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SERUM AND MUCOSAL CYTOKINE PROFILES IN PATIENTS WITH ACTIVE *HELICOBACTER PYLORI* AND ISCHEMIC HEART DISEASE: IS THERE A RELATIONSHIP?

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This study is designed to investigate, for the first time, circulating and gastric mucosal levels of IL1-α, IL-6, IL-8 and TNF-α in patients with ischemic heart disease (IHD) and matched controls, according to the presence or absence of active *Helicobacter pylori* infection. Furthermore, in order to evaluate whether modified lipid profile was associated to an increased cardiovascular risk, this was determined in the same groups. Cytokine levels were measured using ELISA in 58 patients with IHD and 52 controls. Active *H. pylori* infection was assessed if either culture of *H. pylori* or rapid urease test gave a positive result. Our findings indicate increasing cytokine mucosal levels in *H. pylori*-positive patients compared to *H. pylori*-negative subjects. However, the increase was statistically significant only for IL-6 and TNF-α in the gastric mucosa of IHD patients. In *H. pylori*-positive controls, IL-8 mucosal levels positively correlated with both IL-1α (r = 0.98; P = 0.0003) and IL-6 (r = 0.83; P = 0.03) levels. Circulating cytokine levels were comparable in IHD and healthy subjects, regardless of *H. pylori* status. There were no correlations between mucosal and circulating cytokine levels. Active *H. pylori* infection was not associated with a modified lipid profile in either controls or IHD patients, although ApoAI levels were significantly higher in *H. pylori*-positive controls compared to those *H. pylori*-negative. Taken together, the results of the present study provide evidence that active *H. pylori* infection may play a role as a trigger factor in the pathophysiology of IHD by inducing an inflammatory cascade concentrated on gastric mucosa.

Cardiovascular diseases constitute one of the leading causes for mortality and morbidity in industrialized countries (1). In particular, ischemic heart disease (IHD) is the single biggest contributor to cardiac mortality. Major factors associated with an increased risk of IHD are well known, but not all of IHD incidence can be attributed to serum lipids, hypertension, smoking, obesity and diabetes (2). It is, therefore, important to identify additional causes of IHD.

Atherosclerosis is now generally accepted as an inflammatory disorder in the arterial wall (3). Since the first evidence reported by Mendall et al. (4), a number of studies have confirmed the key

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role for the proinflammatory cytokines in several mechanisms that contribute to the development of coronary heart disease (5-7). Hence *H. pylori*-induced cytokines may represent the pathogenic mechanism that explains the associations between *H. pylori* and IHD.

Recent epidemiological, serological and immunohistochemical studies suggest the "infectious theory" of IHD (8-12). Particularly, in 1994 Mendall et al. (13) attracted considerable attention by reporting an association between coronary heart disease and *Helicobacter pylori* infection. Since this report, conflicting data and divergent opinions have appeared in literature to explain the pathomechanism of the heart vessel atherosclerosis in relation to extradigestive manifestation of chronic infection with *H. pylori* (14-20). Despite serological evidence for association between *H. pylori* infection and IHD, the mechanisms by which *H. pylori* may influence cardiovascular risk are not yet well characterized. Various mechanisms by which *H. pylori* could increase the risk of arterial plaque formation have been proposed. At present, the data support both the direct and indirect effect of bacterial infections on atherogenesis: release of acute-phase reactants including fibrinogen, reduction of HDL cholesterol, elevation of homocysteine levels and immunological cross-reactivity between bacterial and human heat shock proteins (15-16, 21-22).

An intriguing possibility concerns the role of *H. pylori* in influencing the systemic inflammatory response, because they offer potential therapeutics targets for nonspecific "anti-inflammatory" treatment of arterial disease. *H. pylori* is a non-invasive microorganism causing intense acute and chronic gastric mucosal inflammatory reactions, resulting in a chronic release of large amounts of pro-inflammatory cytokines which play important roles in the atherosclerotic process. *Helicobacter pylori*-induced gastric mucosal chemokine and cytokine overproduction has been clearly documented (23-27). The stomach has a large surface area and a continuous spill-over of locally produced cytokines into the blood stream is a possibility. Several studies produced conflicting data about the synthesis and secretion of a variety of proinflammatory cytokines in patients with *H. pylori* infection (5, 28-29).

