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Expression of Mucosal Cyto-Chemokine mRNAs in Patients with *Helicobacter pylori* Infection

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Background : *Helicobacter pylori-induced destruction of the gastroduodenal mucosal barrier is initiated with mucosal infiltration of inflammatory cells. Cytokines and chemokines have been suggested to play important roles in the migration and activation of these inflammatory cells into the mucosa. The present study aimed to investigate expression rates of cyto-chemokine mRNAs using gastric mucosal biopsy specimens.*

Methods : *In 98 patients infected with Helicobacter pylori, mucosal mRNA expression rates of cytokines (IL-1β, IL-6, and IL-10), C-C chemokines (macrophage inflammatory protein 1α [MIP-1α], and macrophage inflammatory protein 1β [MIP-1β], monocyte chemotactic and activating factor [MCAF], regulated on activation, normal T cell expressed and presumably secreted [RANTES]) and C-X-C chemokines (IL-8 and growth regulated α [GRO-α]) were examined using reverse transcription polymerase chain reaction (RT-PCR).*

Results : *The expression rates of mRNA for IL-8, GRO-α, MIP-1α and RANTES were significantly more increased in H. pylori-positive patients than in H. pylori-negative patients. However, the expressions of IL-1β, IL-6 and IL-10 mRNA were statistically not different between two groups. After eradication of H. pylori, expressions of mRNA for three cytokines (IL-1β, IL-6 and IL-10), four C-C chemokines (MIP-1α, MIP-1β, MCAF and RANTES) and two C-X-C chemokines (IL-8 and GRO-α) were significantly decreased.*

Conclusion : *These results suggest that C-X-C chemokines and some C-C chemokines play important roles in H. pylori-associated peptic ulcer diseases.*

Key Words : *Helicobacter pylori; Cytokine; C-C chemokine; C-X-C chemokine.*

INTRODUCTION

Helicobacter pylori (*H. pylori*) is recognized as a major cause of chronic gastritis, peptic ulcer diseases, gastric adenocarcinoma and gastric MALToma. *H. pylori*-induced destruction of the gastroduodenal mucosal barrier is initiated with mucosal infiltration of inflammatory cells and *H. pylori*-associated gastritis begins as an acute neutrophilic inflammatory response which, in the majority of individuals, progress to chronic gastritis. Bacterial eradication results in significant reduction in these inflammatory cell infiltrations in the gastric mucosa and healing of the gastritis. The migration and activation of inflammatory cells into the mucosa are thought to be related to the expression of various cytokines[1-8]. Of these, IL-8 is now proved to be a definite chemoattractant and activation signal for neutrophils in acute as well as more prolonged *H. pylori* infection[2-5,7,8].

Chemotactic cytokines or "chemokines" are classified into two major families on the basis of the arrangement of the first two of four conserved cysteine residues. Chemokine α or C-X-C chemokine is located on chro-

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mosome 4 (q12-21) and the first two of their cysteine groups are separated by one amino acid. This C-X-C chemokine group includes IL-8, melanoma growth-stimulating activity/growth regulated (MGSA/GRO), platelet factor 4 (PF4), β thrombo-globulin (βTG), IP-10 and ENA-78. The chemokine β or C-C chemokine group is located on chromosome 17 (q11-32), has no intervening amino acid between the first two cysteines and includes macrophage chemotactic and activating factor (MCAF/MCP-1), regulated on activation, normal T cell expressed and presumably secreted (RANTES), LD-78 (also known as human MIP-1α, ACT-2 or huMIP-1β) and I-309. In general, C-C chemokines mainly show chemotactic activities for neutrophils but not monocytes, whereas C-X-C chemokines show chemotactic activities for monocytes and lymphocytes but have little effect on neutrophils[9].

The present study aimed to clarify any relationship between *H. pylori* infection and expression rates of cyto-chemokine mRNAs in the gastric mucosa by using the reverse transcription polymerase chain reaction (RT-PCR) method.

MATERIALS AND METHODS

Population studied

This study was prospectively performed at the Department of Internal Medicine, Chung-Ang University, Yong-San Hospital from June 1997 to June 1999.

The study population was made up of 98 patients (74 males and 24 females; mean age, 45.7 years; range 22-86) with endoscopically and histologically confirmed benign gastric (GU, 29 patients) or duodenal ulcers (DU, 69 patients) who were infected with *H. pylori*. The control group consisted of 18 peptic ulcer patients (10 GU patients and 8 DU patients) who were not infected with *H. pylori*. Patients were excluded if they had a history of gastric surgery, active gastrointestinal bleeding, exposure to steroids or NSAIDs, H_2-receptor antagonists, proton pump inhibitor, or antimicrobial agents within 30 days prior to the study. Patients with any other chronic illness were also excluded.

