that discovery of surrogate markers for BT, especially soluble factors that can be easily detected in biological fluids, will be of great significance in this clinical setting. One such marker that has been found to be increased in patients with cirrhosis is the soluble form of CD14 (sCD14) [6].

The pathophysiology of BT still remains largely unknown. Small intestinal bacterial overgrowth [7], alterations in gut microbiota [8,9], and defective epithelial barrier function with increased intestinal permeability have all been pointed out as the responsible factors alone or in combination. Recently, attention was also drawn to deficits in innate immunity that may occur in patients with cirrhosis [10]. As a large proportion...
of microorganisms implicated in cirrhosis-related infections are inhabitants of the gut microflora, focus has been placed on gut defensive mechanisms and their dysregulation in cirrhosis. An integral component of this intestinal antimicrobial barrier is the system of the natural antimicrobial peptides (AMPs). These are endogenous molecules, which are produced in all eukaryotic organisms and demonstrate activity against any type of microorganism [11]. Several classes of intestinal AMPs have been described, including defensins, cathelicidin, and lysozyme [12]. Human defensins are cationic peptides that are further subdivided in α- and β-defensins [13]. The first group constitutes lysozyme [12]. Several classes of intestinal AMPs and have been described, including defensins, cathelicidin, and lysozyme [11].

Patients and methods

Patients

Patients with chronic viral hepatitis and cirrhosis were recruited from the patient population of the Academic Department of Gastroenterology at Laikon Hospital, Athens, Greece. Demographic and clinical information are shown in Table 1. Diagnosis of chronic hepatitis was confirmed by standard criteria (HBsAg or anti-HCV positivity with viremia for more than 6 months and persistent or intermittent aspartate aminotransferase or alanine aminotransferase elevations). When liver biopsy was available, all patients with chronic viral hepatitis had minimal liver fibrosis and mild-to-moderate inflammation (stage=0-1, grade=2-6, Ishak score).

| Parameter | Chronic hepatitis | Cirrhosis |
|-----------|-------------------|-----------|
| Patients  | 47                | 51        |
| Age (yrs, median±SD, range) | 41±12.4 (27-70) | 66±13 (35-89) |
| Male      | 34                | 34        |
| HBV-related | 22               | 5         |
| HCV-related | 25               | 15        |
| Alcoholic | 20                |           |
| Cryptogenic | 7                |           |
| Other etiology* | 4              |           |
| TBil (mean±SD, range) | 0.89±0.66 (0.2-2.8) | 2.17±3 (0.28-17.52) |
| AST (mean±SD, range) | 70.6±145.6 (13-750) | 53.4±46.4 (17-248) |
| ALT (mean±SD, range) | 73±124.3 (12-660) | 41±44.3 (8-237) |
| y-GT (mean±SD, range) | 69.9±100.7 (10-436) | 82±81 (10-448) |
| Crea (mean±SD, range) | 0.82±0.14 (0.6-1.1) | 1±0.42 (0.6-2.2) |
| INR (mean±SD, range) | 1.07±0.09 (1-1.33) | 1.36±0.33 (1-2.59) |
| MELD score (median±SD, range) | 13±5.9 (6-28) |           |

*other etiology, including non-alcoholic liver disease and primary biliary cirrhosis

Decompensated cirrhosis was defined by the presence of at least one major complication (variceal bleeding, ascites, hepatic encephalopathy, or jaundice). Four patients had compensated cirrhosis, detected via liver biopsy and/or elastography, using a cutoff of 12 kPa.

The control group included healthy volunteers who underwent endoscopy for screening reasons in our department and were age and gender matched to the chronic hepatitis and cirrhosis groups. As expected, patients with viral hepatitis were younger than cirrhotics. To match both groups, our control population varied from 32 to 81 years of age.

Patients with diseases known to affect the expression of the molecules under study, such as chronic obstructive pulmonary disease, inflammatory bowel disease, psoriasis and other immunological disorders, were excluded from the study.

The study protocol was approved by the Laikon Hospital Ethics Committee and all subjects gave written informed consent.

