Cloning of Mongolian gerbil cDNAs encoding inflammatory proteins, and their expression in glandular stomach during *H. pylori* infection

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(Received June 29, 2004/Revised August 23, 2004/Accepted August 24, 2004)

Mongolian gerbils are considered to be a good animal model for understanding the development of *Helicobacter pylori*-associated diseases. However, limitations regarding the genetic information available for this animal species hamper the elucidation of underlying mechanisms. Thus, we have focused on identifying the nucleotide sequences of cDNAs encoding Mongolian gerbil inflammatory proteins, such as interleukin-1 (IL-1β), tumor necrosis factor α (TNF-α), cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS). Furthermore, we examined the mRNA expression of these genes in the glandular stomach by RT-PCR at 1–8 weeks after *H. pylori* infection. The deduced amino acid homologies to mouse, rat and human proteins were 86.2%, 83.6% and 67.8% for IL-1β, 87.2%, 85.1% and 78.4% for TNF-α, 91.9%, 90.2% and 84.8% for COX-2 and 90.8%, 89.1% and 80.1% for iNOS, respectively. The average stomach weight of Mongolian gerbils inoculated with *H. pylori* was increased in a time-dependent manner at 1, 2, 4 and 8 weeks after inoculation. In the pyloric region, mRNA expression levels of IL-1β, TNF-α and iNOS were increased in *H. pylori*-infected animals at the 2 weeks time point, while in the fundic region, expression levels of IL-1β, TNF-α and iNOS were elevated at 4 and 8 weeks. The COX-2 expression level in the fundic region was clearly elevated in infected animals compared with control animals at 4 and 8 weeks, but in the pyloric region, expression levels were similar in both infected and control animals. Thus, our results indicate that oxidative stress occurs from an early stage of *H. pylori* infection in the glandular stomach of Mongolian gerbils. (Cancer Sci 2004; 95: 798–802)

It is known that *Helicobacter pylori* infection is associated with upper gastrointestinal diseases, such as peptic and duodenal ulcers,[1–3] as well as gastric cancer development.[4–6] Mongolian gerbils can be readily colonized by *H. pylori*, with associated development of chronic gastritis, gastric ulcers and intestinal metaplasia after prolonged infection.[7, 8] Furthermore, it has been reported that *H. pylori* infection greatly enhances *N*-methyl-*N*-nitrosourea-induced stomach carcinogenesis in Mongolian gerbils.[9] Thus, they have been used as an animal model for development of *H. pylori*-associated diseases. However, genetic information about Mongolian gerbils is relatively limited, which hampers elucidation of the underlying mechanisms.

H. pylori infection is associated with activation and infiltration of monocytes, neutrophils and lymphocytes, which produce various inflammatory factors. Inflammatory cytokines, such as interleukin-1β (IL-1β) and tumor necrosis factor α (TNF-α) are potent inhibitors of gastric acid secretion,[10, 11] and the acid secretion level in turn influences *H. pylori* colonization and development of gastritis.[12] In epidemiological studies, IL-1β polymorphisms have been found to be related to gastric cancer risk.[13, 14] Prostaglandins, which are synthesized from arachidonic acid by cyclooxygenases (COXs), are associated with protection of gastric mucosa.[15] There are two isomers of COX, COX-1, which is constitutively expressed, and COX-2, which is induced by various cytokines, gastric injury[16] and *H. pylori* infection.[17, 18] It has also been reported that COX-2 plays an important role in the recovery from gastric ulceration.[16, 17] Activated monocytes and neutrophils produce reactive oxygen species such as superoxide anion radicals, hydrogen peroxide and subsequently hydroxyl radicals in gastric mucosa.[19] The inducible nitric oxide synthase (iNOS), which is upregulated by inflammatory stimuli, produces large amounts of NO,[20] which reacts with oxygen and superoxide and produces nitrogen oxides and peroxynitrite, respectively, and these radicals are potentially genotoxic oxidants with oxidizing, nitrating and nitrosating activities.

