Role of Activated Protein C in *Helicobacter pylori*-Associated Gastritis

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The protein C (PC) pathway has recently been suggested to play a role in the regulation of the inflammatory response. To further extend the anti-inflammatory effect of activated PC (APC) in vivo, particularly its biological relevance to human disease, the activity of APC in the mucosa of patients with *Helicobacter pylori*-associated gastritis and the effect of vacuolating cytotoxin (VacA), cytoxin-associated antigen (CagA), and *H. pylori* lipopolysaccharide (LPS) on PC activation were evaluated. This study comprised 35 patients with chronic gastritis. There were 20 patients with and 15 without *H. pylori* infection. The levels of PC and APC-PC inhibitor (PCI) complex were measured by immunohasyses. The level of PC was significantly decreased and the level of APC-PCI complex was significantly increased in biopsy specimens from gastric corpus and antrum in patients with *H. pylori*-associated gastritis as compared to *H. pylori*-negative subjects. The concentrations of VacA, CagA, and LPS were significantly correlated with those of the APC-PCI complex in biopsy mucosal specimens from the gastric corpus and antrum. *H. pylori* LPS, VacA, and CagA induced a dose-dependent activation of PC on the surface of monocytic cells. APC inhibited the secretion of tumor necrosis factor alpha (TNF-α) induced by *H. pylori* LPS. Overall, these results suggest that *H. pylori* infection is associated with increased APC generation in the gastric mucosa. The inhibitory activity of APC on TNF-α secretion may serve to protect *H. pylori*-induced gastric mucosal damage.

*Helicobacter pylori* is the major causative factor of chronic atrophic gastritis and peptic ulcer disease (5, 18). Infection by this bacterium has more recently been identified as a risk factor for gastric cancer and as a causative factor of mucosa-associated lymphoid tissue lymphoma (15, 35). The virulence of *H. pylori* in the gastric mucosa has been associated with its ability to express cytotoxins (vacuolating cytotoxin [VacA], cytoxin-associated antigen [CagA]) and various enzymes (urease, protease) and with its ability to induce the secretion of various cytokines from host cells (31). Most clinical isolates of *H. pylori* produce VacA, which causes vacuolar degeneration in several mammalian cell lines; VacA- and CagA-producing strains are associated with the more severe forms of disease, such as peptic ulcer and gastric cancer (31). Urease derived from *H. pylori* may induce tissue damage by catalyzing the formation of ammonia or indirectly by inducing oxidative bursts of neutrophils or by stimulating monocytes to secrete proinflammatory cytokines (7, 25, 31). The production of cytokines has an important role in *H. pylori*-associated gastric-duodenal disease. Increased expression of tumor necrosis factor alpha (TNF-α), interleukin (IL)-1β, IL-8, and IL-6 has been reported in culture supernatants of *H. pylori*-infected gastric biopsy specimens (10). The mRNA expressions of IL-7, IL-8, and IL-6 were also found to be significantly higher in *H. pylori*-infected subjects than in controls (49). Cytokines can function in the acute-phase response, in wound healing, and in defense mechanisms by amplifying the host immune response. However, increased and persistent production of cytokines may exaggerate the inflammatory response, thus exerting a deleterious effect on the host. Acute and chronic inflammation, intravascular thrombosis, tissue atrophy, and remodeling are among the pathologic conditions that have a significant cytokine component (10).