Collection of serum and plasma

Blood was collected from peripheral veins of patients and controls and centrifuged at 3000 rpm. In a subset of patients, blood was collected from the hepatic veins of cirrhotic patients (Seldinger technique-transdermal catheterization of femoral vein, inferior vena cava and right hepatic vein). Serum and plasma were separated, aliquoted, and stored at -80°C until use.
Measurement of soluble hBD-1 and soluble human CD14

Concentration of hBD-1 in the plasma and sCD14 in the serum was measured by enzyme-linked immunosorbent assay (ELISA). For hBD-1, primary and secondary (biotinylated) anti-hBD-1 antibodies were purchased from Peprotech, Inc, UK. Briefly, 0.5 μg/mL of rabbit anti-human anti-BD-1 antibody (capture) was coated to flat-bottomed microplates and incubated overnight at room temperature. Non-specific binding was prevented by adding blocking solution (1% BSA in PBS) for 2 h. Wells were washed and 100 μL of diluted patient’s plasma was added at a dilution of 1:50 and incubated for 2 h. After further washing, 0.5 μg/mL of biotinylated rabbit anti-human anti-BD-1 antibody (detection) was added and incubated for the next 2 h. Finally, after washing, avidin-HRP Conjugate (Peprotech, UK) was added for 30 min in dark, the excess was washed off, and 100 μL of ABTS Liquid Substrate Solution (Sigma, USA) was added. Absorbance was measured at 405 nm and corrected at 650 nm. Absorbance of each sample was plotted against a standard curve produced by serial dilutions of recombinant human h-BD-1 (Peprotech, UK).

For sCD14, human CD14 ELISA Development kit (R&D Systems, Abingdon, UK) was used following manufacturer’s instructions. Briefly, 2 μg/mL of mouse anti-human anti-CD14 antibody (capture) was coated to flat-bottomed microplates and incubated overnight at room temperature. Non-specific binding was prevented by adding blocking solution (1% BSA in PBS) for 2 h. Wells were washed and 100 μL of diluted patient’s serum was added at a dilution of 1:10000 and incubated for 2 h. After further washing, 200 ng/mL of biotinylated sheep anti-human anti-CD14 antibody (detection) was added and incubated for the next 2 h. After washing, streptavidin-HRP was incubated for 20 min in dark and then H₂O₂ and Tetramethylbenzidine (R&D Systems) were added, and after 30 min optical absorbance was measured at 450 nm and corrected at 570 nm. Absorbance of each sample was plotted against a standard curve produced by serial dilutions of recombinant human CD14 (R&D Systems).

For both hBD-1 and sCD14, concentrations were calculated via logarithmic analysis. All samples with an absorbance of less than the average of the zero standards + 2 SD were considered as non-detectable. In addition, to avoid any inter-assay variability, in each individual ELISA assay, similar numbers of samples from patients with chronic hepatitis B and C, cirrhosis and healthy controls were run in parallel.

Lipopolysaccharide binding protein (LBP) was measured by a commercially available ELISA kit (USCN), according to manufacturer’s instructions.

Real-time reverse transcription polymerase chain reaction (RT-PCR)

For mRNA studies, intestinal biopsies were obtained from terminal ileum and colon during colonoscopy, which were performed for diagnostic and/or therapeutic reasons. Biopsy specimens were directly immersed into RNA stabilization solution (RNAlater® AmbionInc., CA), kept for 1-2 days at 4˚C, and then stored at -80˚C until further use. Samples were homogenized using a rotor-stator homogenizer and total RNA was isolated by using Purelink RNA extraction kit (Ambion, Carlsbad, Ca, USA), according to manufacturer’s instructions. To diminish possible genomic DNA contamination total RNA was treated with DNase I, Amplification grade (Invitrogen, Carlsbad, Ca, USA). Next, 250 ng of total RNA were converted to complementary DNA (cDNA) with the aid of the Transcriptor High Fidelity cDNA Synthesis Kit (Roche Diagnostics GmbH, Mannheim, Germany). Relative gene expression in each sample was measured by quantitative PCR using Kapa SYBR Fast enzyme (Kapa Biosystems, Boston, MA, USA) on a CFX96 Thermocycler (Biorad, USA). Amplification of the target genes was performed by primers that span an exon-exon junction (shown Table 2). Thermal cycling conditions for all genes were as follows: 95˚C for 3 min, then 40 cycles of 95˚C for 3 sec and 63˚C for 30 sec, followed by melting curve analysis, from 70˚C to 95˚C with measurements taken every 0.5˚C. The measurements for every target gene were normalized to those of the reference gene ribosomal phosphoprotein P0 (RPLP0) [22]. All samples were assayed in duplicates. Quantification of relative gene expression was calculated by the ΔΔCt method.

Statistical analysis

Kolmogorov–Smirnov tests were performed to determine if the variables (hBD-1, sCD14) under study followed a normal distribution. Both hBD-1 and sCD14 concentrations were found to present with highly skewed distributions. Thus, non-parametric tests were used for statistical analysis. Comparison between groups was performed by Mann-Whitney (2-groups compared) and Kruskal-Wallis (more than 2 groups compared) tests. Spearman’s r-test was used to assess correlations between variables. In all cases an alpha level of <0.05 was considered significant.

