• *Helicobacter pylori* •

High concentrations of human β-defensin 2 in gastric juice of patients with *Helicobacter pylori* infection

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Abstract

AIM: Human β-defensin (HBD)-1 and HBD-2 are endogenous antimicrobial peptides. Unlike HBD-1, the HBD-2 expression is augmented by *Helicobacter pylori* (*H pylori*). We sought to determine HBD-1 and HBD-2 concentrations in gastric juice during *H pylori* infection.

METHODS: HBD-1 and HBD-2 concentrations were measured by radioimmunoassay in plasma and gastric juice of 49 *H pylori*-infected and 33 uninfected subjects and before and after anti-*H pylori* treatment in 13 patients with *H pylori*-associated gastritis. Interleukin (IL)-1β and IL-8 concentrations in gastric juice were measured by enzyme-linked immunosorbent assay (ELISA). Histological grades of gastritis were determined using two biopsy specimens taken from the antrum and corpus. Reverse phase high performance liquid chromatography (RP-HPLC) was used to identify HBD-2.

RESULTS: HBD-2 concentrations in gastric juice, but not in plasma, were significantly higher in *H pylori*-positive than -negative subjects, albeit the post-treatment levels were unchanged. Immunoreactivity for HBD-2 was exclusively identified in *H pylori*-infected mucosa by RP-HPLC. HBD-2 concentrations in gastric juice correlated with histological degree of neutrophil and mononuclear cell infiltration in the corpus. IL-1β levels correlated with those of IL-8, but not HBD-2. Plasma and gastric juice HBD-1 concentrations were similar in *H pylori*-infected and uninfected subjects.

CONCLUSION: Our results place the β-defensins, especially

HBD-2, in the front line of innate immune defence. Moreover, HBD-2 may be involved in the pathogenesis of *H pylori*-associated gastritis, possibly through its function as immune and inflammatory mediator.

INTRODUCTION

Helicobacter pylori (*H pylori*) infection is the major cause of chronic gastritis and peptic ulcer disease and is a risk factor for gastric cancer[1,2]. The non-invasive organism colonizes the gastric epithelium and elicits specific antibodies against various immunogenic proteins derived from the bacteria[3]. *H pylori*-associated gastritis is characterized by intense infiltration of neutrophils and mononuclear cells into the lamina propria[4,5]. However, despite these humoral and cellular immune responses, the infection usually lasts a lifetime in the absence of antibiotic treatment[5]. Moreover, current anti-*H pylori* combination regimens are rather complicated and do not always result in cure of the infection because *H pylori* strains often develop resistance to antibiotic drugs[3,6].

Recently, various endogenous anti-microbial peptides have been identified as key elements of innate host defence against infection[7,9]. Defensins, single chain cationic peptides of molecular weight ranging from 3 000 to 4 500 Da, are one of the most extensively studied classes of such naturally occurring antibiotics[7,9]. They exhibit a wide variety of microbicidal activities against Gram-positive and -negative bacteria, mycobacteria, fungi and certain enveloped viruses[7,9]. Human defensins are divided into α- and β-defensins, based on the arrangements of three intra-molecular disulfide bridges[7,9]. At present, four members of β-defensins have been isolated in humans[7-11]. They are essentially synthesized in the epithelial compartment at various mucosal sites[7-11].

Recent *in vitro* studies showed the constitutive expression of human β-defensin (HBD)-1 and induced expression of HBD-2 in several gastric cancer cell lines in response to

H pylori infection[12-14]. Exclusive enhancement of HBD-2 expression upon *H pylori* infection was also noted in patients with chronic gastritis[15,16]. However, there is no information on the secretion of HBD-2 into the gastric lumen *in vivo* or its concentrations in gastric juice during *H pylori* infection.

We have developed a sensitive, specific radioimmunoassay (RIA) for HBD[17]. Employing this assay system, we measured HBD-2 concentrations in gastric juice of *H pylori*-infected and uninfected individuals. This study paves the way for further understanding of the mechanisms involved in host immune response to this pathogen.