Methods

Endoscopic procedure and the diagnosis of *H. pylori* infection

Gastroscopic examination was performed and the endoscopic findings were recorded. Eight mucosal biopsy specimens were obtained from each patient: one biopsy specimen each from the gastric antrum within 2 cm proximal to the pylorus and from the gastric midbody were submitted for histological evaluation by a single pathologist who was unaware of the PCR results. Histological confirmation of *H. pylori* infection was done by the Warthin-Starry silver staining of individual mucosal specimen. Microaerophilic culture under 37℃ and rapid urease test were performed on each patient. The remaining one biopsy specimen each from the gastric antrum and midbody were frozen at -70℃ for RNA extraction and RT-PCR to detect the presence of *H. pylori* genes and cyto-chemokine mRNAs.

Patients were classified as *H. pylori*-positive when the culture was positive or if at least two of the three examinations, that is, the rapid urease test, histological examination and presence of the 16S-rRNA, gave positive results for *H. pylori*. Both GU and DU patients infected with *H. pylori* were treated with omeprazole (10 mg twice daily), amoxycillin (1,000 mg twice daily) and clarithromycin (500 mg twice daily), for the first two weeks. Thereafter, ranitidine (150 mg twice daily) and antacid were given for four or six more weeks to the GU patients. Four weeks after cessation of treatment, the patients were endoscoped again and biopsy specimens were taken as before. Successful eradication of *H. pylori* was determined if all of the above mentioned test results were negative.

Preparation of RNA and cDNA for measurement of *H. pylori*-specific, 16S-rRNA and cyto-chemokine mRNA in gastric biopsy specimens

Individual gastric biopsy specimens were placed in a sterile vial and stored at -70℃ until they were used for RNA extraction for RT-PCR. Samples were homogenized with tissue homogenizer (Bio-Spec, Bartlesville, OK, USA) and total RNA was extracted and purified by the guanidiumthiocyanate-phenol-chloroform method using ULTRA™-II RNA Resin Purification System kit (Biotecx Lab., Inc. Houston, TX, USA) in 500 μL of homogenization buffer. Individual cDNA was synthesized by reverse transcription of RNA using Perkin Elmer GenAmp RNA PCR kit (Applied Biosystems Division, Roche, Foster City, CA, USA).

PCR amplification reaction

Detection of *H. pylori* 16S-rRNA of cDNA from gastric

mucosal biposy specimens was performed by PCR amplification by using oligonucleotide primers described in detail previously[10]. The sense and antisense primers specific for each cyto-chemokine are shown in Table 1. Amplification reaction of each cyto-chemokine was carried out in a total volume of 100 μL containing 10 μL of cDNA, 8 μL of 10x PCR reaction buffer (50 mM KCl, 1 mM Tris-HCl [pH 8.3]), 2.0 mM $MgCl_2$, 200 μM for each of dATP, dCTP, dGTP, and dTTP, and 2.5 U AmpliTaq DNA polymerase (Perkin Elmer Citus, Foster, CA, USA). Primers of each cyto-chemokine were used as a final concentration of 0.1 μM. Each reaction mixture was overlayed with mineral oil and was amplified for 35 cycles, each of which consisted of 1 minute at 95℃ for denaturation, 1 minute at 60℃ for annealing and 1 minute at 72℃ for extension with final extension of 7 minutes at 72C. Fifteen microliter aliquots of each PCR product were analyzed using electrophoresis on 1.5% agarose gel containing ethidium bromide (Sigma-Aldrich, St. Louis, MO, USA) and the bands were examined under UV light for the presence of the amplified DNA.

Table 1. Oligonucleotide primers specific for *H. pylori* gene and for human cyto-chemokines

Gene and Cyto-chemokine		Primer	cDNA (bp)
16SrRNA	Sense	GCTAAGAGATCAGCCTATGTCC	522
	Antisense	TGGCAATCAGCGTCAGGTAATG	
IL-1β	Sense	ATAAGCCCACTCTACAGCT	443
	Antisense	ATTGGCCCTGAAAGGAGAGA	
IL-6	Sense	GTACCCCCAGGAGAAGATTC	819
	Antisense	CAAACTGCATAGCCACTTTC	
IL-8	Sense	GCTTTCTGATGGAAGAGAGC	585
	Antisense	GGCACAGTGGAACAAGGACT	
IL-10	Sense	ATGCCCCAAGCTGAGAACCAAGAC	353
	Antisense	TCTCAAGGGGCTGGGTCAGCTATCCCA	
MIP-1α	Sense	CCTTGCTGTCCTCCTCTGGA	254
	Antisense	CACTCAGCTCTAGGTCGCYG	
MIP-1β	Sense	TGTCTCTCCTCATGCTAGTA	233
	Antisense	GTACTCCTGGACCCAGGAT	
GROα	Sense	TTGCAGACCCTGCAGGGAAT	184
	Antisense	TGGATTTGTCACTGTTCAGC	
MCAF	Sense	CAATAGGAAGATCTCAGTGC	188
	Antisense	GTGTTCAAGTCTTCGGAGTT	
RANTES	Sense	TGCCTCCCATATTCCTCGG	211
	Antisense	CTAGCTCATCTCCAAAGA	

Statistical analysis

Pearson chi-square test and paired t-test were used to determine the significance of differences between two groups with a difference in *p* value less than 0.05 being considered significant.