The protein C (PC) pathway constitutes the most important anticoagulant system that regulates the activation of blood coagulation (13, 42). The anticoagulant PC zymogen is converted to the serine protease activated PC (APC) by the thrombomodulin (TM)-thrombin complex on the phospholipid surface of endothelial cells, monocytes, and platelets. Classically, APC has been described to exert anticoagulant activity by catalyzing the proteolytic inactivation of the coagulation factors Va and VIIIa and probrinolytic activity by inactivating plasminogen activator inhibitor type-1 (34). Recent studies suggested that, in addition to modulating the activation of blood coagulation, the PC pathway may also regulate the inflammatory response. Animal studies have demonstrated that systemic administration of APC prevents the lethal effects of *Escherichia coli*-induced sepsis and that it is effective for the treatment of patients with disseminated intravascular coagulation associated with meningococcemia and acquired PC deficiency (19, 38, 44). Data from these studies showed that APC may play a role in the inflammatory response by modulating the effects of cytokines, such as TNF-α, and by blocking neutrophil activation (32, 33). These observations have been supported by more recent in vitro studies in which it was shown that APC inhibits lipopolysaccharide (LPS), phorbol ester, and gamma-interferon-induced production of proinflammatory cytokines and that APC suppresses E-selectin-mediated inflammatory cell adhesion to endothelial cells (20, 23). Exacerbation of the response of primates to sublethal levels of *E. coli* and the increased circulating levels of TNF-α after inhibition of protein S, a glycoprotein that enhances the effect of APC, also support the thesis that PC has a regulatory role in the inflammatory response (45).

To further extend the anti-inflammatory effect of APC in vivo, particularly its biological relevance to human disease, in the present study, we evaluated the activity of APC in the mucosa of patients with *H. pylori*-associated gastritis. The effect of cytotoxins and LPS derived from *H. pylori* on PC acti-
viation and the inhibitory activity of APC on H. pylori-derived LPS-induced secretion of TNF-α were also investigated.

MATERIALS AND METHODS

Reagents. Cary-Blair medium was purchased from Oxoid Unipath Ltd. (Hampshire, United Kingdom), and M-BHM pylori agar was purchased from Nikken Chemicals (Kyoto, Japan). Recombinant VacA toxin, recombinant CagA from H. pylori, and polyclonal anti-VacA and anti-CagA antibodies were purchased from Austral Biologicals (San Ramon, Calif.). Bovine serum albumin (BSA), RPMI 1640 medium, and recumbent hirudin and apronin (an inhibitor of APC) were from Sigma Chemical (St. Louis, Mo.), and the APC chromogenic substrate (nitrophenyl-β-D-galactoside, 0.1 mM, pH 5.5, Chromogenic Substrate, Sweden). Penicillin/streptomycin were from Nakalai Tesque (Kyoto, Japan), and fetal bovine serum (FBS) was from Gibco BRL (Grand Island, N.Y.). WST-1-(iodonitrotetrazolium)-3(4-iodonitrophenyl)-5(4,4-disulfophenyl)-2H-tetrazolium-Na was purchased from Dojindo (Kumamoto, Japan). PC and thrombin were prepared from plasma as previously described (11, 43). All other chemicals and reagents used in this study were of the best quality commercially available.

Subjects and gastric endoscopy. This study comprised 35 patients (16 men and 19 women; age, 59.1 ± 12.2 [mean ± standard deviation] years) with chronic gastritis. They consulted in our institution because of dyspepsia. The patients were categorized into H. pylori-positive (12 men and 8 women; age, 53.5 ± 10.5 years) and H. pylori-negative (4 men and 11 women; age, 47.2 ± 13.0 years) patients after serological test, for H. pylori, which was also confirmed by bacteriological studies as described below. Classification and grading of gastritis was done according to the Updated Sydney System (12). In the latter group of patients, dietary habits, alcohol intake, duodenal regurgitation, and stress were considered potential causative factors of gastritis. Further, for comparison, patients with gastric (n = 13) and duodenal (n = 12) ulcer were also examined. None of the patients has undergone upper gastrointestinal surgery or had taken any drug over the previous 6 weeks. Gastric mucosal biopsy was performed by endoscopy in all subjects. Gastric endoscopy was carried out in the morning before breakfast, using an endoscope (Olympus Co., Tokyo, Japan). Patients lasted from 9:00 p.m. of the previous day until the time of endoscopy. Before endoscopy, the patients received pharyngeal anesthesia with lidocaine hydrochloride and an intramuscular injection of atropine sulfate (0.5 mg) and scopolamine butylyl bromide (20 mg). An intravenous injection of diazepam (5 mg) was additionally administered to some patients showing reactivity during the endoscopy study. The study protocol was approved by the Mic University Hospitals Institutional Review Board, and it was carried out following the principles of the Helsinki Declaration.