MATERIALS AND METHODS

Patients and sampling

A total of 82 patients referred for diagnostic upper gastro-intestinal endoscopy between September 2002 and August 2003 were enrolled in the present study. The following exclusion criteria were applied for enrollment in the study. (1) The use of non-steroidal anti-inflammatory drugs, proton pump inhibitors, histamine H_2-receptor antagonists or antibiotics within 4 wk prior to the present study; (2) History of severe concomitant diseases, upper gastro-intestinal surgery, peptic ulcer diseases and gastric cancer. On the day of endoscopy, blood samples were taken, transferred into tubes containing EDTA-2Na and aprotinin, centrifuged, plasma separated, and stored at -80 ℃ until assay.

At the beginning of endoscopy (XQ 200; Olympus Optical Co., Tokyo, Japan), a sample of the gastric juice was aspirated into collection tube containing EDTA-2Na and aprotinin using an aspiration instrument (PW-6P-1, Olympus) under endoscopic guidance. Gastric juice samples were immediately neutralized to pH 7.0 with 1 N NaOH and frozen at -80 ℃ until measurement. Two biopsy specimens were endoscopically obtained from both the antrum within 2 cm of the pyloric ring and the middle portion of the corpus along the greater curvature, fixed in 10% buffered formalin and embedded in paraffin. One was used for rapid urease test (Helicocheck, Otsuka Pharmaceutical Co., Tokushima, Japan) and another for histopathological and immunohistochemical assessments.

Fourteen patients with *H pylori*-associated gastritis were treated with eradication therapy consisting of lansoprazole (30 mg twice daily), amoxicillin (750 mg twice daily) and clarithromycin (400 mg twice daily) for 7 d[18]. Four weeks after cessation of the treatment, patients were examined by endoscopy again, and gastric juice and biopsy specimens were taken in a similar fashion to that performed before treatment.

Eradication of *H pylori* was considered successful when ^{13}C-urea breath test was negative[18].

Histopathological examination

Paraffin-embedded biopsy specimens were cut into 4-μm thick sections and stained with hematoxylin and eosin. Each histological parameter of activity (neutrophils), chronic inflammation (mononuclear cells), glandular atrophy and intestinal metaplasia in the antrum and corpus of patients with *H pylori* infection was scored as 0, 1, 2 or 3 corresponding to none, mild, moderate or severe, respectively, based on

the Sydney system[19,20]. Intestinal metaplasia was defined by the presence of goblet cells in glandular mucosa with Alcian blue (pH 2.50)/periodic acid-Schiff staining[21].

Furthermore, the density of *H pylori* colonization assessed with Giemsa staining was also scored from 0 to 3, based on the above classification system[19,20]. The biopsy specimens were examined blindly without knowledge of the results of β-defensins measurement.

Diagnosis of H pylori infection

H pylori status was assessed by serology (anti-*H pylori* immunoglobulin G antibody, HEL-p TEST, AMRAD Co., Melbourne, Australia), rapid urease test and histology with Giemsa staining. Patients were considered positive for *H pylori* infection when at least two of these examinations yielded positive results[20,22]. On the other hand, patients were defined as *H pylori*-negative, if all test results were negative[23].

Measurement of interleukin (IL)-1β and -8 levels in gastric juice

The concentrations of interleukin (IL)-1β and -8 in gastric juice were measured as described previously[20,22,24]. The samples were assayed for total protein by a modified Lowry method, diluted to 0.5 mg/mL total protein concentration, and frozen at -80 ℃ until assay. Measurement of the two cytokines in the aliquots was performed using commercially available assay kits (Research and Diagnostics, Minneapolis, MN, USA), which employ the quantitative immunometric sandwich enzyme immunoassay technique. These assays were performed in duplicate according to the instructions provided by the manufacturer. In our study, inter- and intra-assay variabilities were <10%, respectively[20,22].

