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Involvement of Endothelin-1 in Up-regulation of Gastric Mucosal Inflammatory Responses to *Helicobacter pylori* Lipopolysaccharide

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In this study, we investigated gastric mucosal inflammatory responses during *Helicobacter pylori* lipopolysaccharide-induced gastritis by analyzing the interplay between mucosal expression of endothelin-1 (ET-1), interleukin-4 (IL-4) and tumor necrosis factor-α (TNF-α). The assays conducted 4 days after intragastric dose of *H. pylori* lipopolysaccharide demonstrated a pattern of acute mucosal reaction characterized by the inflammatory infiltration of the lamina propria, hyperemia, and epithelial hemorrhage. This was accompanied by a 3.1-fold increase in the mucosal expression of ET-1 and a 9-fold enhancement in TNF-α, while the level of IL-4 showed a 20.8% decline. The results implicate ET-1 in gastric mucosal responses to *H. pylori*, and suggest that an increase in its level, combined with a loss of compensatory action by IL-4, may be responsible for the induction of TNF-α and triggering apoptotic events that exacerbate the inflammatory process. © 1999 Academic Press

***Key Words: Helicobacter pylori;* lipopolysaccharide; acute gastritis; ET-1; IL-4; TNF-α.**

Helicobacter pylori is recognized as a primary etiologic factor in the development of gastric disease as well as eliciting mucosal inflammatory responses that characterize gastritis, and the product of particular significance to the virulent action of the bacterium is its cell wall lipopolysaccharide (1–5). The pathogenic effects of *H. pylori* lipopolysaccharide are manifested by a marked enhancement in gastric epithelial cell apoptosis, disturbances in nitric oxide signaling pathway, apoptotic caspase activation, and induction of inflammatory TNF-α, IL-6 and IL-8 expression (6–11). Other mediators intimately involved in the progression of mucosal inflammatory process include regulatory cytokine, IL-4, and a potent vasoactive peptide, ET-1 (8, 13–16).

The endothelins (ET) are a family of cysteine-rich peptides consisting of 21 amino acids and containing two intramolecular disulfide bridges (17). At the present, the existence of three active isoforms of endothelin, ET-1, ET-2, and ET-3, and two distinct receptors, ET_A and ET_B, is well documented (17, 18). The ET receptors, aside of vascular tissues, are also found in gastric and intestinal mucosa where ET-1 plays a major role in the pathogenesis of mucosal injury, and the increase in gastric mucosal ET-1 expression is a characteristic feature of stress ulcer (12, 19, 20). Moreover, enhanced ET-1 levels accompany local and systemic inflammations, and there are data indicating that ET-1 stimulates formation of several proinflammatory cytokines, including TNF-α (13–15). The expression of these mediators of inflammatory process is controlled at the translational level by the regulatory pleiotropic cytokine IL-4 (16, 21).

Indeed, the results of several studies demonstrated that IL-4 down-regulates a wide variety of TNF-induced effects, including suppression of TNF-induced NFκB and AP-1 activation, inhibition of caspase-3 activity, and abrogation of TNF-induced c-Jun N-terminal kinase activation (21–23). Moreover, IL-4 through the involvement of a specific metalloprotease is capable of inducing TNF receptor shedding (21, 24). The role for phosphatidylinositol 3-kinase in down-regulation of TNF receptors by IL-4 has also been suggested (25).

In this study, we investigated the course of inflammatory events elicited in gastric mucosa by *H. pylori* lipopolysaccharide by analyzing the interplay between mucosal expression of ET-1, IL-4 and TNF-α.