Preparation of biopsy specimen homogenates. During the gastric endoscopy, four biopsy samples were obtained from the middle portion of the gastric body and the antrum along the greater curvature. Two specimens were used for H. pylori culture and histological examination, and the remaining specimens were used for preparing homogenates. After sampling, biopsy specimens for preparing homogenates were frozen in liquid nitrogen and then homogenized in phosphate-buffered saline (PBS) and stored at –80°C until use. Homogenization of biopsy specimens was carried out in 1 ml of PBS containing leupeptin (1 μg/ml), pepstatin-A (1 μg/ml), aprotinin (1 μl/ml), and phenylmethylsulfonfyl fluoride (1 mM). H. pylori homogenates were prepared by the method of Westphal and Jann (36, 48). In brief, the bacteria were scraped from blood agar into saline, centrifuged at 10,000 × g for 5 min, and the supernatants were then used successively with biotin-labeled rabbit anti-mouse immunoglobulin G, peroxidase-labeled streptavidin, and peroxidase substrate by using the Catalyzed Signal Amplification System from DAKO.

H. pylori LPS preparations. LPS was prepared from H. pylori ATCC 43504 by the hot-phenol-water method of Westphal and Jann (36, 48). In brief, the bacteria were scraped from blood agar into saline, centrifuged at 10,000 × g for 15 min, and then resuspended in distilled water with an equal volume of 90% phenol at 60°C for 15 min. The mixture was then cooled to 4°C and centrifuged at 10,000 × g for 15 min. The aqueous layer was pooled and the same procedure was repeated twice. The pooled water-extracted layers were then dialyzed for 72 h against several changes of distilled water. The structure of the LPS from H. pylori ATCC 43504 has been described to be composed of a hydrophobic lipid A moiety, a core oligosaccharide region, and an O-polysaccharide chain; the last one is a partially fucosylated N-acetyllactosaminoglycan chain containing a terminal Lewis antigen (1). As a control, LPS purified by the hot-phenol-water from Escherichia coli O55:B5 (Difeo Laboratories, Detroit, Mich.) was used in each experiment.

Identification of H. pylori. After sampling, biopsy specimens were immediately placed in Cary-Blair medium and stored at 4°C until use. Within 3 h of collection, specimens were plated onto M-MEM pylori agar and then incubated at 37°C for 5 days in a low aerobic atmosphere created by using 10% of CO2 incubator. The presence of H. pylori in milky white semitransparent colonies suspected of containing H. pylori was examined by observing various biochemical tests (urease, oxidase, catalase) and citrate utilization tests. H. pylori was identified by examining, under a light microscope, formalin-fixed biopsy specimens stained with May Giemsa. H. pylori cells appeared as spiral rods with a width of about 0.5 μm and a length of about 3 μm. Patients were classified as H. pylori-positive if their biopsy specimen was positive in culture or histological examination and as H. pylori-negative when the organism was not detected in culture, histologically, or by biochemical tests.

Culture of THP-1 cells. THP-1 cells (American Type Culture Collection, Rockville, Md.) were cultured in RPMI medium supplemented with 10% FBS, 100 μg of penicillin/ml, 100 μg of streptomycin/ml, and 2 nM l-arginine under an atmosphere of 95% air and 5% CO2.

Preparation of peripheral blood mononuclear cells. Peripheral blood mononuclear cells (PBMC) were obtained by density gradient centrifugation using a Ficoll-Hypaque (Lymphoprep Tube (Nycomed Pharma Diagnostica, Oslo, Norway). The mononuclear cell phase, comprising monocytes and lymphocytes, was harvested, washed twice with RPMI, and resuspended in RPMI (107 cells/ml). Monocytes (1 × 107 cells/liter) were depleted by using the antibody (pan-CD14, Pharmingen, San Diego, CA, USA) and a magnetic bead (Miltenyi Biotec, Bergisch Gladbach, Germany). The purity of the monocytes was confirmed by FACS analysis, allowing the isolation of >95% monocytes.