Measurement of HBD-1 and -2 levels in plasma and gastric juice

The concentrations of HBD-2 in plasma and gastric juice samples were measured by RIA established in our laboratory[17]. Briefly, full-length HBD-2 was synthesized using a peptide synthesizer (model 430, Applied Biosystems, Foster City, CA, USA) and purified by reverse phase high performance liquid chromatography (RP-HPLC). Synthetic HBD-2 was used for immunizing New Zealand white rabbits by multiple intracutaneous and subcutaneous injections. It was radio-iodinated and the ^{125}I-labelled peptide was purified by RP-HPLC on a TSK ODS 120A column (Tosoh Co., Tokyo). The diluted sample or a standard peptide solution (100 μL) was incubated for 24 h with 100 μL antiserum diluent (final dilution 1:4 200 000). The ^{125}I-labelled solution (16 000 cpm in 100 μL) was added and the mixture was incubated again for another 24 h. Normal rabbit serum and anti-rabbit IgG goat serum were then added and stored for 16 h. Bound and free ligands were separated by centrifugation. All procedures were performed at 4 ℃ and duplicate assays were carried out. Volumes of 0.5 mL plasma and 1-2 mL gastric juice were used to determine the levels of HBD-2. The concentrations of HBD-1 were also measured in similar fashions. The intra-assay and inter-assay coefficients of variation were <10%, respectively, in both the RIA analyses[15,23].

Chromatographic characterization of HBD-2

Chromatographic characterization of HBD-2 was performed as described previously[26,27]. Each 10 mg wet weight of non-cancerous mucosal tissue was sampled from surgically resected stomach of three patients with gastric cancer and *H pylori* infection. The samples were immediately homogenized and aliquots of homogenate supernatants, obtained by centrifugation (10 000 *g* for 10 min), were used for RP-HPLC on a TSK ODS SIL 120A column (Tosoh Co.). A linear gradient of acetonitrile (CH_3CN) from 10% to 60% in 0.1% trifluoroacetic acid (pH 2.0) was used at a flow rate of 1.0 mL/min for 50 min. All fractions were analyzed for HBD-2 employing RIA.

Statistical analysis and ethical considerations

Statistical analyses were performed using Fisher's exact, χ^2, Student's *t*, Mann-Whitney *U*, Kruskal-Wallis, Spearman rank and Wilcoxon signed ranks tests, as appropriate. A *P* value of less than 0.05 was accepted as statistically significant. Data were expressed as mean±SD.

All examinations were conducted according to Good Clinical Practice and the Declaration of Helsinki, and were approved by the university ethics committees. All samples were obtained with written informed consent of the patients prior to their inclusion in the study. All experiments involving animals were approved by the ethics review committees for animal experimentation of participating universities.

RESULTS

Patient demographics

The study population consisted of 49 *H pylori*-positive and 33 *H pylori*-negative subjects. They included 38 men and 44 women, with mean age of 50 years (range, 25-78 years). There were no significant differences between the *H pylori*-positive and -negative groups in background data on age, sex, body mass index, current tobacco use and alcohol intake (Table 1).

Table 1 Baseline characteristics

	H pylori-positive (n = 49)	*H pylori*-negative (n = 33)
Mean age, ys (range)	49.9 (25-72)	49.6 (28-78)
Male/female	24/25	14/19
Smoker	17 (34.7%)	12 (36.4%)
Alcohol drinker	16 (32.7%)	10 (30.3%)
Body mass index (range)	24.4 (17.1-29.0)	23.7 (16.4-28.8)

Plasma and gastric juice β-defensins concentrations according to H pylori status

HBD-2 levels in gastric juice of patients with *H pylori* infection were significantly higher than those of *H pylori*-negative subjects (*P*<0.0005, Table 2), whereas infection had no significant impact on plasma concentrations of HBD-2 (Table 2). There were no significant differences in HBD-1 concentrations both in plasma and gastric juice with respect to *H pylori* status (Table 2). The levels of β-defensins in gastric juice did not correlate with those in plasma

(correlation coefficient, *r* = 0.036 and -0.005 for each HBD-1 and HBD-2). In addition, there were no significant correlations between HBD-1 and HBD-2 levels in plasma (*r* = -0.077) and gastric juice (*r* = -0.092).

