Human β-defensin-2 induction in Helicobacter pylori-infected gastric mucosal tissues: antimicrobial effect of overexpression

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INTRODUCTION

Defensins are antimicrobial peptide components of the innate immune system against micro-organisms. Two subfamilies, α- and β-defensins, distinguished according to structural features at the gene and protein levels, have been identified in vertebrates (Diamond & Bevins, 1998). Three β-defensins were recently characterized in various human epithelial tissues. Human β-defensin (hBD)-1 mRNA is expressed constitutively in various epithelial tissues (Harder et al., 1997). hBD-2 mRNA expression is detected in epithelial cells of the skin, lung, trachea and urogenital tract and can be induced by treatment with tumour necrosis factor (TNF)-α or interleukin (IL)-1 or by exposure to micro-organisms (Wada et al., 1999, 2001; Hamanaka et al., 2001; O’Neil et al., 2000). Both hBD-1 and hBD-2 show antimiicrobial activity, predominantly against Gram-negative bacteria. hBD-3 mRNA, expressed in the skin, tonsil and trachea, can be induced in epithelial cells by treatment with TNF-α or contact with Pseudomonas aeruginosa and shows antimicrobial activity against Gram-negative and Gram-positive bacteria (Harder et al., 2001).

Recently, induction of hBD-2 mRNA expression by Helicobacter pylori has been shown in human gastric cancer cell lines (MKN45 and AGS) (Wada et al., 1999, 2001; Hamanaka et al., 2001; O’Neil et al., 2000). 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-2 mRNA expression in gastric mucosal tissues has not been fully characterized.

To understand the innate immune response to H. pylori, we determined hBD-2 expression in various gastric mucosal tissues with or without H. pylori infection using a semi-quantitative TaqMan RT-PCR assay as well as immunohistochemistry. Additionally, the antimicrobial effect of hBD-2 against H. pylori was evaluated in transfection experiments.

METHODS

Bacterial strains. H. pylori ATCC 43504T, Salmonella typhimurium ATCC 14028, Escherichia coli ATCC 25922, Staphylococcus aureus ATCC 25923 and Enterococcus faecalis ATCC 33186 were used for hBD-2 mRNA induction.

hBD-2 mRNA induction in MKN45 gastric cancer cells. MKN45 gastric cancer cells were cultured in RPMI 1640 medium (BioWhitaker) supplemented with heat-inactivated fetal bovine serum (FBS) (JRH Biosciences) at 37°C in an atmosphere including 5% CO2. Induction of hBD-2 mRNA was carried out as described by Wada et al. (1999). Briefly, 106 MKN45 cells were seeded into dishes 60 mm in diameter and incubated for 12 h. Culture medium was replaced with 2 ml fresh RPMI 1640 medium without FBS. Bacterial suspensions
Infection was defined as positive when CLO test was positive.

Incubation at 37°C by a limiting-dilution method.

The pre-incubation mixture was diluted 100-fold immediately and fixed in 10% formalin. All histological factors were evaluated according to the criteria of the Japanese Research Society for Gastric Cancer (1995).

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 from the prepyloric antrum, greater curvature of the corpus and fundus (Marshall et al., 1987). H. pylori infection was defined as positive when H. pylori was detected and/or the CLO test was positive.

Quantitative RT-PCR assay for hBD-2 mRNA. ISOGEN (Nippon Gene) was used to extract total RNA from cells or tissues and this extract was assayed for RNA with the Gene Quant DNA/RNA calculator (Amersham Pharmacia Biotech). For quantitative RT-PCR, fluorescent hybridization probes and the 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-2 mRNA was quantified by methods reported previously (Yajima et al., 1998). Primers and the TaqMan probe for hBD-2 mRNA were as follows: forward primer, 5′-ATCGAGCTCTTTTGCCCTAGAAGGTA-3′; reverse primer, 5′-GGCTTTTGGACAGATTGTGTTGTC-3′; TaqMan probe, 5′-AACAAAATTCGACCTGGGTGTCC-3′. Aliquots of 25 ng total RNA from samples were 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 for hBD-2 mRNA were normalized to data for glyceraldehyde-3-phosphate dehydrogenase.

Immunostaining. Formalin-fixed, paraffin-embedded tissue sections were stained with polyclonal goat antibody against hBD-2 (Santa Cruz Biotechnology) or non-immune goat serum using an indirect immunoperoxidase technique.