On this basis, the aim of this comparative study is to investigate, for the first time, IL-1α, IL-6, IL-8 and TNF-α concentrations in both gastric mucosa and serum of IHD patients, as well as in healthy subjects, with or without active *H. pylori* infection. In addition, in order to evaluate whether modified lipid profile is associated to an increased cardiovascular risk, we measured lipoproteins known to have an atherogenic effect in cases as well as in controls, with regard to *H. pylori* infection.

MATERIALS AND METHODS

Patients

The study population consisted of 110 (mean age, 60.6 ± 13.0 years) men, enrolled from January 2000 to December 2003: 58 (mean age, 62.8 ± 9.6 years) cases scheduled for elective coronary artery bypass grafting and 52 (mean age, 58.2 ± 16.2 years) controls. Control subjects, recruited among patients undergoing endoscopy because of clinical symptoms attribuitable to the upper gastrointestinal tract, had no history of IHD and exhibited normal ECG; further, they matched with the cases for age and educational levels. No patient or control had evidence of active infections, inflammatory diseases or cancer, nor had received anti-inflammatory drugs (i.e. aspirin) within the preceeding 2 weeks or drugs known to affect *H. pylori* status. The study was approved by the Ethics Committee of "G. d'Annunzio" University of Chieti-Pescara, and all patients gave their written informed consent. All patients were asked about age, sex, cigarette smoking (current smoker), obesity, hypertension and diabetes by using a structured questionnaire.

Assessment of H. pylori status

At endoscopy, seven biopsy specimens were collected from the antrum of each patient: two for histology (Giemsa), one for rapid urease test (CP test: Yamanouchi Pharma S.p.A., Milan, Italy), two were streaked in parallel onto egg yolk emulsion agar and modified chocolate agar for culture (30), and two for cytokine assays. Patients were classified as *H. pylori*-positive (that is, active *H. pylori* infection) if either culture of *H. pylori* or rapid urease test gave a positive result. We also determined CagA status of *H. pylori* by a commercial PCR kit (Multigen Pylori: Diatech srl, Jesi, Italy) in pooled gastric biopsy specimens, as previously described (31).

Serologic assays

Before endoscopy, serum blood samples of each 12h-fasted patient were obtained for measurement of cytokine levels and lipid profile (HDL and total cholesterol, triglicerides, Apo-AI, Apo-B). All serum

samples were prepared within 1 h and kept frozen at -80°C until analysis. Total cholesterol and triglicerides were measured enzymatically with reagents from Wako Chemicals GmbH. HDL cholesterol was measured in the supernatant after precipitation of apoB-containing lipoproteins with phosphotungstate acid and $MgCl_2$ obtained from Roche Diagnostics. ApoAI, and B were determined by immunoturbidimetry with antisera from Greiner Biochemicals. The apolipoprotein assays were calibrated with N apolipoprotein standard serum (Behring). All analyses were performed on a Wako R-30 automated analyzer in a laboratory setting certified according to ISO 9001.

Mucosal and serum cytokine assays

IL-1, IL-6, IL-8 and TNF-α levels were determined on serum and gastric biopsy specimens collected for each patients. Biopsy specimens were immediately placed in RPMI-1640 with 10% fetal bovine serum (FBS) and transported to the Clinical Microbiology Laboratory where the specimens' wet weights were determined. The samples were subsequently placed in 1 ml of RPMI-1640 with 10% FBS and incubated in the presence of CO_2 at 37°C for 24 h. At the end of incubation, the specimens were homogenized in the culture supernatant by means of a Potter tube and clarified by centrifugation (3.500 x g, 10 min, +4°C). The supernatant was aliquoted and stored at –20°C until its use. All specimens were measured in duplicate for cytokine concentration by commercially available ELISA kits (Biotrak; Amersham Pharmacia Biotech, Little Chalfont, UK) according to the manufacturer's instructions. Levels of cytokines were expressed as: picograms per 100 milligrams of biopsy tissue per 24 h (pg/100mg/24h), for biopsy specimens; and picograms per milliliter (pg/ml) for serum.