RESULTS

Relationship between *H. pylori* infection and expression of mRNAs for cyto-chemokines.

Of the ninety-eight patients infected with *H. pylori*, 48 patients (GU 10 and DU 38) were not followed up. While 35 patients (GU 16 and DU 19) attained successful eradication of *H. pylori* after the two-weeks' therapy of antibacterial drugs, the remaining 15 patients (GU 3 and DU 12 patients) did not.

H. pylori infection was associated with significantly increased rates of expression of mRNA for IL-8, GRO-α, MIP-1α and RANTES. However, there was no difference in the expression rates of IL-1β, IL-6, IL-10, MIP-1β or MACF mRNA between *H. pylori*-positive patients and *H. pylori*-negative control patients ($p > 0.05$), (Table 2, 3). There was no significant difference in cyto-chemokine mRNA expression rates between patients with GU and those with DU (Table 3).

Table 2. Relationship between expression of cytokine mRNA and *H. pylori* infection

H. pylori Infection	Diseases	No. Studied	Cytokines (%)		
			IL-1β	IL-6	IL-10
Absent	GU & DU	18	12 (66.7)*	2 (11.1)†	1 (5.6)‡
Present	GU	29	21 (71.4)	10 (34.5)	4 (13.8)
	DU	69	64 (92.8)	17 (24.6)	21 (30.4)
	Total	98	85 (86.7)*	27 (27.6)	25 (43.9)

GU, Gastric ulcer; DU, Duodenal ulcer *,†,‡ $p > 0.05$

Effect of *H. pylori* eradication on expression rates of cyto-chemokines.

Expression of mRNA of three cytokines, two C-X-C chemokines, four C-C chemokines and mucosal concentrations of IL-1β, IL-6, IL-8, GRO-α and RANTES protein in patients infected with *H. pylori* were compared before and after the eradication therapy.

Fifty patients were treated for bacterial eradication. Nineteen patients had GU and 31 had DU. Of 50 patients, 35 patients became *H. pylori*-negative and 15 patients

Table 3. Relationship between expression of chemokine mRNA and *H. pylori* infection

H. pylori Infection	Diseases	No. Studied	C X-C Chemokine (%)		C-C Chemokine (%)			
			IL-8	GRO-α	MIP-1α	MIP-1β	MCAF	RANTES
Absent	GU & DU	18	3 (16.7)*	1 (0.6)†	3 (16.7)‡	14 (77.8)	15 (83.3)	3 (16.7)§
Present	GU	29	21 (72.4)	22 (75.9)	17 (58.6)	26 (89.7)	19 (65.5)	15 (51.7)
	DU	69	55 (79.7)	51 (73.9)	51 (58.6)	50 (72.5)	48 (69.6)	43 (62.3)
	Total	98	76 (67.1)*	73 (74.5)†	68 (69.6)‡	76 (77.6)	67 (68.4)	58 (59.2)§

GU, Gastric ulcer; DU, Duodenal ulcer *,†,‡,§, $p < 0.002$

Table 4. Expression of cyto-chemokine mRNA before and after *H. pylori* eradication

	No. Studied	Cytokine (%)			C-X-C Chemokine (%)		C-C Chemokine (%)			
		IL1-β	IL-6	IL-10	IL-8	GRO-α	MIP-1α	MIP-1β	MCAF	RANTES
Eradicated										
Before	35	34 (97.1)	13 (8.6)	7 (20.0)	30 (85.7)	28 (80.0)	27 (77.1)	29 (82.9)	26 (74.3)	26 (74.3)
After	35	3 (8.6)	1 (2.9)	0 (0)	0 (0)	1 (2.9)	0 (0)	1 (2.9)	4 (11.4)	0 (0)
p value		<0.001	0.001	0.017	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Not-Eradicated										
Before	15	13 (86.7)	3 (20.0)	4 (26.7)	11 (73.3)	8 (53.3)	9 (71.4)	14 (93.3)	10 (66.7)	8 (53.3)
After	15	7 (46.7)	3 (20.0)	1 (6.7)	11 (73.3)	16 (6.6)	4 (26.7)	1 (6.6)	9 (60.0)	2 (13.3)
p value		0.186	0.669	0.353	0.799	0.032	0.219	<0.001	1.0	0.088

were still positive for *H. pylori* after eradication therapy.