MATERIALS AND METHODS

This study was conducted with 180 to 200 g Sprague-Dawley rats cared for by the professional personnel of the Research Animal Facility. The animals were deprived of food for 24 h before the experiment, and water was withheld 2 h before the procedure (9). All

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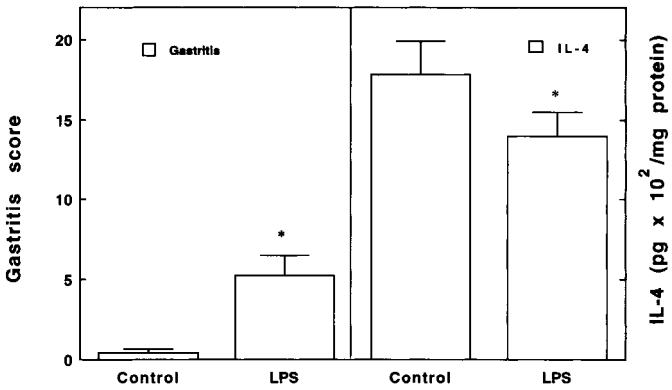

FIG. 1. Scores of acute gastritis and the expression of IL-4 in gastric mucosa of rats exposed to *H. pylori* lipopolysaccharide at 50 μg/animal. The assays were carried out 4 days after the lipopolysaccharide application. Values represent the means ± SD obtained with 8 animals in each group.

experiments were carried out with groups consisting 8 animals per treatment, and the animals received intragastrically either 100 μl of saline or *H. pylori* lipopolysaccharide at 50 μg per animal (8, 9). The rats in each group were killed 4 days after the treatment, their stomachs dissected, and the mucosal tissue used for histologic assessment and quantification of ET-1, IL-4 and TNF-α.

TNF-α was quantitated with an enzyme-linked immunosorbent assay according to the manufacturer's (Genzyme) instructions. The wells were precoated with monoclonal anti-TNF-α to capture TNF-α from the mucosal homogenates, and, after washing, the retained complex was probed with horseradish peroxidase-conjugated anti-TNF-α. The complex was then incubated with tetramethylbenzidine (TMB) reagent for TNF-α quantitization (8).

ET-1 assays were carried out on the individual specimens of gastric mucosa following lyophilization and homogenization with 4 volumes of 1 M acetic acid containing 10 μg/ml of pepstatin (12, 26). The homogenates were heated for 5 min at 100°C, centrifuged, and the resulting supernatants applied to a Sep-Pack C-18 reverse phase cartridges. After initial washing with 0.1% trifluoroacetic acid, the adsorbed ET-1 was eluted with methanol-water-trifluoroacetic acid (90:10:0.1, v/v/v). The eluates were dried under vacuum, reconstituted in the assay buffer, and subjected to ET-1 quantitization using double-antibody sandwich technique according to the manufacturer's (Alexis Corporation) instruction.

IL-4 measurements were conducted using a solid-phase enzyme-linked immunosorbent system (Bio-Source International). The individual specimens of gastric mucosa were homogenized with 5 volumes of the sample buffer, centrifuged, and the resulting supernatant diluents were pipetted to the microtitrator wells precoated with antibody specific for rat IL-4 (27). Following incubation, the complex was probed with biotinylated second antibody, reacted with streptavidin-peroxidase, and processed with TMB reagent for IL-4 quantitization (27).

Helicobacter pylori ATCC No. 4350 clinical isolate was used for lipopolysaccharide preparation (5), and the protein content of samples was measured with BCA kit. The changes in mucosal histology were quantified on the basis of the scoring system of Rauws et al. (28). The sections of gastric mucosa were cut into 4 μm strips, fixed with formalin, stained with hematoxylin and eosin, and examined by a person unaware of the type of treatment (5). The intact mucosa with no infiltration was graded as 0; the density of the inflammatory infiltrate in the lamina propria, 0–2; the density of polymorphonuclear leukocytes in the lamina propria, 0–3; the presence of intraepithelial polymorphonuclear leukocytes, 0–3; and superficial erosions, 0–2 (5, 28).

All experiments were carried out in duplicate, and the results are expressed as the means ± SD. The significance level was set at $p < 0.05$ and the tests were performed using Soft Stat, STATISTICA, software.

RESULTS

The course of inflammatory events elicited in gastric mucosa during *H. pylori*-induced gastritis was assessed in the animal model, using rats exposed to *H. pylori* lipopolysaccharide (5). The results of histologic examination of the mucosa 4 days after exposure of the animals to intragastric surface epithelial application of *H. pylori* lipopolysaccharide at 50 μg per animal revealed a pattern of inflammatory responses resembling that of acute gastritis with the mean grade of mucosal pathologic condition of 5.3 (Fig. 1).