Statistical analysis

Results are given as the mean ± standard deviation. Comparisons between groups and within groups were made using chi-square test (discrete variables), unpaired t test with Welch's correction (continuous variables with normal distributions), or Mann-Whitney U non-parametric test (continuous variables with non-normal distributions). Correlations between cytokine levels were calculated using Pearson correlation coefficient. P-values of < 0.05 were considered indicative of statistically significant difference. All statistical analyses were performed using GraphPad Prism (version 4: GraphPad Software Inc., San Diego, CA).

RESULTS

Baseline characteristics and H. pylori infection

Basal clinical and laboratory data of patients according to IHD status are shown in Table I. Patient and control groups resulted pair-matched for age and all risk factors for IHD except hypertension which was significantly more prevalent in cases than controls (58.6 vs 19.2%, respectively; P = 0.006).

Active *H. pylori* infection was observed in 60 out of 110 (54.5%) subjects enrolled in this study: 26 (50%) in controls and 34 (58.7) in cases. Among *H. pylori*-positive subjects, 40 (66.6%) showed active chronic gastritis and 20 (33.3%) peptic ulcer. All of 52 subjects negative for *H. pylori* infection showed chronic gastritis. *H. pylori* infection was present in 58.7% of cases and in 50% of controls (P = 0.08).

Gastric mucosal cytokine levels

According to the *H. pylori* status, IL-6 and IL-8 mucosal levels were significantly higher in *H. pylori*-positive than in negative subjects (IL-6, 1700 ± 1080 vs 410 ± 370 pg/100mg/24h, respectively, P = 0.008; IL-8, 225350 ± 162680 vs 55610 ± 88970 pg/100mg/24h, respectively; P = 0.02). In both *H. pylori*-positive and negative subjects, IL-1α levels positively correlated with IL-8 levels (r_s=0.86 and r_s=0.91, respectively; P = 0.005). Cytokine levels were higher in IHD patients than in controls, although these differences were not statistically significant (IL-1α, 560 ± 380 vs 370 ± 430 pg/100mg/24h; IL-6, 1300 ± 760 vs 780 ± 600 pg/100mg/24h; IL-8, 169780 ± 121230 vs 106530 ± 103160 pg/100mg/24h; TNF-α, 5110 ± 1580 vs 660 ± 500 pg/100mg/24h). Cytokine mucosal levels (pg/100mg/24h) according to the combined status of IHD and *H. pylori* are shown in Fig. 1. Positivity to *H. pylori* infection was associated with higher cytokine levels in both patients and controls, although the increase resulted being statistically significant only for IL-6 in IHD patients (2040 ± 1120 vs 570 ± 400 pg/100mg/24h, respectively; P = 0.03). In *H. pylori*-positive controls, IL-8 levels positively correlated with both IL-1α (r = 0.98; P = 0.0003), and IL-6 (r = 0.83; P = 0.03) levels. Accordingly, TNF-α levels were also significantly higher in *H. pylori*-positive IHD patients than in *H. pylori*-negative IHD patients (6920 ± 2120 vs 3300 ± 1040 pg/ml, respectively; P = 0.002).

Serum cytokine levels

Circulating cytokine levels are shown in Fig. 2. There were no statistically significant differences between IL-1α, IL-6, IL-8 and TNF-α levels with

regard to *H. pylori* status, IHD status and *H. pylori*-IHD combined status. No statistically significant correlation was found among cytokine circulating levels and between circulating and mucosal cytokine levels.

