PAPERS

Gastric leptin and *Helicobacter pylori* infection

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Abstract

Background—Leptin regulates feeding behaviour and therefore may be a mediator of anorexia associated with acute and chronic inflammation. Recently, leptin mRNA and leptin protein were found in the gastric epithelium.

Aim—The aim of the present study was to examine the effect of *Helicobacter pylori* infection on gastric leptin expression to investigate the pathophysiological role of gastric leptin.

Methods—Surgically resected human stomach tissues were subjected to immunohistochemistry and reverse transcriptase-polymerase chain reaction (RT-PCR) to check for the presence of leptin in the human gastric epithelium. A total of 201 *H pylori* positive patients with chronic gastritis underwent eradication therapy for *H pylori* and were examined for the effect of infection cure in terms of body mass index (BMI) and serum leptin levels. Biopsy specimens from the gastric fundic mucosa were obtained from 40 of the 201 patients before and three months after eradication therapy. These samples were subjected to quantitative RT-PCR to examine the effect of eradication therapy on leptin expression in the gastric fundic mucosa.

Results—Leptin immunoreactive cells were detected in the lower half of the gastric fundic glands and a leptin PCR product was also found in the gastric fundic mucosa. *H pylori* infection significantly increased gastric leptin expression. In addition, cure of *H pylori* infection significantly reduced gastric leptin expression, with a concomitant increase in BMI. In contrast, serum leptin levels did not change significantly after cure of *H pylori* infection.

Conclusion—Leptin is present in the human gastric mucosa. Gastric leptin may play a role in weight gain after eradication of *H pylori* infection. Gastric leptin may have a local rather than systemic action.

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Keywords: *Helicobacter pylori*; leptin; gastric mucosa; body mass index; gastritis

a crucial role in the homeostasis of body weight by regulating food intake and energy expenditure. Fasting and starvation decrease leptin mRNA and protein levels, and this substantial fall produces a signal to eat.¹⁻⁴ Leptin was initially reported to be secreted only by adipocytes and the placenta.¹⁻⁵ Recently, Bado *et al* reported that leptin mRNA and leptin protein were found in the rat gastric epithelium, and that cells in the glands of the gastric fundic mucosa were immunoreactive for leptin.⁶ They also reported that both feeding and administration of CCK-8 (the biologically active carboxy terminal end of cholecystokinin (CCK)) resulted in a rapid and marked decrease in both leptin cell immunoreactivity and leptin concentration in the fundic epithelium, with a concomitant increase in the concentration of leptin in plasma, suggesting that gastric leptin may be involved in early CCK mediated effects activated by food intake, possibly including satiety. In contrast, anorexia and loss of lean body mass are hallmark manifestations of acute or chronic disease, including infection or cancer.⁷⁻¹¹ *Helicobacter pylori* infection is a cause of chronic active gastritis.¹²⁻¹³ *H pylori* gastritis causes alarm symptoms such as anaemia, weight loss, and dysphagia in an as yet undefined subset of infected subjects.¹⁴⁻¹⁶ It has also been reported that *H pylori* infection influences growth rate in children.¹⁷⁻¹⁸ In addition, weight gain after curing *H pylori* infection has been reported.¹⁹ It is possible that gastric inflammation induced by *H pylori* infection affects expression of gastric leptin which may play a role in the regulation of food intake. However, the relationship between *H pylori* infection and gastric leptin expression has not been examined.

Therefore, we examined the effect of *H pylori* infection on gastric leptin expression to investigate the pathophysiological role of gastric leptin.

Methods**EXPERIMENTAL DESIGN**

Firstly, surgically resected human stomach tissue samples obtained from early gastric cancer patients (two cases; a 56 year old man and a 52

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Leptin, a product of the *ob* gene, is a 16 kDa protein synthesised by adipose tissue that plays

Abbreviations used in this paper: RT-PCR, reverse transcriptase-polymerase chain reaction; CCK, cholecystokinin; BMI, body mass index; UBT, urea breath test; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; TNF, tumour necrosis factor; IL, interleukin.

