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 antimicrobial 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 35186 were used for hBD-2 mRNA induction. 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 with obtained from hBD-2-3T3J2-1 cells. To determine the number of c.f.u., (500/ml) to the manufacturer's instructions. After selection with G418 neomycin-resistance gene using LipofectAmine (Gibco-BRL) according cells were co-transfected with pCAhBD-2 and a plasmid containing a orientation of the hBD-2 gene in the vector were confirmed by of vector pCAcc (Yoshida & Hamada, 1997). The sequence and amplified product was digested with extracted from the human oral cancer cell line HSC2 as a template. The specific primers (forward primer, 5'-ATCGTCAGCTTGGGTCATGAGGGTCTTG-3'; reverse primer, 5'-GGCTTTATCGATGGCATCG-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 immuno- peroxidase 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'-GGGAATTCCATGGATCCTTGTTGCTTCCT-3'; reverse primer, 5'-GGGAAATTCCATGGATCCTTGTTGCTTCCTG-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 pCABB-2. Briefly, ST3J2 cells were co-transfected with pCABB-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 using a dilution-diffusion method.

Antimicrobial assay. To evaluate the antimicrobial effect of hBD-2 on H. pylori, 25 µl of a suspension of 4 × 10⁶ c.f.u. H. pylori strain ATCC 43504/ml was cultured on HP agar (Eiken Kagaku) after 1–4 h of 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 0–10⁶ 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 10⁶ 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 typhiurium, 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...
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 43504'. Moreover, exposure of MKN45 cells to *Salmonella typhimurium* ATCC 14028, *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 25923 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 10⁻⁵ M, chemically synthesized hBD-2 inhibits growth of *H. pylori* completely (Hamanaka et al., 2001). Schroder & Harder (1999) reported that the LD₉₀ of incubation with the culture supernatant (or with control medium) were respectively approximately 10⁻⁵ (10⁻⁵), 0 (10⁻⁵), 0 (10⁻⁵) and 0 (82). Thus, growth of *H. pylori* was inhibited completely after 1 h of incubation with the culture supernatant.
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