Lipid profile

Lipoprotein and apolipoprotein levels in relation to *H. pylori* infection in healthy subjects and patients with IHD are shown in Table II. Mean Apo-AI concentration was significantly (P = 0.02) higher in healthy subjects with *H. pylori* infection compared with those without infection. Other lipoprotein concentrations were not appreciably different between controls and patients, with or without *H. pylori* infection.

DISCUSSION

To the best of our knowledge, this study is the first designed to investigate serum and gastric mucosal cytokine levels in patients with and without IHD, referred to an active *H. pylori* infection. In fact, up to now, studies assessing the presence of *H. pylori* infection in IHD patients have been based on the measurement of serum specific antibody levels (14-20), although serology is not able to discriminate between past or persistent infection. For this reason, in this study we have specifically investigated the presence of an active infection by culture and rapid-urease-test from gastric mucosa.

Direct and indirect effects of TNF-α and IL-6 shown *in vitro* and *in vivo* could have important effects on the development of the atherosclerotic lesion. TNF-α is mainly produced by macrophages and has been detected in human atheromas (32). This cytokine has been shown to stimulate endotheial cell activation, procoagulant activity and angiogenesis, and cause cell necrosis (32). IL-6, a multifunctional cytokine known as a potent stimulator of acute-phase protein synthesis (33-34), has been proposed to be another mediator of cardiovascular risk (35). The results of the present study provide the evidence that mucosal IL-6 and TNF-α levels were significantly higher in IHD patients with *H. pylori* infection compared to those without infection. Our findings were discordant with previous studies showing that *H. pylori* infection was associated with higher serum and mucosal IL-6 and TNF-α concentrations in dyspeptic *H. pylori*-positive patients (4, 23, 36), but in agreement with findings by Bayraktaroglu (28) and Isomoto (25). The lack of correlation between *H. pylori* infection and cytokine levels in healthy subjects might indicate that proinflammatory contribution becomes significant only when combined with other risk factors for the development of IHD.

The proinflammatory interleukin (IL)-1

Table I. *Basal and clinical data in healthy subjects (controls) and patients with ischemic heart disease (IHD).*

	Controls (n = 52)	**IHD (n = 58)**	**P (controls vs IHD)**
Age (mean ± SD)	58.2 ± 16.2	62.8 ± 9.6	ns[a]
HP+	26 (50.0%)	34 (58.7%)	ns
CagA+	16 (30.7%)	14 (24.1%)	ns
Smoking	10 (19.2%)	14 (24.1%)	ns
Diabetes	8 (15.4%)	12 (20.7%)	ns
Hypertension	10 (19.2%)	34 (58.6%)	0.006
Obesity	8 (15.4%)	8 (13.8%)	ns

Table II. *Lipid profile in relation to active H. pylori (Hp) infection in healthy subjects (controls) and patients with ischemic heart disease (IHD). Values are expressed as mean (mg/dL) ± SD.*

Variable	Controls		IHD	
	Hp- (n=26)	Hp+ (n=26)	Hp- (n=24)	Hp+ (n=34)
Total cholesterol	179 ± 37.75	182.20 ± 44.90	165.7 ± 29.39	167.5 ± 31.65
HDL cholesterol	38.67 ± 4.51	45 ± 5.87	47 ± 12.72	44.5 ± 9.09
Triglicerides	144 ± 18.68	103.80 ± 45.17	110.2 ± 18.46	119.5 ± 40.49
ApoAI	108 ± 10.54	124.40 ± 7.96 [b]	121 ± 10.67	121 ± 12.04
ApoB	101.67 ± 24.42	96.40 ± 30.24	83.7 ± 16.21	88.33 ± 23.60

[a] *Hp+ vsHp-, P<0.05.*

polypeptides (IL-1α and IL-1β) are pleiotropic cytokines that affect, through the same receptors, a wide variety of cells (37- 38). However, contrarily to IL-1β, IL-1α is cell-associated and thus its levels correlate better than those of IL-1β with disease severity (37). Recent findings support the role of IL-1 and other cytokines in the pathogenesis of atherosclerosis (39-41), probably because these cytokines are expressed mainly within the endothelium of atherosclerotic plaques that respond by mediating inflammation and also influencing hemostasis (42). Jung et al. (43) found that IL-1α mRNA is expressed more frequently than IL-1 β in gastric epithelial cells infected with *H. pylori.* Straubinger et al. (44) found IL-1α upregulated as an early consequences of *H. pylori* in cats.