The analysis of gastric mucosal expression of IL-4 revealed that the inflammatory changes associated with *H. pylori* lipopolysaccharide-induced gastritis were accompanied by a 20.8% reduction in this regulatory cytokine level. The mean value attained for IL-4 in gastric mucosa of the control animals was 176.8 pg/mg protein, while that in the animals exposed to *H. pylori* lipopolysaccharide averaged 140.1 pg/mg protein (Fig. 1).

The data on the gastric mucosal expression of ET-1 and TNF-α during acute gastritis induced by inflammatory responses to *H. pylori* lipopolysac-charide are summarized in Fig. 2. The assays established the mean value for ET-1 in normal gastric mucosa at 7.9 pg/mg protein and for TNF-α at 2.7 pg/mg protein. Compared with controls, the intragastric application of *H. pylori* lipopolysaccharide elicited a 3.1-fold enhancement in the mucosal expression of ET-1, while the mucosal level of TNF-α showed a 9-fold increase.

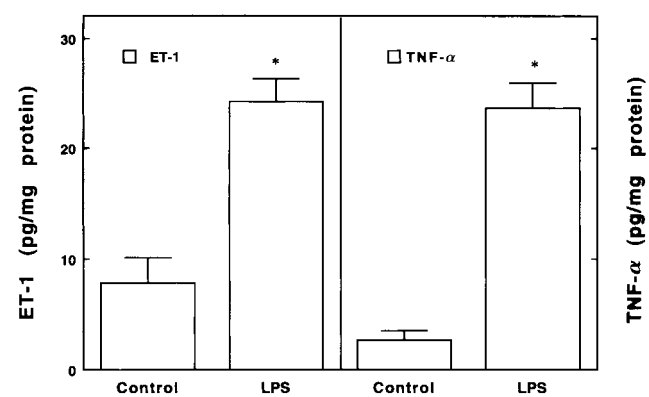

FIG. 2. Expression of ET-1 and TNF-α in gastric mucosa of rats exposed to *H. pylori* lipopolysaccharide at 50 μg/animal. The assays were carried out 4 days after the lipopolysaccharide application. Values represent the means ± SD of duplicate analyses performed with 8 animals in each group.

DISCUSSION

Our previous studies with animal model demonstrated that gastric mucosal responses to *H. pylori* lipopolysaccharide are histologically similar to those observed in patients with *H. pylori*-associated gastritis and include such recognizable feature of acute gastritis as inflammatory infiltration of the lamina propria, hyperemia, and epithelial hemorrhage (1–5). These inflammatory effects of the lipopolysaccharide have been linked to the enhancement in proinflammatory cytokine expression, induction of nitric oxide synthase-2, disturbances in regulatory cytokines release, up-regulation of caspase-3 activation, and a massive epithelial cell apoptosis (5, 8, 9). As the expression of TNF-α remains under the stimulation by ET-1, and is controlled at the translational level by IL-4 (16, 21, 29, 30), in this study we investigated further the effect of *H. pylori* lipopolysaccharide on the course of events associated gastric mucosal inflammatory responses by analyzing the interplay between the expression of ET-1, IL-4 and TNF-α.

The results obtained revealed that gastric mucosal inflammatory responses to *H. pylori* lipopolysaccharide, accompanied by a massive epithelial cell apoptosis, were characterized by a 3.1-fold increase in mucosal expression of ET-1 and a 9-fold enhancement in TNF-α, while the mucosal level of IL-4 fell by 20.8%. These findings, together with our recent data on the enhanced mucosal expression of NOS-2 and caspase-3 activity in response to *H. pylori* (9), underscore the importance of ET-1 and IL-4 in regulation of the events involved in gastric mucosal inflammatory reaction to *H. pylori* lipopolysaccharide. Indeed, the available literature indicates that ET-1 affects formation of such proinflammatory cytokines as IL-2 and IL-6, and stimulates biosynthesis of TNF-α (14, 15). The later process apparently involves the activation by ET-1 of ET_A receptor which, in turn, leads to the activation of tyrosine kinase intracellular protein cascade and ultimately culminates in translation of the TNF-α gene (15, 30). Thus, our present findings on the enhanced expression of ET-1 and TNF-α in response to challenge by *H. pylori* lipopolysaccharide, strongly imply a key involvement for ET-1 in triggering the prolonged mucosal inflammatory reaction that interferes with cessation of apoptotic events required for mucosal repair.