Correlation between HBD-2 and cytokine levels in gastric juice

There were significant differences in IL-1β and IL-8 concentrations in gastric juice between *H pylori*-positive and -negative subjects (*P*<0.05 and *P*<0.005, respectively, Table 2). HBD-2 concentrations in gastric juice did not correlate with IL-1β and IL-8 levels (*r* = -0.055 and *r* = 0.121, respectively). There was a positive correlation between IL-1β and IL-8 concentrations (*r* = 0.575, *P*<0.0001).

Table 2 Plasma and gastric juice HBD-1 and HBD-2 levels and gastric juice IL-1β and -8 concentrations

	H pylori-positive (n = 49)	*H pylori*-negative (n = 33)	*P*
Plasma			
HBD[1] -1 levels (ng/mL)	10.1±0.9	9.6±1.8	NS
HBD-2 levels (pg/mL)	276.2±61.0	257.2±56.0	NS
Gastric juice			
HBD-1 levels (pg/mL)	384.2±101.5	357.9±79.6	NS
HBD-2 levels (pg/mL)	230.3±77.4	118.1±87.7	<0.0005
Interleukin 1β levels (mg/mL)	53.6±16.9	5.3±4.4	<0.05
Interleukin 8 levels (pg/mL)	17.9±3.1	8.6±1.7	<0.005

[1]Human β-defensin, NS: not significant.

Correlation between gastric juice HBD-2 concentrations and histopathological parameters

As shown in Table 3, HBD-2 concentrations in gastric juice correlated positively with the scores of activity and chronic inflammation in the corpus (*P*<0.005 and *P*<0.05, respectively), but not with glandular atrophy and intestinal metaplasia scores in the corpus. On the other hand, there were no significant correlations between HBD-2 concentrations and each score of gastritis in the antrum. In addition, *H pylori* density in the antrum and corpus did not correlate with HBD-2 concentrations in gastric juice.

Table 3 Correlation coefficients between gastric human-β-defensin 2 levels and each histological parameter

	Correlation coefficient	*P*
Antrum		
Activity	0.048	NS
Chronic inflammation	0.258	NS
Glandular Atrophy	0.148	NS
Intestinal Metaplasia	-0.063	NS
H pylori density	0.011	NS
Corpus		
Activity	0.499	<0.005
Chronic inflammation	0.341	<0.05
Glandular Atrophy	0.294	NS
Intestinal Metaplasia	0.081	NS
H pylori density	0.237	NS

NS: not significant.

Table 4 Gastric juice HBD-2 and IL-1β and -8 levels before and after anti-*H pylori* therapy

	Successful eradication of *H pylori* ($n = 10$)			Failed eradication ($n = 4$)		
	Before therapy	After therapy	P	Before therapy	After therapy	P
Gastric juice						
HBD[1]-2 levels (pg/mL)	182.2±57.6	210.1±66.4	NS	137.4±65.2	112.9±79.3	NS
Interleukin 1β levels (pg/mL)	17.7±10.2	0.5±0.3	<0.05	88.5±80.9	64.7±64.1	NS
Interleukin 8 levels (pg/mL)	22.3±2.1	12.8±1.8	<0.005	18.1±6.1	18.6±4.5	NS

[1]Human β-defensin, NS:not significant.