IL-8 is an extremely potent and ubiquitous neutrophil chemotactic peptide synthesized predominantly by monocytes/macrophages in response to bacterial endotoxin. IL-8 may play an important role in recruitment and activation of inflammatory cells, which are the key factors in initiation and progression of atheromathic processes (44). Recently, Kowalski et al. (45) proposed that cytokines such as IL-8 are significantly higher in *H. pylori* infected coronary artery disease patients than in control subjects. IL-8 levels have been also shown to be significantly increased in serum and gastric mucosa of *H. pylori*-infected subjects (24-25, 27, 36). *H. pylori* induces increased IL-8 production in gastric mucosa by heat-shock proteins 60 and 90 (46-47).

Our results showed that active *H. pylori* infection increased mucosal levels of IL-1α and IL-8 in both IHD and controls, although these differences did not reach statistical significance. Furthermore, our study failed to show any statistical significant differences in mucosal and circulating IL-1α and IL-8 levels between *H. pylori*-positive and negative subjects in either IHD patients or controls. In *H. pylori*-positive controls, we found a significant correlation between mucosal IL-1α and IL-8 levels, suggesting that a common pathway of synthesis for both cytokines.

Recently, focus has been on the role played by the more virulent strains of *H. pylori* possessing the cytotoxin-associated gene-A (CagA) in IHD pathogenesis. It has been shown that these strains induce a stronger inflammatory response (48-49) and are strongly associated with IHD (50). However, in agreement with previous findings (18, 51), the results of the present study show that virulent strains are equally prevalent in both controls and cases, suggesting that infection with CagA-positive strains of *H. pylori* does not represent a major risk factor for IHD. Recently, Aceti et al. (52), evaluating the possible role of *H. pylori* infection in acute IHD, suggested that virulent strains other than CagA-strains may be involved.

Recent reports propose that active infection with *H. pylori* is associated with an atherogenic lipid profile

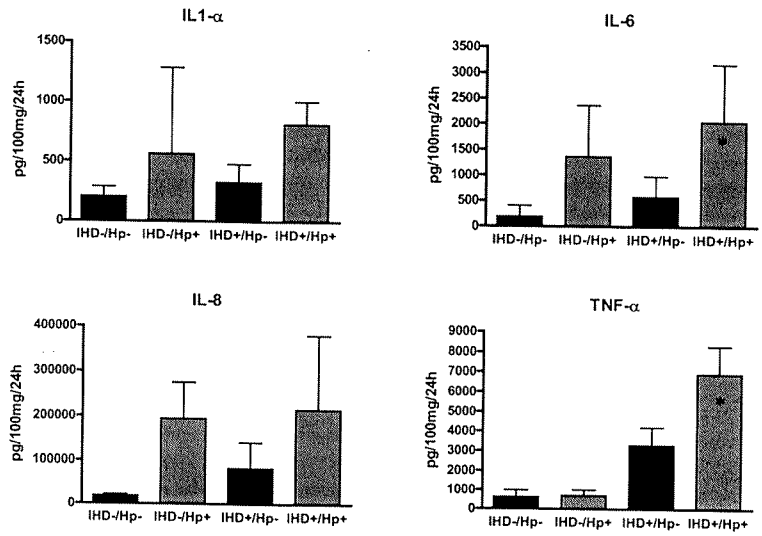

Fig. 1. *Mean mucosal levels (pg/100mg/24h) of IL-1α, IL-6, IL-8, and TNF-α in healthy subjects (IHD-) and patients (IHD+), with (Hp+) or without (Hp-) active H. pylori infection.*