Vector construction and generation of hBD-2-producing cells. The full-length hBD-2 gene was amplified by RT-PCR using hBD-2-specific primers (forward primer, 5′-GGGATCCATGAGGGTCTTGACATGCGATGCCT-3′; reverse primer, 5′-GGGAAATTCGGGATGCCTCATGCGCTTTTGAGCAATTTGTTGTGC-3′). Total cellular RNA was extracted from the human oral cancer cell line HSC2 as a template. The amplified product was digested with BamHI and inserted at the BglII site of vector pCAcC (Yoshida & Hamada, 1997). The sequence and orientation of the hBD-2 gene in the vector were confirmed by sequencing. This construct was designated pCAhBD-2. Briefly, ST3T2 cells were co-transfected with pCAhBD-2 and a plasmid containing a neomycin-resistance gene using LipofectAmine (Gibco-BRL) according to the manufacturer’s instructions. After selection with G418 (500 μg ml−1; Gibco-BRL), hBD-2 gene-transfected cells were cloned by a limiting-dilution method.

Antimicrobial assay. To evaluate the antimicrobial effect of hBD-2 on H. pylori, 25 μl of a suspension of 4 × 105 c.f.u. H. pylori strain ATCC 43504T ml−1 was cultured on HP agar (Eikenkagaku) after 2–4 h pre-incubation at 37°C in the presence or absence of culture supernatant obtained from hBD-2-3T3J2-1 cells. To determine the number of c.f.u., the pre-incubation mixture was diluted 100-fold immediately with culture medium and samples were cultured in triplicate. Viable cells (c.f.u. ml−1) were counted after 5 days in culture at 37°C.

RESULTS AND DISCUSSION

Induction of hBD-2 mRNA expression in MKN45 cells and expression of hBD-2 in various gastric mucosal tissues

To clarify the effect of H. pylori on hBD-2 mRNA expression by using TaqMan RT-PCR, MKN45 cells were first incubated for 1–20 h with H. pylori. hBD-2 mRNA expression was detected in MKN45 cells 1 h after starting incubation with H. pylori and reached a maximum at 10 h (Fig. 1).

To determine a suitable number of H. pylori bacteria for induction of hBD-2 mRNA expression, 100 μl aliquots of suspensions containing 0–108 c.f.u. H. pylori ml−1 were incubated with MKN45 cells for 7.5 h. hBD-2 mRNA expression was up-regulated in a manner dependent on numbers of bacteria (Fig. 2), being first detectable at 107 c.f.u. ml−1.

To determine whether other species of bacteria could induce hBD-2 mRNA expression in MKN45 cells, the cells were exposed to Salmonella typhimurium, Escherichia coli, Staphylococcus aureus or Enterococcus faecalis for 7.5 h. hBD-2 mRNA expression in MKN45 cells was induced by all species of bacteria assessed in this study (Fig. 3). Gram-negative bacteria were more effective than Gram-positive bacteria in inducing hBD-2 mRNA expression in MKN45 cells (Fig. 3).

To evaluate the effect of H. pylori colonization in gastric tissues on hBD-2 expression, gastric cancer and paired adjacent mucosa showing gastritis from four H. pylori-positive and three H. pylori-negative patients were assessed by TaqMan RT-PCR analysis and immunostaining. In H. pylori-positive specimens, the mean expression of hBD-2 was significantly higher than in H. pylori-negative specimens (Fig. 4).

Fig. 1. Time-course of hBD-2 mRNA expression induced by H. pylori in a gastric cancer cell line. MKN45 cells (105) were seeded into dishes 60 mm in diameter and incubated for 12 h. Culture medium was replaced with 2 ml fresh RPMI 1640 medium without FBS. The cells were incubated for 1–20 h with 100 μl of a suspension of 108 c.f.u. H. pylori ml−1. hBD-2 mRNA expression was measured by TaqMan RT-PCR.
mRNA was 26.5 (Fig. 4). In contrast, the mean expression of hBD-2 mRNA in H. pylori-negative specimens was 0.27 (Fig. 4). The difference was significant (P = 0.028; Mann–Whitney U test).

hBD-2 protein was detected in gastric cancers and paired adjacent non-neoplastic tissue showing gastritis from H. pylori-positive patients, but not in specimens from two of three H. pylori-negative patients (Fig. 5).