## Effects of eradication treatment on gastric juice concentrations of HBD-2, IL-1β and IL-8

Successful eradication of *H pylori* was achieved in 10 of 14 (71%) with the triple therapy. At 4 wk after completion of the 7-d treatment, HBD-2 concentrations in gastric juice of these patients were not significantly different from those measured before eradication therapy (Table 4). However, *H pylori* eradication was associated with a significant fall in IL-1β, and IL-8 levels of gastric juice compared with pre-treatment levels (*P*<0.05 and *P*<0.005, respectively, Table 4).

In patients who were still infected with *H pylori* after antibiotic treatment, HBD-2, IL-1β and IL-8 levels in gastric juice remain unchanged compared to the pre-treatment concentrations (Table 4).

Chromatographic characterization of HBD-2

Immunoreactive HBD-2 was identified in the gastric mucosa infected with *H pylori* on RP-HPLC coupled with RIA (Figure 1). The peak was situated at a position identical to that of synthetic HBD-2 peptide. Each sample prepared yielded the same chromatographic pattern.
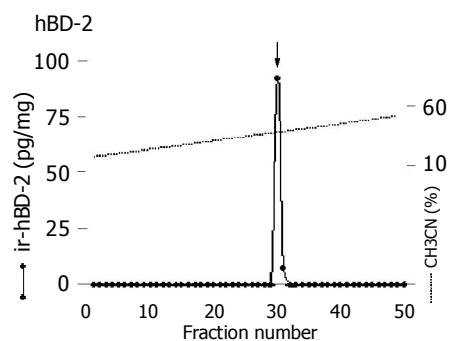
Figure 1 Representative RP-HPLC profiles of the HBD-2 molecules in *H pylori*-infected gastric mucosa.

DISCUSSION

Recent studies have shown the inducible expression of HBD-2 messenger ribonucleic acid (mRNA) in response to *H pylori* infection in cultured gastric epithelial cells[12-14]. In clinical setting of gastritis, the HBD-2 mRNA and peptide expression is evidently increased in gastric mucosa infected with the organism[15,16]. There are a few *in vitro* observations of the secretion of HBD-2 peptide upon *H pylori* infection. George *et al.*[26], showed its enhanced release in supernatants from infected gastric cancer cell lines by Western blotting. However, the actual concentrations of such antimicrobial peptides at the local site of human stomach during *H pylori* infection remain unknown. Our sensitive RIA system allowed us to determine the HBD-2 concentrations in gastric juice, as well as in other body fluids and blood[17,25]. Thus, we demonstrated, for the first time, that the HBD-2 levels in gastric juice of *H pylori*-infected patients were significantly higher than those of uninfected ones, suggesting its physio-pathological significance in the bacterial infection. In contrast, *H pylori* status did not have a significant impact on plasma HBD-2 levels. Immunoreactivity for HBD-2 was identified in *H pylori*-infected gastric mucosa by RT-HPLC combined with RIA. In agreement with the results of previous studies[15,16], immunohistochemical staining against anti-HBD-2 antibody within the infected mucosa was confined to the superficial epithelial cells. Taken together, we believe that *H pylori* infection stimulates the biosynthesis of HBD-2 in gastric mucosa, primarily in epithelia confronting this organism[15], and the product is mainly released into the gastric lumen.

In the present study, however, there was no significant difference in gastric juice, HBD-2 levels before and 4 wk after cure of the infection, and in certain cases, post-treatment HBD-2 levels were rather increased compared to the pre-treatment ones. In contrast, Hamanaka *et al.*[15], reported that epithelial HBD-2 expression assessed by immunohistochemistry was markedly decreased in gastric specimens obtained 4 wk after eradication of *H pylori*, concomitant with the pronounced reduction of its transcripts to undetectable levels by reverse transcription-polymerase chain reaction (RT-PCR). These results must be interpreted within the context of studies' limitations including their small sample sizes, differences in samples assessed-gastric biopsy tissues *vs* gastric juice- and diverse methodological approaches to HBD-2. Nevertheless, one might postulate the mingling of HBD-2 secreted by the other cells than gastric epithelial cells, such as esophageal squamous cells, in the gastric juice. In fact, more than 95% of biopsy specimens from the esophagus were positive for the β-defensin mRNA on RT-PCR analysis[16]. Several recent studies have shown that elimination of *H pylori* infection would lead to the development of gastro-esophageal reflux disease, probably in some cases, in association with post-treatment increase in acid secretion[27-29]. At present, however, there is no information available as to whether the esophageal HBD-2 expression is altered during this condition or upon such stimuli as refluxates with increased acidity.

