METHODS

Bacterial strains. *H. pylori* ATCC 43504, Salmonella typhimurium ATCC 14028, Escherichia coli ATCC 25922, Staphylococcus aureus ATCC 25923 and Enterococcus faecalis ATCC 33186 were used for hBD-2 mRNA induction. The objective of this study was to understand more of the innate immune response to *Helicobacter pylori* by determining the expression of human β-defensin-2 (hBD-2) in various gastric mucosal tissues and MKN45 gastric cancer cells with or without *H. pylori*. Semi-quantitative TaqMan RT-PCR and immunohistochemistry were carried out. The antimicrobial effects of a transfected hBD-2 gene against *H. pylori* were also evaluated. The results showed that hBD-2 was expressed in inflamed gastric mucosal tissues with *H. pylori* infection, but not in the absence of *H. pylori* infection. Expression was also detected in gastric cancers in patients with *H. pylori* infection. Expression was induced in the MKN45 gastric cancer cell line by *H. pylori* in a manner dependent on the abundance of bacteria. hBD-2-transfected 3T3J2-1 cells secreted hBD-2 protein into the culture medium and this protein inhibited growth of *H. pylori*, completely. The results suggest that hBD-2 may be involved in the pathophysiology of *H. pylori*-induced gastritis.
Infection was defined as positive when CLO test was positive.

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).

To evaluate the antimicrobial effect of hBD-2 on H. pylori on 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 lower than that in H. pylori-negative specimens, indicating the suppressive effect of H. pylori on hBD-2 expression.

**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 0–10^10 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 10^7 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).

**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'-GGGATCCATGAGGGTCTTGACATTTGCCTGCTCCTC-3'; reverse primer, 5'-GGGAAATTGCGATCCCGTGGCTTTGTCGTGATGATTTG-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 pCABD-2. Briefly, ST312 cells were co-transfected with pCABD-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 dilution-plating method.
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 1) 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 cag A (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 2923 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.](image-url) 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.](image-url) 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.
values of natural hBD-2 preparations against Escherichia coli, Pseudomonas aeruginosa and Candida albicans were respectively 10, 10 and 25 μg ml⁻¹.

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


