Human β -defensin-3 induction in *H. pylori*-infected gastric mucosal tissues

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Abstract

AIM: To examine human β -defensin-3 (hBD-3) expression in inflamed gastric mucosal tissues or MKN45 gastric cancer cells with or without *H. pylori* infection for better understanding the innate immune response to *H. pylori*.

METHODS: We used reverse transcription-polymerase chain reactions and immunohistochemistry to examine hBD-3 expression in inflamed gastric mucosal tissues or MKN45 gastric cancer cells with or without *H. pylori*. Effects of hBD-3 against *H. pylori* were also evaluated.

RESULTS: The mean mRNA expression of hBD-3 in *H. pylori*-positive specimens was significantly higher than that in *H. pylori*-negative specimens ($P = 0.0002$, Mann-Whitney). In addition, unlike uninfected samples, 8 of 15 (53.33%) infected mucosal samples expressed hBD-3 protein. *H. pylori* dose-dependently induced mRNA expression of hBD-3 in MKN45 cells, an effect inhibited by adding anti-toll-like receptor (TLR)-4 antibody. hBD-3 protein completely inhibited *H. pylori* growth.

CONCLUSION: Our results suggest that like hBD-2, hBD-3 may be involved in the pathophysiology of *H. pylori*-induced gastritis.

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Key words: Human β -defensin-2; Human β -defensin-3; *H. pylori*; Gastric mucosa

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INTRODUCTION

Defensins are antimicrobial peptide components of the innate immune system. Three subfamilies, α -defensins, β -defensins, and θ -defensins, distinguished according to structural features at the gene and protein levels, have been identified in vertebrates^[1-3]. Four human β -defensins (hBD) have recently been characterized in various human epithelial cells. hBD-1 mRNA is expressed constitutively in various epithelial tissues^[1-2]. hBD-2 mRNA expression has been detected in epithelial cells of skin, lung, trachea, and urogenital tract; expression can be induced by treatment with tumor necrosis factor (TNF)- α or interleukin (IL)-1, or by exposure to microorganisms^[1-9]. Both hBD-1 and hBD-2 show antimicrobial activity, predominantly against Gram-negative bacteria. In skin, tonsil, and trachea, hBD-3 mRNA expression can be induced in epithelial cells by treatment with TNF- α or contact with *Pseudomonas aeruginosa*^[9]. hBD-3 protein shows antimicrobial activity against both Gram-negative and Gram-positive bacteria^[9]. Intense hBD-4 mRNA expression has been found in testis and gastric antrum, with lower, presumably constitutive, expression observed in uterus, thyroid gland, lung, kidney, and neutrophils^[10]. hBD-4 mRNA expression can be induced by treatment with phorbol 12-myristate 13-acetate (PMA) or contact with *P. aeruginosa* or *Streptococcus pneumoniae* in SAEC 6043 small airway epithelial cells, but not by IL-1, IL-6, interferon (IFN)- γ , or TNF- α . hBD-4 shows antimicrobial activity against both Gram-negative and Gram-positive bacteria^[10]. However, isolation of natural hBD-4 has not yet been reported.

Recently, induction of hBD-2 mRNA expression by *H. pylori* has been shown in human gastric cancer cell lines (MKN 45 and AGS)^[4-8]. Gastric colonization by *H. pylori*, which is Gram negative, is pathogenetically important in gastritis, peptic ulcer, gastric cancer, and gastric mucosa-associated lymphoid tissue lymphoma. However, hBD-3 mRNA expression in gastric mucosal tissues has not been fully characterized^[1].

To better understand the innate immune response to *H. pylori*, we determined hBD-3 expression in various gastric mucosal tissues with or without *H. pylori* infection using a semiquantitative TaqMan reverse transcription-

polymerase chain reaction (RT-PCR) assay as well as immunohistochemistry. Additionally, the antimicrobial effect of hBD-3 against *H. pylori* was evaluated.

MATERIALS AND METHODS

Bacterial strain and antibodies

H. pylori (ATCC49504) was used for hBD-2 mRNA and hBD-3 mRNA induction. Anti-toll-like receptor (TLR)-4 antibody (Clone: HTA125) and non-immune subclass-matched antibody (IgG_{2a}) were purchased from BD Biosciences Pharmingen. Polyclonal goat antibodies against hBD-2 and hBD-3 were purchased from Santa Cruz Biotechnology.