Figure 1 Immunostaining of leptin in the human fundic mucosa. Leptin immunoreactivity was seen in the lower half of the fundic epithelial glands (A). Higher magnification (B). Scale bars 10 µm.

year old woman) were subjected to immunohistochemistry and reverse transcriptase-polymerase chain reaction (RT-PCR) to check for the presence of leptin in the gastric epithelium.

Secondly, 201 *H pylori* positive patients with chronic gastritis (121 men and 80 women, mean age 41.5 years) were chosen from subjects who visited multiphasic health testing services held by the Second Department of Internal Medicine, Fukui Medical University. The services included upper gastrointestinal endoscopy or radiography to screen for gastric cancer. Chronic gastritis was diagnosed using these examinations.^{20, 21} Patients who had a history of peptic ulcers, reflux oesophagitis, malignant diseases, gall stones, or surgery, as well as those receiving non-steroidal anti-inflammatory drugs or antibiotics were excluded from the study. *H pylori* infection was diagnosed using the ¹³C urea breath test (UBT). Subjects fasted overnight prior to the UBT. Breath samples were collected before and 15 minutes after intake of a ¹³C urea solution (100 mg ¹³C urea in 100 ml water) and analysed using isotope ratio mass spectrometry. A rise in the delta value of ¹³CO₂ of more than 2.5‰ over baseline values was considered positive for *H pylori* infection. A standardised questionnaire was used to assess abdominal symptoms. This questionnaire addressed seven persistent or recurrent symptoms for more than three months which served as the primary target variables (upper abdominal pain, heart burn, regurgitation, nausea, vomiting, upper abdominal fullness, loss of appetite). The intensity of the respective symptoms was scored as 0 (absent), 1 (low intensity), 2 (moderate intensity), or 3 (severe). A total of 121 of

Figure 2 Reverse transcriptase-polymerase chain reaction analysis of leptin mRNA: negative control (lane 1), gastrocolic omentum (lane 2), and fundic mucosa (lane 3).

201 patients (60.2%) had dyspepsia symptoms. All 201 patients underwent eradication therapy for *H pylori* infection with omeprazole (20 mg twice daily), amoxicillin (750 mg twice daily), and clarithromycin (200 mg twice daily) for one week for gastric cancer prophylaxis. Cure of *H pylori* infection was evaluated using the UBT three months after treatment. Body weights and symptom scores of the patients were measured before and three months after eradication therapy. The change in body mass index (BMI kg/m²) after eradication therapy was compared between the eradicated and non-eradicated groups. Serum samples were obtained before and three months after eradication therapy in the morning after an overnight fast, and stored at -20°C until analysed.

Thirdly, biopsy specimens from the gastric fundic mucosa were obtained before and three months after eradication therapy from 40 of 201 patients (33 patients were successfully eradicated: 20 men and 13 women, mean age 42.2 years; and seven patients were not eradicated: four men and three women, mean age 41.6 years). These samples were subjected

to quantitative RT-PCR to examine the effect of eradication therapy on leptin expression in the gastric fundic mucosa. Biopsy specimens of the gastric fundic mucosa obtained from 20 *H. pylori* negative normal controls (13 men and seven women, mean age 40.8 years) were also subjected to quantitative RT-PCR to examine gastric leptin expression in controls.

These studies were performed according to the principles of the Declaration of Helsinki, and consent was obtained from each individual after a full description of the nature and protocol of the study.