We found the association of HBD-2 concentrations in gastric juice with histological degree of mucosal infiltration of neutrophils and mononuclear cells in the corpus, but not in the antrum. In line with this, Wehkamp *et al.*[16], reported that *H pylori* colonization was significantly related to higher percentage of positive biopsies taken from the corpus with respect to HBD-2 transcripts. The sparse distribution and confined localization to cells of the apical foveolae of this peptide[15,16] may be attributable to the topographical differences in HBD-2 expression. In addition to the direct anti-microbial activity, evidence is accumulating for the role of defensins as immune and inflammatory mediators[7-9,30]. In fact, β-defensins display chemotactic activities for dendritic cells and T lymphocytes through the chemokine receptor CCR6[30]. It is possible that β-defensins, especially HBD-2, may be involved in the pathogenesis of *H pylori*-associated gastritis, in part, via their action as chemoattractant. Again, the HBD-2 levels in gastric juice can provide useful information on histological severity of corporal mucosal inflammation.

Recent *in vitro* studies have demonstrated that HBD-2 mRNA expression is stimulated by pro-inflammatory cytokines such as IL-1β and tumor necrosis factor α in various cell lines including gastric epithelial cells[9,12,13]. We have also reported a positive correlation between IL-1β and HBD-2 concentrations in broncho-alveolar lavage fluid of patients with pneumonia and diffuse panbronchiolitis[17,23]. In the present study, however, there was no significant correlation between gastric juice HBD-2 and IL-1β concentrations, whereas the IL-1β levels strongly correlated with those of IL-8. IL-1β is known to activate nuclear factor kappa B (NF-κB)[31], an important transcription factor regulating a plethora of genes including those of IL-8 and HBD-2[9,31,32]. In this regard, we demonstrated that mucosal IL-8 levels of patients with *H pylori*-associated gastritis correlated strongly with the grades of activated NF-κB expression in gastric epithelial cells[20,22]. Considered together, these data suggest that IL-8 expression is probably enhanced by IL-1β-induced NF-κB activation within the *H pylori*-infected mucosa. On the other hand, we suggest that the increased expression of HBD-2 in the gastric epithelium may result from the direct contact between this pathogen and epithelial cells *in vivo*.

H pylori status had no impact on HBD-1 concentrations in gastric juice, providing further evidence for its constitutive nature[12-14,16]. Since ingestion of contaminated food or water exposes the gastric mucosa to a wide variety of microbial pathogens, the constitutive expression of HBD-1 could play a 'surveillance-like' role during normal homeostasis of human stomach[33].

The *in vitro* minimal inhibitory concentrations against a panel of micro-organisms range from 0.1 to 100 μg/mL for defensin peptides[9]. Hamanaka *et al.*[15], reported that the growth rate of cultured *H pylori* was suppressed by 50% on incubation with 0.3 μg/mL of HBD-2 and was completely inhibited at 30 μg/mL of this peptide. It remains to be determined whether β-defensins are microbicidal for the organism in gastric milieu *in vivo*. However, it is tempting to speculate that the *H pylori* strains that are able to establish persistent infection may be rather insensitive to such innate mucosal defense machinery.

In conclusion, we demonstrated the presence of significantly high levels of HBD-2 in gastric juice in patients with *H pylori* infection. The enhanced production/release of HBD-2 may contribute to the perpetuation of mucosal inflammation of the corpus. Our results highlight the importance of β-defensins, especially HBD-2, not only in innate defense but also in adaptive immune response to the organism.

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