HBD-2 mRNA and hBD-3 mRNA induction in MKN45 gastric cancer cells

MKN-45 gastric cancer cells were cultured in RPMI 1640 medium (Bio Whittaker) supplemented with heat-inactivated fetal bovine serum (FBS) (JRH BIOSCIENCES) at 37°C in an humidified atmosphere containing 50 mL/L CO₂. Induction of hBD-2 mRNA and hBD-3 mRNA was carried out as described previously^[7,8]. Briefly, 10⁶ MKN45 cells were seeded into dishes 60 mm in diameter and incubated for 12 h. Culture medium was replaced with 2 mL of fresh RPMI 1640 medium without FBS. Bacterial suspensions (100 µL; 0 to 10⁹ CFU/mL in RPMI 1640 medium) were added to the dishes, and incubation was continued for various time periods.

Tissue samples

Samples of non-cancerous mucosa with or without chronic gastritis were obtained from 25 patients with previously untreated gastric cancer following surgery at Sapporo Medical University Hospital. Informed consent was obtained from all patients. After tissue removal, all samples were immediately frozen and fixed in 100 mL/L formalin.

Determination of *H. pylori* infection

Sections were Giemsa-stained, and the rapid urease test (CLO test, Tri-Med Specialties Inc) was performed with fresh samples taken from the prepyloric antrum, greater curvature of the corpus, and fundus^[12]. *H. pylori* infection was defined as positive when *H. pylori* was detected and/or the CLO test was positive.

Quantitative RT-PCR assays for hBD-2 mRNA and hBD-3 mRNA

ISOGEN (Nippon Gene) was used to extract total RNA from cells or tissues, and this extract was assayed for RNA with the GeneQuant DNA/RNA calculator (Amersham Pharmacia Biotech). For quantitative RT-PCR, fluorescent hybridization probes, TaqMan PCR Core Reagents kit with AmpliTaq Gold (Perkin-Elmer Applied Biosystems) were used with the ABI Prism 7700 Sequence Detection System (Perkin-Elmer Applied Biosystems). Expression of hBD mRNA was quantified as previously described^[8,13]. Primers and TaqMan probe for hBD-2 mRNA were

as follows: 5'-TGGTGGTATAGGCGATCCTGTT-3' (forward) and 5'-GGAGACCACAGGTGCCAATTT-3' (reverse); 5'-CCATATGTCATCCAGTTCTT-3' (TaqMan probe). Primers and TaqMan probe for hBD-3 mRNA were as follows. 5'-AGTGACCAAGCACACCTTTTCA-3' (forward) and 5'-CCAAAAACAGGAAGAGCAAAGC-3' (reverse); 5'-TATGAGGATCCATTATCTTCTGTT-3' (TaqMan probe). Aliquots of 25 ng of total RNA from samples was used for one-step RT-PCR. Conditions of one-step RT-PCR were as follows: 30 min at 48°C (stage 1, reverse transcription), 10 min at 95°C (stage 2, RT inactivation and AmpliTaq Gold activation), and then 40 cycles of amplification for 15 s at 95°C and 1 min at 60°C (stage 3, PCR). Data were normalized as the ratio of hBD-2 optical densities relative to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and were represented as arbitrary units.

Immunostaining

Formalin-fixed, paraffin-embedded tissue sections were stained with polyclonal goat antibodies against hBD-2, hBD-3 or non-immune goat serum using an indirect immunoperoxidase technique.

Antimicrobial assay

To evaluate the antimicrobial effects of hBD-2 and hBD-3 on *H. pylori*, 25 µL of 4 × 10⁶ CFU/mL *H. pylori* strain (ATCC49504) was cultured on HP agar (Eikenkagaku) after a 1-h pre-incubation at 37°C in the presence or absence of hBD-2 protein and hBD-3 protein (Peptide Institute). To determine the CFU number, the pre-incubation mixture was immediately diluted 100-fold with culture medium, and samples were cultured in triplicate. Viable cells (CFU/mL) were counted after 3 d in culture at 37°C.