IMMUNOHISTOCHEMISTRY FOR LEPTIN

The surgically resected tissue samples were fixed in Bouin's solution and embedded in paraffin. Immunohistochemical staining was performed using the avidin-biotin-peroxidase complex technique (Vectastain ABC kit; Vector, Burlingame, California, USA) described by Hsu and colleagues.²² All incubations were done at room temperature. Each section (4 μ m thick) was immersed in 0.3% H_2O_2 in methanol for 30 minutes to destroy endogenous peroxidase activity, washed for 10 minutes with 0.05 M Tris buffered saline, pH 7.5, and then incubated for 30 minutes with normal goat serum to block non-specific Ig binding sites. The goat serum was removed and the slides were incubated overnight with a 1:100 dilution of the antihuman leptin rabbit antibody 18545S (IBR, Gumma, Japan). This was followed by a 30 minute wash in Tris buffered saline, 30 minute incubation with biotinylated goat anti-rabbit IgG, repeat wash in Tris buffered saline, and a 30 minute incubation with avidin DH biotinylated horseradish peroxidase. After a final wash in Tris buffered saline, the slides were incubated for two minutes in a solution containing 0.05% diaminobenzidine and 0.015% H_2O_2 . Nuclear staining was carried out with haematoxylin. The specificity of the immunohistochemical reaction was first established by showing that non-immune rabbit serum did not reveal positive staining. In addition, the specificity of the antibody was evaluated by absorption controls. The diluted antibody was incubated with human leptin in concentrations of up to 10 nmol/ml for 24 hours at 4°C and then applied to the tissue section.

LEPTIN ASSAY

Serum leptin concentrations were determined using radioimmunoassay kits (Linco Research, St Charles, Missouri, USA) according to the manufacturer's protocol.²³

Figure 3 Relationship between the change in symptom score and change in body mass index (BMI). There was no significant correlation ($r=0.10$, $p=0.189$).

QUANTITATIVE RT-PCR

Sections of surgically resected tissue samples of the gastric fundic, antral mucosa, and gastrocolic omentum were frozen and stored in liquid nitrogen. mRNA was purified from tissues using Micro FastTrack kits (Invitrogen, Carlsbad, California, USA) according to the manufacturer's protocol.²⁴ RT-PCR for leptin was done using a novel real time quantitative RT-PCR method using an ABI 7700 sequence detector system (PE Applied Biosystems, Foster City, California, USA).²⁵ The sense primer for leptin was 5'-CACCAAAACCTCA TCAAGACA-3' and the antisense primer was 5'-CTTTCTGTTTGAGGAGACTGACT-3'. The reaction mixture was prepared according to the manufacturer's protocol using TaqMan Gold RT-PCR Kits (PE Applied Biosystems). The reactions also contained the target hybridisation leptin probe (5'-CACCA GGATCAATGACATTTCACACACG-3'). The probe was labelled with a reporter fluorescent dye 6-carboxyfluorescein at the 5' end. The thermal cycling conditions included 48°C for 30 minutes and 95°C for 10 minutes, followed by 50 cycles of amplification at 95°C for 15 seconds and 60°C for one minute. The PCR products were also examined by 2% agarose gel electrophoresis. The gels were stained with ethidium bromide to confirm successful amplification of the expected sequences. mRNA was also purified from biopsy specimens and subjected to real time quantitative RT-PCR for measurement of leptin and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as controls using TaqMan GAPDH control reagents (PE Applied Biosystems). For relative quantification of leptin expression, a

Table 1 Effect of *Helicobacter pylori* eradication therapy on body mass index (BMI)

Patient group	n	Sex M/F	Age (y)	BMI (kg/m ²)	Change in BMI after therapy (kg/m ²)	BMI change more than 0.5 kg/m ² after therapy
Eradicated patients	161	96/65	41.3 (7.0)	22.0 (2.5)	0.20 (0.60) ^a	48/161 (29.8%) ^b
Non-eradicated patients	40	25/15	42.8 (7.6)	22.7 (2.6)	0.017 (0.50)	5/40 (12.5%)

Values are mean (SD) or number.

Significantly different from non-eradicated patients: ^a $p=0.033$; ^b $\chi^2=4.90$, $p=0.026$.