Assay of PC activation on monocyte cell surface. The ability of monocytes to generate APC in the presence of PC and thrombin were evaluated as described (21, 23). Human mononuclear cells (1 × 107 to 2 × 107/well) were washed three times in reaction buffer (50 mM Tris-HCl, pH 7.5, containing 2 mM CaCl2 and 0.1% BSA). Cells were then incubated in 96-well plates in the presence of PC (5 μg/ml), thrombin (0.12 U/ml), and reaction buffer in a final volume of 120 μl at 37°C under an atmosphere of 95% air and 5% CO2. Then, the plates were centrifuged at 11,000 × g for 5 min and the generation of APC was measured in the supernatants. Generation of APC was detected by cleavage of APC substrate S2366 by using a microplate ELISA reader. To prevent nonspecific cleavage of S2366 by thrombin, hirudin (250 antithrombin units/ml) was added to each supernatant for 5 min at room temperature before testing for APC activation. PC activation was markedly expressed on both THP-1 and peripheral blood mononuclear cells (data not shown); thus, subsequent experiments were done by using only THP-1 cells.

Effect of biopsy specimen homogenates on PC activation in monocyte THP-1 cells. To determine the effect of gastric mucosal homogenate on PC activation, THP-1 cells (1 × 107 cells/well) were cultured in RPMI medium (300 μl) containing heat-inactivated 10% FBS for 24 h in duplicate wells of 48-well tissue culture trays in the presence of supernatants of gastric mucosal homogenate (30 μl). Then, the cells were washed three times with reaction buffer, and then APC generation was measured as described above. APC values were extrapolated from graphs, and therefore, results were given as the percentage of control.

Effect of VacA, CagA, and LPS from H. pylori on PC activation in THP-1 cells. To determine the effect of H. pylori-derived cytotoxins or LPS on PC activation, THP-1 cells (1 × 107 cells/well) were cultured for 24 h in duplicate wells of 96-well tissue culture trays. The cytotoxins of VacA, CagA, or LPS from H. pylori. The cells were then washed three times with reaction buffer, and then PC activation was measured as described above. The
Effect of inactive toxins on PC activation on THP-1 cells was also assessed; for these experiments, 10 μg of the toxins per ml were heat inactivated by incubating in reaction buffer at 100°C for 15 min and then used in the assays. VacA was also inactivated by treating with formaldehyde for 48 h at 37°C as described previously (28).

Effect of APC on LPS-induced expression of TNF-α by THP-1 cells. Supernatants were collected from THP-1 cells that were cultured in 96-well flat-bottom tissue culture plates in medium for 24 h in the presence of LPS (10 μg/ml) and various concentrations of APC and stored at −80°C until use. To evaluate the LPS dose dependency of APC effect on TNF-α expression, THP-1 cells were cultured in medium for 24 h in the presence of APC (15 μg/ml) and various concentrations of LPS (15 to 0 μg/ml). After centrifuging, the supernatants were collected and stored at −80°C until use. The concentration of human TNF-α in supernatants was measured by using a commercial immunoassay kit purchased from Biosource International (Camarillo, Calif.). The minimum detectable level of TNF-α was <0.09 pg/ml. The intra-assay and the inter-assay coefficients of variation of TNF-α were <5 and <10%, respectively.

Statistical analysis. Data are expressed as the mean ± the standard error. The difference between the mean of two variables was calculated by Student’s t test and that between three or more variables by analysis of variance. A P value of <0.05 was considered statistically significant.