RESULTS

Induction of hBD-2 mRNA and hBD-3 mRNA expression in MKN45 cells and various gastric mucosal tissue specimens

To clarify the effects of *H. pylori* on hBD-2 mRNA and hBD-3 mRNA expression, according to TaqMan RT-PCR, MKN45 cells first were incubated for 0 to 72 h with *H. pylori*. hBD-2 mRNA and hBD-3 mRNA expression was detected in MKN45 cells 12 h after initiating incubation with *H. pylori*, and then increased gradually (Figure 1).

To determine a suitable number of *H. pylori* bacteria for induction of hBD-2 mRNA and hBD-3 mRNA expression, 100 µL of 0 to 10⁹ CFU/mL *H. pylori* was incubated with MKN45 cells for 48 h, representing multiple inoculum sizes. hBD-2 mRNA and hBD-3 mRNA expression was up-regulated in a manner dependent on bacterial number (Figure 2), an effect first detectable at 10⁷ CFU/mL.

To evaluate the effect of *H. pylori* colonization in gastric tissues on hBD-2 and hBD-3 expression, mucosal samples showing gastritis from 15 *H. pylori*-positive and 10 *H. pylori*-negative patients were assessed by TaqMan RT-PCR

Figure 1 Time course of human β -defensin mRNA expression induced by *H. pylori* in a gastric cancer cell line MKN45. MKN45 cells (10^6) were seeded into dishes 60 mm in diameter and incubated for 12 h. Culture medium was replaced with 2 mL of fresh RPMI 1640 medium without FBS. The cells were incubated for 0 to 72 h with 100 μ L of 10^8 CFU/mL *H. pylori*. Human β -defensin mRNA expression was measured using TaqMan RT-PCR assay. hBD-2 ($\square$), hBD-3 ($\blacksquare$).

Figure 2 Induction of human β -defensin mRNA expression by various numbers of *H. pylori*. MKN45 cells (10^6) were seeded into dishes 60 mm in diameter and incubated for 12 h. Culture medium was replaced with 2 mL of fresh RPMI 1640 medium without FBS. Bacterial suspensions (100 μ L; 0 to 10^9 CFU/mL in RPMI 1640 medium) were added to the dishes, and incubation was continued for 48 h. Human β -defensin mRNA expression was measured using TaqMan RT-PCR assay. hBD-2 ($\square$), hBD-3 ($\blacksquare$).

Figure 3 Human β -defensin mRNA expressions in gastric mucosal tissues from 15 *H. pylori*-positive patients ($\circ$) and 10 *H. pylori*-negative patients ($\bullet$). Human β -defensin mRNA expression was measured using TaqMan RT-PCR assay. A: hBD-2; B: hBD-3.

analysis and immunostaining. Mean mRNA expression of hBD-2 (8.5) and hBD-3 (45.0) in *H. pylori*-positive specimens was significantly higher than that in hBD-2 (1.6) and hBD-3 (3.9) in *H. pylori*-negative specimens, respectively (Figure 3A and B; $P = 0.002$, Mann-Whitney). However, levels of hBD-3 mRNA expression did not correlate with those of hBD-2 mRNA expression in *H. pylori*-positive specimens. In addition, 7 and 8 of 15 *H. pylori*-positive patients with gastritis showed hBD-2 and hBD-3 protein expression in their mucosa, respectively, while none of the *H. pylori*-negative patients showed hBD-2 and hBD-3 protein expression (Figure 4A-D).

Inhibition of induction of hBD-2 mRNA and hBD-3 mRNA expression in MKN45 cells by treatment with anti-TLR-4 antibody

To determine whether hBD-2 and hBD-3 mRNA expression in MKN45 cells was induced via TLR-4, MKN45 cells were incubated for 40 h with 100 μ L of 10^9 CFU/mL of *H. pylori* in the presence or absence of anti-TLR-4 antibody (100 μ g/mL) and control antibody

(100 μ g/mL). Expression of hBD-2 and hBD-3 mRNA in *H. pylori*-exposed MKN45 cells treated with anti-TLR-4 antibody was reduced to 55.7% and 64.1% of the expression in untreated MKN45 cells, respectively.