Table 2 Sequences of oligonucleotides used in real-time PCR

| Gene   | Oligonucleotide sequence (5’-3’)            |
|--------|-------------------------------------------|
| DEFB1  | Forward  GAG ATG GCC TCA GGT GGT AA       |
|        | Reverse  ACA GGT GCC TTG AAT TTT GG       |
| DEFA5  | Forward  ACC TCA GGT TCT CAG GCA AGA G    |
|        | Reverse  GGG ACT CAC GGG TAG CAC AA       |
| DEFA6  | Forward  GCA GGC AAA AGC TTA TGA GG       |
|        | Reverse  TTG TTT AGC CCA AAG CCT TAA     |
| REG3A  | Forward  CCA TAT CCC ACC AGA GAG TGA     |
|        | Reverse  TGG GGT TCT TCA CCT TGA AC      |
| RPLP0  | Forward  GCG ACC TGG AAG TCC AAC TA      |
|        | Reverse  TCT CCA GAG CTG GGT TGT TT      |
Results

Patient characteristics

In the present study, we included 47 patients with chronic viral hepatitis [age 41±12.4 (27-70), median ± SD, range, male/female 34/13, 22 HBV-related, 25 HCV-related], 51 patients with cirrhosis, [age 66±13 (35-89, median ± SD), male/female 34/17, 5 HBV-related, 15 HCV-related, 20 alcoholic, compensated/decompensated 4/47]. Median values of the viral loads were: HBV DNA (3181, 56.6-990000, IU/mL, median, range) and HCV RNA (1.399 × 10^6, 0.0138-9.585*10^6, IU/mL, median, range). The control group consisted of 40 healthy controls [age 48±15.29 (32-81, median ± SD), male/female 25/15].

Soluble hBD-1 is increased in cirrhotic patients

We first measured the concentration of hBD-1 in the plasma of patients with cirrhosis in comparison to patients with chronic viral hepatitis or healthy controls. We found that plasma concentration of hBD-1 was significantly higher in cirrhosis (18.26 ng/mL, 16.65-21.94, median, 95%CI) in comparison to healthy controls (8.85 ng/mL, 7.46-9.69, P<0.001), and to patients with chronic viral hepatitis (8.87 ng/mL, 9.09-12.88, P<0.001) (Fig. 1A). No significant difference was observed between chronic viral hepatitis and healthy controls. Moreover, we compared cirrhotic and non-cirrhotic viral hepatitis (Fig. 1B) as shown, plasma concentration of hBD-1 is statistically significantly elevated in cirrhotic viral hepatitis (20.46 ng/mL, 15.65-26.51, median, 95%CI) in comparison to non-cirrhotic cohorts (8.87 ng/mL, 9.09-12.88, P<0.0001).

We also examined whether the increase in hBD-1 in the plasma was associated with a specific etiology of cirrhosis or the latter was the reason for the elevated concentration. To test this, we divided cirrhotic patients into groups according to the underlying cause and compared hBD-1 concentrations between groups (Fig. 2). As shown, there were no statistically significant differences for any comparison. We also compared patients with chronic viral hepatitis B or C. Again, no statistical significant difference in the levels of hBD-1 was seen (Fig. 2). In contrast, when the groups of HBV- or HCV-related liver disease were analyzed separately, in both cases there was a significant increase of plasma hBD-1 concentration in cirrhotic patients as compared to those with chronic hepatitis.

Taken together, these results indicate that the increased levels of hBD-1 are in direct association with the presence of cirrhosis itself, irrespectively of its etiology.

Plasma hBD-1 correlates with the levels of sCD14, a marker of BT in cirrhosis

We next asked whether the increased plasma hBD-1 concentration in cirrhotic patients may reflect the presence of BT. To examine this possibility, we first measured the levels of sCD14 in the same populations of patients. As already mentioned, sCD14 is a marker of lipopolysaccharide (LPS) bioactivity as its secretion follows LPS-induced activation of myeloid cells; hence its presence is an indicator of bacteremia due to BT. Our results are shown in Fig. 3. Similar to hBD-1, the concentration of CD14 in the systemic circulation was significantly higher in patients with cirrhosis (2777 ng/mL, 2701-3133, median, 95%CI) as compared to healthy controls (1671 ng/mL, 1574-2026, median, 95%CI) (P<0.001). In contrast to results for hBD-1, patients with chronic viral hepatitis also showed higher concentrations than healthy controls (Fig. 3). Similar to hBD-1, the levels of sCD14 were not affected by the specific etiology of cirrhosis (data not shown).