The studies into endothelial cell biology indicate that the vasoactive form of ET-1 is a 21 amino acid peptide, derived from the initial gene product, inactive big ET-1, by a series of proteolytic steps culminating in the hydrolysis of $Tryp^{21}$-Val^{22} bond by a specific metallopeptidase, known as endothelin-converting enzyme ECE (17, 18). Hence, the activity of this cell surface metalloprotease is not only of paramount importance to ET-1 generation, but also to the production of TNF-α and the apoptotic signal propagation. Interestingly, the inflammatory responses involving up-regulation of metalloproteinase gene expression are controlled at the translational level by IL-4, a pleiotropic cytokine that exerts its effects on target cells through the specific bipartite IL- 4 receptor consisting of a distinct α-chain and a common γc-chain that is shared by several other interleukins (16, 31). The interaction of IL-4 with the α-chain and γc-chain of the receptor leads to the receptor subunit dimerization and the activation of Janus kinase-signal transducer and activator of transcription (JAK-STAT) pathway resulting in the transcriptional activation of specific genes (20, 32). Activation of Janus protein tyrosine kinases JAK1, associated with α-chain, and JAK3, associated with γc-chain of the receptor, occurs by transphosphorylation and leads to the recruitment of STAT6, which becomes phosphorylated at critical tyrosine residues (29, 31, 32). The phosphorylated STAT factors then dimerize, translocate to the cell nucleus, and modulate transcription of target genes leading to such cellular events as suppression of apoptosis, and blocking the synthesis and processing of metalloproteinases (16, 18, 29 31). Conversely, tyrosine dephosphorylation of JAK-STAT6 proteins is a key for down regulation of IL-4 signaling.

From the results of our study, it is apparent that *H. pylori* lipopolysaccharide exerts detrimental effect on the gastric mucosal IL-4 expression, causing dysregulation of ET-1 production, induction of TNF-α, and triggering the apoptotic events that further exacerbate the inflammatory process. Hence, *H. pylori* may well be affecting the efficiency of gastric mucosal responses by suppressing the mucosal ability to generate adequate level of IL-4 required for the inhibition of ECE expression.

REFERENCES

1. Stolte, M., and Edit, S. (1996) *Scand. J. Gastroenterol.* **31**(Suppl. 214), 13–16.
2. Rune, S. J. (1996) *Scand. J. Gastroenterol.* **31**(Suppl. 214), 2–4.
3. Piotrowski, J. (1998) *J. Physiol. Pharmac.* **49,** 3–24.
4. Slomiany, B. L., Piotrowski, J., and Slomiany, A. (1997) *Arzneim. Forsch. Drug Res.* **47,** 475–482.
5. Piotrowski, J., Piotrowski, E., Skrodzka, D., Slomiany, A., and Slomiany, B. L. (1997) *Scand. J. Gastroenterol.* **32,** 203–211.
6. Crabtree, J. E., Shallcross, T. M., Heatley, R. V., and Wyatt, J. L. (1991) *Gut* **32,** 1472–1477.
7. Crabtree, J. E., Peichl, P., Wyatt, J. L., Stachl, U., and Lindley, I. J. (1993) *Scand. J. Gastroenterol.* **37,** 65–70.
8. Slomiany, B. L., Piotrowski, J., and Slomiany, A. (1998) *Scand. J. Gastroenterol.* **33,** 916–922.
9. Slomiany, B. L., Piotrowski, J., and Slomiany, A. (1998) *Biochem. Mol. Biol. Int.* **46,** 1063–1070.
10. Mustafa, S. B., and Olson, M. S. (1998) *J. Biol. Chem.* **273,** 5073–5080.
11. Chen, C. C., Wang, J. K., Chen, W. C., and Lin, S. B. (1998) *J. Biol. Chem.* **273,** 19424–19430.
12. Michida, T., Kawano, S., Masuda, E., Kobayashi, I., Nishimura, Y., Tsujii, M., Takei, Y., Tsuji, S., Nagano, K., Fasumoto, H.,