Table 2 Change in symptom scores before and after eradication therapy

	n	Symptom score		p Value
		Before eradication therapy	After eradication therapy	
Eradicated patients	161	3.1 (3.3)	1.8 (2.9)	p<0.0001
Non-eradicated patients	40	2.4 (2.7)	2.7 (2.9)	NS

Values are mean (SD).

Table 3 Effect of *Helicobacter pylori* eradication therapy on expression of gastric leptin

	n	Relative quantification of gastric leptin		p Value
		Before eradication therapy	After eradication therapy	
Eradicated patients	33	2.8 (0.6)	1.1 (0.4)	p<0.0001
Non-eradicated patients	7	2.6 (0.5)	2.3 (0.6)	NS

Values are mean (SD).

calibration curve was constructed using the mRNA obtained from the gastroduodenal omentum. RNA for GAPDH was used as an endogenous control. The amount of leptin RNA was normalised to the level of GAPDH.

STATISTICAL ANALYSIS

Results are expressed as mean (SD). The significance of differences between groups for mean age, BMI, and change in BMI was evaluated using an unpaired Student's *t* test. Comparison of differences in BMI changes of more than 0.5 kg/m² was performed using a χ^2

Figure 4 A calibration curve was constructed using four different dilutions of the mRNA obtained from the gastroduodenal omentum. (A) Amplification plots of leptin; (B) calibration curve for leptin.

Figure 5 Relationship between the change in relative RNA quantification of gastric leptin and the change in body mass index (BMI). There was a significant correlation between the two ($r=-0.69$, $p=0.0002$).

test with Fisher's exact probability test. Differences in serum leptin concentrations and relative RNA quantification of leptin in the interval before and after eradication therapy were analysed with a paired Student's *t* test. Symptom scores among groups were compared by Wilcoxon's signed rank test. The relationship between the change in BMI and the change in symptom scores or the change in relative RNA quantification of leptin was evaluated by Spearman's rank correlation. Differences with a *p* value of less than 0.05 were considered significant.

Results

DETECTION OF LEPTIN IN THE GASTRIC MUCOSA

Leptin immunoreactive cells were seen in the lower half of the fundic epithelial glands (fig 1). No immunoreactivity was seen after adsorption of the antibody with 10 nmol/ml of a leptin fragment. A leptin PCR product was found in the fundic epithelium (fig 2).

EFFECTS OF ERADICATION THERAPY ON CHANGE IN BMI

The change in BMI after eradication therapy was compared between the eradicated and non-eradicated groups. *H pylori* infection was cured in 161 cases. The mean change in BMI was significantly higher in the successfully eradicated patients compared with that in non-eradicated patients. A BMI change of more than 0.5 kg/m² was observed significantly more frequently in the eradicated patients than in the non-eradicated patients (table 1).

SERUM LEPTIN CONCENTRATION

Serum leptin levels did not change significantly after eradication therapy in either the eradicated (3.5 (2.2) to 3.9 (2.6) ng/ml) or non-eradicated (3.3 (2.6) to 3.8 (2.9) ng/ml) patients groups.

SYMPTOM SCORES

Symptom scores improved significantly after successful eradication therapy. In contrast, in the non-eradicated patients, symptom scores did not improve significantly after therapy (table 2). It is possible that improvement in symptoms after cure of *H pylori* infection

caused the weight gain. However, there were no significant correlations between the change in symptom score and the change in BMI (fig 3).

QUANTITATIVE ANALYSIS OF GASTRIC LEPTIN

Relative RNA quantification of gastric leptin in *H pylori* positive patients with chronic gastritis (2.3 (0.6)) was significantly higher than that in *H pylori* negative normal controls (1.2 (0.5)) ($p < 0.0001$). Relative RNA quantification of leptin significantly decreased after successful eradication. However, levels did not change significantly in the non-eradicated patients (fig 4, table 3). There was a significant correlation between the change in the relative RNA quantification of leptin and the change in BMI ($r = -0.69$, $p = 0.0002$) (fig 5).