Patients with successful eradication of *H. pylori* showed significant decrease in positive rates of mRNA expression of all cyto-chemokines (Table 4). However, patients without *H. pylori* eradication with antibiotic therapy did not show any change in expression rates for mRNAs of cyto-chemokines except for GRO-α and MIP-1β. The positive rates of GRO-α and MIP-1β mRNA were significantly decreased in spite of persistence of *H. pylori* after eradication therapy (53.3% to 6.6% and 93.3% to 6.6%, respectively) (Table 4).

DISCUSSION

Several mechanisms are thought to be involved in *H. pylori*-induced damage of the gastroduodenal mucosa; 1) qualitative and quantitative changes of the mucus layer by bacterial protease, glycosulphatase, phospholipase, urease, toxins and possible pepsin inhibitor[11] 2) reduced mucosal hydrophobicity induced by bacterial phospholipase A2[12] 3) adherence and internalization of epithelial cells by bacterial adhesin from *H. pylori*[13-15] and 4) specific humoral and cell-mediated immunologic damages[16,17]. Bacterial lipopolysaccharide[18], heat-shock protein[19] and gastric acid inhibitory protein[20] also play important roles in breaking the gastroduodenal integrity. The local inflammatory and immune responses seen in *H. pylori* infection are also thought to cause the breakdown of the integrity of the gastroduodenal mucosal barrier.

A number of inflammatory mediators have been shown to be increased in *H. pylori* infection. Crabtree et al.[1,2] detected higher levels of TNF-α, IL-6 and IL-8 in culture supernatants of *H. pylori*-infected gastric biopsy specimens than in specimens from uninfected patients. Noach et al.[3] also detected increased levels of IL-1β, IL-8 and TNF-α in culture supernatants of antral biopsy specimens from *H. pylori*-infected patients. Yamaoka et al.[7,8,21] also reported similar results.

The present study showed that mRNAs for IL-1β, IL-6 and IL-10 were detected in gastric mucosal biopsy

specimens both from *H. pylori*-positive and *H. pylori*-negative patients. Although pre-treatment expression rates of mRNAs were not different between the two groups, detection rates of these mRNAs were significantly decreased when *H. pylori* were successfully eradicated. Our results were not in agreement with those of other studies[7, 11, 21] which showed thlat expression rates of mRNAs for IL-1β, IL-6 and IL-10 were significantly higher in *H. pylori*-positive patients than in *H. pylori*-negative patients. However, the number of *H. pylori*-negative cases in our study was too small to compare with *H. pylori*-positive patients (n=18 vs n=98). Further study with a larger number of *H. pylori*-negative patients is needed in order to verify the exact difference between the two groups.

IL-8 is a potent chemoattractant for neutrophils and T lymphocytes. In response to *H. pylori* infection, IL-8 is produced from gastric epithelial cells evidenced by direct expression of IL-8 mRNA in these cells and is localized to the epithelial cell layer[22, 23]. The epithelial cells are the first line of mucosal defense against the *H. pylori* infection and IL-8 acts as a trigger for inflammation. These inflammatory cells then reciprocally produce IL-8 and other cytokines which form the "cytokine network" to induce gastric mucosal inflammation. Our study showed that expression rate of IL-8 mRNA was significantly higher in *H. pylori*-positive patients than in *H. pylori*-negative patients and was significantly decreased after successful eradication of *H. pylori*. Our results were in agreement with those of other studies[2-4, 7, 8]. Another C-X-C chemokine, GRO-α mRNA was also significantly increased in patients with *H. pylori* infection and decreased in all patients after successful bacterial eradication. Patients without bacterial eradication after treatment also showed decreased rate of detection of GRO-α mRNA. These findings suggest that IL-8 and GRO-α play important roles in *H. pylori*-associated gastric inflammation.

With regard to C-C chemokines, positive rates of expression of both MIP-1α and RANTES mRNA were significantly more increased in *H. pylori*-positive patients than in *H. pylori*-negative patients. MIP-1β and MCAF mRNA were detected in the majority of patients regardless of the presence of *H. pylori*. When expression rates of MIP-1α, MIP-1β, MACF and RANTES mRNA were compared before and after antibacterial therapy, detection rates of these four C-C chemokine mRNAs were significantly decreased after eradication of *H. pylori*.

In conclusion, our results support the hypothesis that C-X-C chemokines and some C-C chemokines play important roles in the pathogenesis of gastroduodenal injury in patients infected with *H. pylori*.

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