Kamada, T., and Sugimoto, T. (1998) *Am. J. Gastroenterol.* **92,** 1177–1181.

13. Agui, T., Xin, X., Cai, Y., Sakai, T., and Matsumoto, K. (1994) *Blood* **84,** 2531–2538.
14. Yin, X., Cai, Y., and Agui, T. (1995) *Endocrinology* **136,** 132–137.
15. Ruetten, H., and Thiemermman, C. (1997) *J. Physiol. Pharmac.* **48,** 675–688.
16. Mijatovic, C., Kruys, V., Caput, D., Defrance, P., and Huez, G. (1997) *J. Biol. Chem.* **272,** 1494–1498.
17. Pollock, D. M., Keith, T. L., and Highsmith, R. F. (1995) *FASEB J.* **9,** 1196–1204.
18. Turner, A., and Tanzawa, K. (1997) *FASEB J.* **11,** 355–364.
19. Boros, M., Massberg, S., Baranyi, L., Okada, H., and Messmer, K. (1998) *Gastroenterology* **114,** 103–114.
20. Hassan, M., Kashimura, H., Matsumaru, K., Nakahara, A., Iwata, R., Hayashi, T., Muto, H., Tanaka, N., Goto, K., and Fukutomi, Y. (1997) *Dig. Dis. Sci.* **42,** 1375–1380.
21. Manna, S. K., and Aggarwal, B. B. (1998) *J. Biol. Chem.* **273,** 33333–33341.
22. Bennet, L. R., Cruz, R., Lacson, R. G., and Manning, A. M. (1997) *J. Biol. Chem.* **272,** 10212–10219.
23. Brunet, L. R., Finkelman, F. D., Cheever, A. W., Kopf, M. A., and Pearce, E. J. (1997) *J. Immunol.* **159,** 777–785.
24. Crowe, P. D., Walter, B. N., Mohler, K. M., Otten-Evans, C., Black, R. A., and Ware, C. F. (1995) *J. Exp. Med.* **181,** 1205–1210.
25. Ueno, H., Sasaki, K., Honda, H., Nakamoto, T., Yamagawa, T., and Hirani, H. (1998) *Blood* **91,** 46–53.
26. Slomiany, B. L., Piotrowski, J., and Slomiany, A. (1998) *Biochem. Mol. Biol. Int.* **45,** 681–688.
27. Slomiany, B. L., Piotrowski, J., and Slomiany, A. (1998) *Biochem. Mol. Biol. Int.* **44,** 381–388.
28. Rauws, E. A. J., Langenberg, W., Houthoff, H. J., Zanen, M. C., and Tytgat, G. N. J. (1988) *Gastroenterology* **94,** 33–40.
29. Lishke, A., Moriggl, R., Brandlein, S., Berchtold, S., Kammer, W., Sebal, W., Groner, B., Liu, X., Hennighausen, L., and Fredrich, K. (1998) *J. Biol. Chem.* **273,** 31222–31229.
30. Gallios, C., Habib, A., Tao, J., Moulin, S., Maclouf, J., Mallat, A., and Lotersztain, S. (1998) *J. Biol. Chem.* **273,** 2383–2390.
31. M., Giasi, M., and Krammer, P. W. (1998) *J. Biol. Chem.* **273,** 32460–32466.
32. Haque, S. J., Harbor, P., Tabrizi, M., Yi, T., and Williams, B. R. G. (1998) *J. Biol. Chem.* **273,** 33893–33896.