Antimicrobial effects of hBD-2 and hBD-3 against *H. pylori*

To evaluate the antimicrobial effects of hBD-2 and hBD-3 on *H. pylori*, *H. pylori* was cultured on HP agar for 4 d after a 1-h pre-incubation in the presence or absence of chemically synthesized hBD-2 and hBD-3. At concentrations of 50 μ g/mL or more, hBD-2 and hBD-3 inhibited growth of *H. pylori* (Figure 5A and B).

DISCUSSION

Using TaqMan RT-PCR for hBD-3 mRNA and immunostaining for hBD-3 protein, we demonstrated, probably for the first time, that like hBD-2, hBD-3 is frequently expressed in gastric mucosa with *H. pylori* infection showing gastritis, but not in inflamed mucosa without *H. pylori* infection. The amount of hBD-3 mRNA

Figure 4 Human β -defensin protein expressions in gastric mucosal tissues with or without *H. pylori* infection. Tissues were stained with anti-hBD-2 antibody (A and C) or hBD-3 antibody (B and D). Case 1: A and B, gastric mucosa with *H. pylori*-associated gastritis. Immunostaining was observed in gastric cancer cells in A and gastric epithelial cells in B. Case 2: C and D, gastric mucosa with gastritis but without *H. pylori* infection. No staining was observed in C and D.

Figure 5 Antimicrobial effects of human β -defensin protein on *H. pylori* (ATCC49504). *H. pylori* were cultured on HP agar for 4 d after a 1-h pre-incubation in the presence or absence of hBD-2 (A) and hBD-3 (B).

expression in *H. pylori*-positive specimens was 11.5 times higher than in *H. pylori*-negative specimens. In addition, hBD-3 was induced by contact of MKN45 cells with *H. pylori*. Recent reports indicated that hBD-3 mRNA expression was induced in primary cultures of tracheal epithelial cells by pro-inflammatory cytokines, such as TNF- α , and in the MKN7 gastric cancer cell line by contact with *H. pylori*. Considered together with recent reports, our results implied that contact of gastric epithelial cells with *H. pylori* as well as amounts of pro-inflammatory cytokines are important for induction of hBD-3 mRNA expression.

At 10^{-5} mol/L, chemically synthesized hBD-2 (30 µg/mL) was reported to completely inhibit growth of *H. pylori*, while recombinant hBD-3 began to inhibit growth of *H. pylori* at a concentration of 10^{-7} mol/L^[5,11]. In the present study, we demonstrated that synthesized hBD-3 as well as hBD-2 inhibited *H. pylori* growth at concentrations of 50

µg/mL or more.

Isomoto *et al*^[14] detected activated nuclear factor (NF)- κ B in epithelial cells in gastric mucosa from patients with *H. pylori*-associated gastritis. Diamond *et al*^[15] reported that expression of hBD-3 was regulated by NF- κ B-independent mechanisms, that remain to be characterized. Su *et al*^[16] reported that *H. pylori* up-regulated TLR-4 expression in two gastric cancer cell lines (AGS and MKN45). These reports suggest that *H. pylori* may induce hBD-3 mRNA expression via direct or indirect activation of NF- κ B or other mechanisms. Pathogen-associated molecular patterns in *H. pylori* and pattern recognition receptors in gastric epithelial cells will require further study. In the present investigation, anti-TLR-4 antibody inhibited, but not completely, the expression of hBD-3 mRNA in MKN45 cells induced by contact with *H. pylori*. This suggests that not only *H. pylori* binding to TLR-4, but also other mechanisms may exist for the induction

of hBD-3 mRNA in gastric epithelial cells by *H pylori* infection. In addition, in the present study, levels of hBD-3 mRNA expression did not correlate with those of hBD-2 mRNA expression in *H pylori*-positive specimens. This also suggests that the expression of hBD-3 and hBD-2 mRNA in gastric epithelial cells is likely to be regulated differently.

In conclusion, expression of hBD-3 increases in response to *H pylori* infection with a bacterial effect. Further study is needed to detect the role and regulating mechanism of hBD-3.

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