Next, we presumed that if the elevations of hBD-1 and sCD14 in cirrhotic patients are indeed a reflection of BT, there should be a significant correlation between the two, when values in individual patients were analyzed. Our study confirmed that this was indeed the case in our population of cirrhotic patients. More importantly, the correlation was stronger when the concentrations of these two molecules were measured in blood isolated from hepatic veins (Spearman r=0.6, P=0.0045) (Fig. 4B). The correlation became borderline significant, when

Figure 1 Increased levels of human beta defensin-1 (hBD-1) in cirrhotic patients. Plasma concentration of hBD-1 was measured as described in Patients and Methods. (A) Concentration of hBD-1 compared in various groups of controls (n=40) and patients with liver disease [chronic viral hepatitis (n=47), cirrhosis (n=51)]. Results are shown as Whisker box-plots. (B) Concentration of hBD-1 compared in cirrhotic (n=20) and non-cirrhotic (n=47) viral hepatitis patients
Ileal or colonic mRNA expression of natural AMPs is not altered in patients with cirrhosis

Finally, we tested whether the presence of cirrhosis is associated with differences in the mucosal expression of natural AMPs in the small or large intestines in comparison with healthy controls. To answer this question we compared the relative mRNA expression for several AMPs in total RNA from endoscopically-obtained intestinal biopsies (Suppl. Fig. 2). In line with previous publications, we found significantly increased mRNA expression of A5 defensin in the terminal ileum compared to the colon. However, no differences were found between healthy controls and cirrhotics for any of the AMPs tested in either location (terminal ileum, colon). These results show that a quantitative defect in the production of AMPs does not take place in cirrhosis.

Discussion

In the present study, we report that patients with cirrhosis have increased systemic levels of the antimicrobial peptide hBD-1. We demonstrated that this elevation is driven by the presence of cirrhosis per se and is not associated with any specific etiology. We also report a significant elevation in the levels of sCD14, an indicator of circulating LPS and gram-negative bacteremia in patients with cirrhosis. Finally, we showed that hBD-1 concentration significantly correlated with that of sCD14 in hepatic veins and borderline significant in the peripheral blood, raising the possibility that elevations of hBD-1 may also represent a response to and be a surrogate marker for BT in cirrhosis.

Herein, we confirmed a significant elevation of sCD14 in the peripheral blood of patients with chronic liver diseases, as it was reported in previous works [6,23]. CD14 is a glycoprotein that exists both in membrane (mCD14) and soluble forms (sCD14). It is expressed on monocytes and binds to the LPS-LBP complex, thus participating in immune responses against gram-negative, LPS-producing bacteria [24]. CD14-mediated signaling leads to nuclear translocation of the transcription factor nuclear factor (NF)-κB and promotes cytokine production [25]. This pathway may result in acceleration of fibrogenesis in the liver [26]. The expression of sCD14 is induced by circulating LPS; therefore, sCD14 serves as an indicator of the presence of LPS in the serum and, in extension, of gram-negative bacteremia. Thus, its use as a marker of BT has been proposed.

In our study, we also demonstrated that the natural antimicrobial peptide hBD-1 is highly elevated in the circulation of patients with cirrhosis. Our findings clearly support the notion that this upregulation is a cirrhosis-associated phenomenon. First, cirrhotic patients had significantly higher plasma concentrations of hBD-1 compared to healthy controls as well as to patients with chronic hepatitis. Second, elevation of hBD-1 occurred independently of the etiology of cirrhosis. In fact, within the populations of patients with HBV- or HCV-related liver disease, only patients with cirrhosis (and not those with chronic hepatitis) had elevations of hBD-1. To our knowledge, this is the first report of elevated expression of an antimicrobial peptide in the cirrhotic population. In addition, elevated plasma concentration of hBD-1 has not been previously reported in relation to a specific condition.