RESULTS

Gastric mucosal and plasma concentrations of PC and APC-PCI complex. The concentration of PC was significantly decreased in biopsy specimens from gastric corpus (7.5 ± 3.7 [mean ± standard error] versus 22.9 ± 11.5 pg/μg of protein) and antrum (1.8 ± 0.3 versus 3.1 ± 0.4 pg/μg of protein) in patients with H. pylori-associated gastritis as compared to that of H. pylori-negative subjects (Fig. 1). The concentration of APC-PCI complex, an indicator of ongoing PC activation, was significantly increased in biopsy specimens from gastric corpus (13.9 ± 2.3 versus 7.8 ± 0.9 pg/μg of protein) and antrum (10.2 ± 1.6 versus 6.7 ± 0.7 pg/μg of protein) in H. pylori-positive gastritis patients as compared to those without H. pylori infection (Fig. 1). The patients were also classified according to the degree of gastric mucosal infiltration of neutrophils in active, inactive, and healthy groups. The APC-PCI complex level in mucosal specimens from corpus was significantly higher in the active group (14.5 ± 2.7 pg/μg of protein) than in the inactive (11.1 ± 3.5 pg/μg of protein) and healthy (7.7 ± 0.9 pg/μg of protein) groups. The gastric mucosal concentrations of APC-PCI tended to be higher, but not at a significant level, in patients infected with H. pylori positive for CagA compared to those infected with bacteria negative for this antigen. In addition, there was not a significant difference in the degree of mucosal PC activation among patients with gastritis or gastric and duodenal ulcer (data not shown). The plasma concentrations of PC and APC-PCI were not significantly different between patients with and without H. pylori infection (data not shown).

Gastric mucosal and plasma concentrations of TAT. The concentrations of TAT, a marker of coagulation activation, in gastric mucosal specimens and plasma were not significantly different between H. pylori-positive and H. pylori-negative patients (data not shown).

PC activation on THP-1 cells induced by homogenates of gastric biopsy specimens, LPS, and cytotoxins. PC activation on the surface of THP-1 cells was significantly increased after overnight incubation of these cells with homogenate supernatants prepared from gastric biopsy specimens of patients with H. pylori-positive gastritis as compared to that induced by homogenate supernatants from biopsy specimens of H. pylori-negative gastritis patients and by buffer control (Fig. 2). To assess the effect of endotoxin and cytotoxins from H. pylori on PC activation by mononuclear cells, THP-1 cells were cultured overnight in the presence of various concentrations of H. pylori LPS, VacA, or CagA. H. pylori LPS induced a dose-dependent activation of PC on the surface of THP-1 cells. The degree of this PC activation was similar to that induced by E. coli-derived LPS (Fig. 3). Both VacA and CagA also increased the activation of PC in a dose-dependent manner (Fig. 4). VacA increased PC activation from concentrations of 1 μg/ml, whereas CagA increased PC activation above concentrations of 3 μg/ml. Neither heat-inactivated toxins nor formaldehyde-inactivated VacA affected the activation of PC on THP-1 cells. H. pylori LPS, VacA, and CagA also similarly increased the activation of PC on peripheral blood monocytes from healthy donors (data not shown).

Relationship between the levels of APC-PCI complex and those of VacA, CagA, and LPS in the gastric mucosa of H. pylori-positive patients. In the gastric corpus, the concentrations of VacA, CagA, and LPS were 0.070 ± 0.02 [mean ± standard deviation], 2.3 ± 2.1, and 21.4 ± 2.7 pg/μg of protein, respectively. In antrum, the concentrations of VacA, CagA, and LPS were 0.10 ± 0.04, 1.1 ± 0.4, and 2.2 ± 0.2 pg/μg of protein, respectively. The concentrations of VacA (r = +0.7, P < 0.03), CagA (r = +0.9, P < 0.03), and LPS (r = +0.6, P < 0.04) were significantly correlated with those of APC-PCI complex in biopsy mucosal specimens from the gastric corpus.
concentrations of VacA (r = +0.6, P < 0.01), CagA (r = +0.7, P < 0.002), and LPS (r = +0.5, P < 0.05) were also significantly correlated with those of APC-PCI complex in biopsy mucosal specimens taken from the antrum. The relation of these H. pylori components with the degree of gastric inflammation was also investigated; the gastric mucosal level of VacA (r = +0.7, P < 0.01), but not that of CagA or LPS, was significantly correlated with the number of inflammatory cells in the gastric mucosa (mononuclear cells plus neutrophils).