IHD-/Hp- = 26; IHD-/Hp+ = 26; IHD+/Hp- = 24; IHD+/Hp+ = 34.
* *IHD/Hp+ vs IHD/Hp-, P<0.05.*

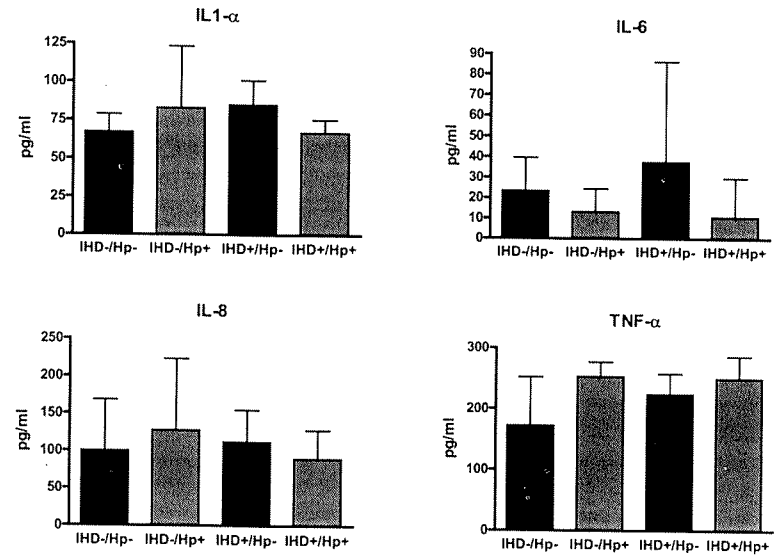

Fig. 2. *Mean circulating (pg/ml) levels of IL-1α, IL-6, IL-8, and TNF-α in healthy subjects (IHD-) and patients (IHD+), with (Hp+) or without (Hp-) active H. pylori infection.*

IHD-/Hp- = 26; IHD-/Hp+ = 26; IHD+/Hp- = 24; IHD+/Hp+ = 34.

(8, 53-54). Feingold et al. (55) postulated that chronic infection may alter lipid profile in an atherogenic direction via the action of proinflammatory cytokines such as IL-1 and IL-6, interferon α and TNFα that are capable of affecting lipid metabolism in different ways. However, our results gave no evidence that active infection is associated with an atherogenic modification of lipoproteins. In particular, in contrast with Hoffmeister et al. (53), we found that *H. pylori* infection in healthy subjects is associated to significantly higher apolipoprotein ApoAI levels than those in subjects without infection. We have no explanations accounting for this experimental evidence.

In this study, among the established risk factors considered, only the frequency of hypertension was significantly higher in IHD patients in comparison with controls subjects. This finding could represent a limitation of our study.

Inflammation represents an important feature of coronary heart disease (3), and several authors have discussed the role of an increased inflammatory response to various infectious stimuli that might represent the pathophysiological link between infection and coronary heart disease (8-12). The results of the present study, taken together, suggest that *H. pylori* active infection may play a role as a trigger factor in the pathophysiology of IHD by inducing, if combined with other risk factors for IHD, an inflammatory cascade to the site of infection where there is a generalized cytokine overproduction, although IL-6 and TNF-α only result significantly increased. However, *H. pylori*-induced cytokine activation becomes concentrated on gastric mucosa and does not influence circulatory levels, suggesting, in agreement with Klausz et al (24), that gastric mucosa could be considered as a separate compartment from the systemic circulation with respect to cytokine diffusion. Furthermore, the lack of a systemic cytokine overproduction could be due to the pharmacological stabilization of IHD patients we considered in the present study.

The possibility of treating *H. pylori* infection leads to the amazing scenario of reducing cardiovascular risk by using antibiotic therapy. The eradication of the bacterium could represent an absolute indication for patients with other risk factors for IHD, and at least relative if *H. pylori* infection is the only one risk factor. In this regard, prospective clinical studies are needed to clarify the role played by active *H. pylori* infection in the pathogenesis of IHD.

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