Figure 2 Increased levels of human beta defensin-1 (hBD-1) in cirrhotic patients are independent of the etiology of liver disease. Concentration of hBD-1 according to specific etiology of liver disease [chronic hepatitis B (Hep B) (n=22), chronic hepatitis C (Hep C) (n=25), HBV cirrhosis (n=5), HCV cirrhosis (n=15), alcoholic cirrhosis (n=20), other etiology of cirrhosis (n=11)]. Results are shown as Whisker box-plots

Figure 3 Elevated soluble CD14 (sCD14) concentration in the serum of cirrhotic patients. Concentration of sCD14 was measured in healthy controls (n=31), patients with chronic viral hepatitis (n=26) and patients with cirrhosis (n=50) as described in patients and methods. Data is presented graphically as Whisker box-plots
Taken together, our current findings and previous studies clearly show that several bacteria-inducible factors are upregulated in cirrhosis and their concentration increases or becomes detectable in the systemic circulation of patients. This provides an indirect evidence for the presence of BT in this patient population. Previous studies have pointed out the role of portal hypertension in the pathogenesis of BT. Experiments in mice with experimental portal hypertension demonstrated increased BT [27], partly as a result of increased epithelial permeability [28]. In humans, portal hypertension is associated with altered immune responses [29] as well as bacterial overgrowth, increased intestinal permeability and altered gut motility resulting in BT [30]. On the other hand, the presence of LPS (a vasodilatory factor) along with activation of fibrogenic factors like NF-κB, as previously mentioned, lead to the deterioration of portal hypertension.

As BT predisposes to serious complications of cirrhosis, its early recognition with the use of easily identifiable and measured biomarkers in biological fluids becomes of paramount importance. This is particularly significant as it is known that BT may exist at a preclinical state, and that, often no microorganism is isolated by microbiological methods [31,32]. Bacterial DNA has been proposed as definitive marker of BT [4,33]; nevertheless, its use is complicated by technical difficulties and inter-laboratory variations [34]. Fecal calprotectin also suffers from validation problems between different laboratories and assays [35]. The high correlation of hBD-1 with sCD14 reported herein makes the former a potential candidate surrogate marker of BT as well. Interestingly, a combination of polymorphisms in the genes encoding for these two proteins, hBD-1 and sCD14 has been found to confer risk for periodontitis [36]. This result indicates that an association between these two markers may take place.

The particular reasons for the elevation of hBD-1 in cirrhosis and its cellular source(s) are not clear. The significant correlation between hBD-1 and sCD14 supports the notion that bacterial stimuli drive their upregulation. Microbial products present in the intestinal lumen may induce the expression of both proteins; hence their significantly higher correlation in hepatic veins than in the systemic circulation. Intestinal epithelial cells produce hBD-1 upon stimulation with bacteria-derived factors [14,16]. Nevertheless, our mRNA expression study failed to detect a significant difference in the intestinal mucosal expression of hBD-1 (or any other AMP) between cirrhotic patients and healthy controls. This, however, does not exclude the possibility that defects in the secretion of proteins or the antimicrobial capacity of excreted defensins may take place in cirrhosis. Such defects were recently shown to take place during intestinal inflammation [37]. Compartmentalization of expression may also be a confounding factor. Recently, Teltschik et al reported upregulation of BD-1 RNA in the cecum but downregulation in the colon of rats with experimental cirrhosis [20]. It is possible that hBD-1 may also be expressed differently in various parts of the large intestine of cirrhotics. Furthermore, we did not detect altered expression of human defensin A5 in the terminal ileum of cirrhotics, whereas, in the aforementioned study, downregulation of α-cryptdin 5 and 7, the rat homolog for human Paneth cells α-defensins, in rats with liver cirrhosis and BT was reported [20].

Additional cellular sources of circulating hBD-1 may also exist. First, monocytes were shown to highly upregulate hBD-1 expression upon stimulation with bacterial factors. In particular, stimulation with muramyl-dipeptide (MDP) that binds to the intracellular bacterial sensor NOD2, upregulated miRNA129-5p, which then induced the expression of hBD-1/DEFB1 [38]. Second, it was shown recently that platelets produce and store hBD-1, which can be released only after stimulation by microbial products that induce cell permeabilization [39]. Therefore, the elevated systemic levels of hBD-1 in cirrhotic patients may also be derived from monocytes and/or platelets in response to the process of BT and the release of pathogen-associated molecular patterns, such as LPS or MDP.

We are aware that our study has certain limitations. First, the number of patients is relatively low; thus our results should be replicated in a larger sample. Second, there is under-representation of important cirrhotic subgroups, most importantly, of patients with non-alcoholic fatty liver disease.
associated cirrhosis. Finally, due to the lack of a definitive marker of BT in cirrhotic patients, a direct association between hBD-1 and sCD14 cannot be firmly established.

In conclusion, we herein provide evidence for increased systemic levels of hBD-1 and sCD14 in cirrhosis, irrespectively of the etiology of liver disease. We propose that hBD-1 may represent another possible marker of BT, with the advantage of being easily measured in peripheral blood. Larger studies are required to validate the present findings and further delineate the role of hBD-1 in cirrhosis and its applicability as a biomarker for BT.

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