**Immunohistochemical staining of TM in the gastric mucosa.** The gastric biopsy specimens from H. pylori-positive patients showed significant expression of immunoreactive TM in the subepithelial region of the gastric mucosa. Capillaries and monocytic phagocytes showed immunoreactivity of TM (Fig. 5A); some inflammatory cells migrating towards the gastric lumen were also found to stain positively for TM. Increased immunoreactivity of the monocytic phagocyte marker CD68 was also mainly observed in the subepithelial region of the gastric mucosa of H. pylori-positive patients (Fig. 5B). Staining of TM (Fig. 5C) or CD68 (Fig. 5D) was relatively weak in mucosal specimens from H. pylori-negative patients.

**Effect of APC on cytokine production induced by H. pylori LPS.** To assess the effect of APC on H. pylori LPS-induced secretion of TNF-α by THP-1 cells, these mononuclear cells were cultured overnight in the presence of LPS and various concentrations of APC. TNF-α levels were measured in the cell culture supernatants. As shown in Fig. 6, APC inhibited the secretion of TNF-α induced by H. pylori LPS in a dose-dependent fashion. The inhibitory activity was significant above APC concentrations of 2 μg/ml. Incubation of APC in the presence of aprotinin (15 μM) blocked the inhibitory activity of APC on TNF-α secretion. The effect of APC on TNF-α secretion by THP-1 cells was LPS dose dependent. The inhibitory activity of APC on TNF-α secretion was found to be significantly effective at LPS concentrations between 10 and 2 μg/ml. The viability of the cells as measured by WST-1 was not affected by APC at any concentration used in the assay.

**DISCUSSION**

Following tissue injury, there is an exquisite interplay between coagulation, anticoagulation proteins, cytokines, adhesion molecules, and inflammatory cells in an attempt to resolve injury. The balance between these multiple interrelated factors are thought to be fundamental for the resolution of tissue injury. The PC natural anticoagulant pathway has been proposed to serve as a link between inflammation and coagulation (14). APC, the enzyme effector of the natural anticoagulant pathway, has also been found to have anti-inflammatory activity and to protect against organ damage by inhibiting the secretion of cytokines at site of inflammation (14, 33). To further extend the biological function of APC, we evaluated the role of this protease in the inflammatory response associated with H. pylori infection. The work reported here supports the concept that APC can play an important role in inflammation, particularly in the regulation of cytokine production. Our present results demonstrate that (i) APC formation is increased in the gastric mucosa of patients infected with H. pylori, (ii) PC activation is induced by LPS, VacA, and CagA derived from the bacterium, and (iii) APC inhibits the secretion of TNF-α induced by H. pylori LPS on monocytic cells.

The rate-limiting event in the generation of APC is the cellular availability of the membrane-bound glycoprotein TM. In the present study, compared to uninfected individuals, a high concentration of APC-PCI complex (an indicator of APC
FIG. 5. Immunohistochemical staining of TM and monocytes/macrophages in the gastric mucosa (×400). (A) Significant expression of immunoreactive TM can be observed in the subepithelial region of the gastric mucosa of patients with H. pylori infection; TM staining was observed on monocytic phagocytes and capillaries. (B) Increased immunoreactivity of the monocytic phagocyte marker CD68 was also observed in the gastric mucosa of H. pylori-positive patients. Immunoreactivity for TM (C) and CD68 (D) was weak in the mucosa of patients without H. pylori infection.
**REFERENCES**


**FIG. 6.** Effect of APC on H. pylori LPS-induced TNF-α secretion from THP-1 cells. APC significantly inhibited the secretion of TNF-α induced by H. pylori LPS in a dose-dependent fashion. Each value represents the mean ± standard error of triplicate determinations performed in four separate experiments. Aprotinin-treated APC did not affect TNF-α secretion by THP-1 cells. *P value of <0.05 when data were compared to data of control medium (medium with H. pylori LPS alone).
PROTEIN C IN HELICOBACTER PYLORI INFECTION