Using TaqMan RT-PCR for hBD-2 mRNA and immunostaining for hBD-2 protein, we demonstrated that hBD-2 is expressed in gastric mucosa with H. pylori infection showing gastritis, but not in inflamed mucosa without H. pylori infection. In addition, hBD-2 mRNA expression was detected in gastric cancers from patients with H. pylori infection and hBD-2 mRNA expression was induced in the MKN45 gastric cancer cell line according to the intensity of H. pylori exposure. However, the level of expression of hBD-2 mRNA was variable in inflamed gastric mucosa and in cancers. A recent report has indicated that IL-1 and TNF-α can induce hBD-2 mRNA expression and that H. pylori, but not culture filtrate, increased hBD-2 mRNA expression in MKN45 cells (Wada et al., 1999). These results imply that contact of gastric epithelial cells with H. pylori and the amounts of proinflammatory cytokines are important in induction of hBD mRNA expression. In addition, the magnitude of gastritis was variable in our cases. Our results might reflect the number of H. pylori cells in the gastric mucosa or influences of other factors such as proinflammatory cytokines.

Isomoto et al. (2000) detected activated NF-κB in epithelial cells in gastric mucosa of patients with H. pylori-associated gastritis. Recent reports suggest that only H. pylori strains (type I) that carry a cag pathogenicity island (PAI) induce activation at the NF-κB site of the hBD-2 promoter (Wada et al., 1999, 2001). In the present study, all clinical H. pylori isolates from four patients with H. pylori infection had a cagA (data not shown), and as did H. pylori ATCC 43504T. Moreover, exposure of MKN45 cells to Salmonella typhimurium ATCC 14028, Escherichia coli ATCC 25922, Staphylococcus aureus ATCC 29523 and Enterococcus faecalis ATCC 33186 resulted in induction of hBD-2 mRNA. These results suggested that H. pylori (cag PAI) and other pathogens may induce hBD-2 mRNA expression via direct or indirect activation of NF-κB. Salmonella species have pathogenicity
islands (SPI 1 and 2) that may be important in induction of hBD mRNA expression. Pathogen-associated molecular patterns in these bacterial species and pattern-recognition receptors in MKN45 cells should be studied.

Assessment of expression of hBD-2 in hBD-2-gene-transfected cells and antimicrobial effect of medium from transfected cells

hBD-2 mRNA expression and secretion of hBD-2 protein into the culture medium were confirmed by the TaqMan RT-PCR for hBD-2 described above and by immunoblot analysis using anti-hBD-2 polyclonal antibody (Fig. 6). A mouse embryonic fibroblast clone showing high production of hBD-2 protein, hBD-2-3T3J2-1, was selected for further study.

Culture supernatants from hBD-2-3T3J2-1 cells were used to evaluate the antimicrobial effect of overexpressed hBD-2 against H. pylori. Aliquots of 25 µl (4 × 10⁶ c.f.u. ml⁻¹) of H. pylori ATCC 43504 were cultured on HP agar for 3 days. The mean numbers of c.f.u. of H. pylori after 0, 1, 2 and 4 h of pre-incubation with the culture supernatant (or with control medium) were respectively approximately 10⁵ (10⁵), 0 (10⁵), 0 (10⁵) and 0 (82). Thus, growth of H. pylori was inhibited completely after 1 h of incubation with the culture supernatant.

It has been reported that, at 10⁻³ M, chemically synthesized hBD-2 inhibits growth of H. pylori completely (Hamanaka et al., 2001). Schroeder & Harder (1999) reported that the LD₅₀

Fig. 5. hBD-2 protein expression in gastric cancer and paired adjacent tissue showing gastritis with or without H. pylori infection. Tissues were stained with anti-hBD-2 antibody. (a) and (b) Case 1: gastric cancer (moderately differentiated adenocarcinoma) and mucosa with gastritis with H. pylori infection. Positive staining was observed in gastric cancer cells in (a) and gastric epithelial cells in (b). (c) and (d) Case 2: gastric cancer (poorly differentiated adenocarcinoma) and mucosa with gastritis but without H. pylori infection. No positive staining was observed in (c) or (d). Magnification, ×120.

Fig. 6. Detection of hBD-2 protein in culture medium of HBD-2 gene-transfected 3T3J2 cells (lane 3), designated hBD-2-3T3J2-1, and parent 3T3J2 cells (lane 2) by immunoblot analysis using anti-hBD-2 antibody. Lane 1, RPMI 1640 medium control.

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values of natural hBD-2 preparations against *Escherichia coli*, *Pseudomonas aeruginosa* and *Candida albicans* were respectively 10, 10 and 25 μg ml<sup>-1</sup>. In the present study, we demonstrated that hBD-2-3T3J2-1 cells could secrete hBD-2 protein into the culture medium and that this protein inhibited growth of *H. pylori* completely. In conclusion, hBD-2 originating from the epithelium clearly can be bactericidal for *H. pylori*, yet is elevated in infection. This suggests a role for hBD-2 in the pathophysiology of *H. pylori* infection that has yet to be defined.

REFERENCES