Discussion

Leptin has been reported to be secreted only by adipocytes and the placenta.¹⁻⁵ Bado *et al* first reported that leptin mRNA and leptin protein were present in the rat gastric epithelium, and that cells in the glands of the gastric fundic mucosa were immunoreactive for leptin.⁶ Recently, immunoreactive leptin cells were found in the lower half of the gastric fundic glands in humans, and leptin mRNA was also detected in human gastric mucosa by RT-PCR.²⁶⁻²⁷ In the present study, we confirmed the presence of leptin in the human gastric fundic mucosa. The physiological function of gastric leptin is unknown. Bado *et al* reported that both feeding and administration of CCK-8 resulted in a rapid and large decrease in both leptin cell immunoreactivity and the leptin content of the fundic epithelium, with a concomitant increase in the concentration of leptin in plasma. They suggested that gastric leptin may be involved in early CCK mediated effects activated by food intake, possibly including satiety.⁶

In contrast, anorexia and loss of lean body mass are hallmark manifestations of acute or chronic disease, including infection or cancer. The role of tumour necrosis factor (TNF), interleukin (IL)-1, and IL-6 as endogenous mediators of the host response to infection or malignancy has been extensively studied.⁷⁻⁹ It has also been reported that multiple cytokines and inflammation raise leptin levels. Leptin regulates feeding behaviour and therefore may be a mediator of anorexia associated with acute and chronic inflammation.²⁸⁻³⁰ Several studies have shown the involvement of cytokines in the pathogenesis of gastric inflammation. Crabtree *et al* detected higher levels of TNF- α , IL-6, and IL-8 in the culture supernatants of *H pylori* infected gastric biopsy specimens than in specimens from uninfected patients.³¹ Noach *et al* also detected increased levels of IL-1 β , IL-8, and TNF- α in culture supernatants of antral biopsy specimens from *H pylori* infected patients.³² Yamaoka *et al* reported that expression levels of IL-6, IL-7, IL-8, and IL-10 mRNA were significantly higher in mucosa with *H pylori* positive gastritis than in *H pylori* negative normal mucosa, and that this increased cytokine expression due to *H pylori* infection was reduced significantly after *H*

pylori infection was cured.³³ In the present study, *H pylori* infection increased gastric leptin expression, and cure of *H pylori* infection induced a reduction in gastric leptin expression. These findings suggest that gastric inflammation induced by *H pylori* infection raises gastric leptin expression.

Interestingly, cure of *H pylori* infection also induced significant weight gain, and there was a significant correlation between the change in relative RNA quantification of gastric leptin and change in BMI. Gastric leptin may play a role in weight gain after cure of *H pylori* infection. However, serum leptin levels did not change significantly after cure of *H pylori* infection. Gastric leptin may have a local rather than a systemic action, exerting paracrine effects within the gastric mucosa or stimulating vagal afferents to signal the central nervous system. Recently, several investigators reported the presence of leptin and its receptors in the human stomach, and suggested a paracrine and/or autocrine effect of leptin on gastric epithelial cell function.²⁶⁻²⁷ In addition, leptin sensitive vagal afferent terminals have been identified in the rat stomach.³⁴ On the other hand, symptom scores improved significantly after successful eradication therapy. It is possible that the improvement in symptoms, rather than a change in gastric leptin expression, caused weight gain. However, there was no significant correlation between the change in BMI and the change in the dyspepsia symptom score.

In conclusion, gastric inflammation induced by *H pylori* infection raised gastric leptin expression. Cure of *H pylori* infection reduced gastric leptin expression, with a concomitant increase in BMI. Gastric leptin may play a role in weight gain after curing *H pylori* infection. Evidence for the presence of leptin in the gastric fundic mucosa opens new avenues in the study of the regulatory mechanism of satiety. Further examination of the physiological role of gastric leptin is warranted.

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