From these observations, it seems that expression of inflammatory-associated genes, including IL-1β, TNF-α, COX-2 and iNOS, might be closely related with gastric lesions and cancer development, and it is important to analyze changes in their expression in *H. pylori*-induced gastritis. In the present study, we therefore identified the nucleotide sequences of cDNAs encoding Mongolian gerbil inflammatory factors, IL-1β, TNF-α, COX-2, iNOS, as well as β-actin (a house-keeping gene). Furthermore, we examined the mRNA expressions of these genes in the glandular stomach at 1–8 weeks after *H. pylori* infection. Based on the data obtained, the roles of IL-1β, TNF-α, COX-2, and iNOS in *H. pylori*-induced gastritis are discussed.

Materials and Methods

Bacteria. *H. pylori* (ATCC 43504; American Type Culture Collection, Manassas, VA) was grown in brucella broth supplemented with 10% heat-inactivated horse serum for 24 h at 37°C under microaerobic conditions (5% O_2, 10% CO_2 and 85% N_2), as previously described.[21]

Cell culture. Macrophages were isolated from the peritoneal cavity after injection of 5 ml aliquots of 3% proteose peptone solution into male Mongolian gerbils of 10 weeks of age (Seac Yoshitomi, Ltd., Fukuoka, Japan). After 3 days, the peritoneal cavity was washed with 15 ml of cold Hank's balanced salt solution without calcium and magnesium. The peritoneal exudate cells were collected by centrifugation and the macrophage fraction was purified by the Percoll gradient method.[22] The isolated macrophages were plated onto 24-well plates (1.0×10^6 cells/well) and maintained in RPMI 1640 medium (Gibco Industries, Inc., Langley, OK) supplemented with 10% heat-inactivated fetal bovine serum (HyClone Laboratories, Inc., Logan, UT), antibiotics (100 μg/ml of streptomycin and 100 units/ml of penicillin) and 1 μg/ml of *Escherichia coli* lipopolysaccharide (LPS, Sigma Chemical Co., St. Louis, MO) at 37°C in 5% CO_2

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for 18 h.

cDNA cloning of inflammatory factors in Mongolian gerbils. Total RNA was isolated from LPS-stimulated macrophages for the cloning of IL-1β, TNF-α, COX-2 and β-actin, and from the glandular stomach of *H. pylori*-infected Mongolian gerbils for the cloning of iNOS using ISOGEN (Nippon Gene, Tokyo). A cross-species RT-PCR method was used to obtain Mongolian gerbil cDNA fragments. Briefly, 0.5 μg aliquots of total RNA were subjected to the reverse transcription reaction with random 9-mer primer using an RNA "LA PCR" Kit (AMV) Ver.1.1 (TaKaRa Bio, Inc., Otsu, Japan). After reverse transcription, PCR was carried out with LA *Taq* polymerase (TaKaRa Bio), according to the manufacturer's instructions. Primers for IL-1β, TNF-α, COX-2, iNOS and β-actin PCR were designed from the cDNA sequences that matched perfectly between mouse and rat. PCR amplifications were performed in a thermal cycler (Gene Amp PCR System 9600, Perkin-Elmer Applied Biosystems, Foster City, CA), with an initial denaturation (94°C for 2 min) followed by 40 cycles of denaturation at 94°C for 30 s, annealing at optimum temperature for 30 s and extension at 72°C for 1 min, with a final extension process for 5 min. The PCR products were electrophoresed on 2% agarose gels, and the amplified DNA fragments were eluted and subjected to direct DNA sequencing using an "ABI PRISM" "BigDye" Terminator Cycle Sequencing Ready Reaction Kit and an ABI 310 PRIZM DNA Sequencer (PE Applied Biosystems, Foster City, CA). Mongolian gerbil-specific primers were designed from these identified sequences and rapid amplification of cDNA ends (RACE) was employed to identify the sequences of 5′ and 3′-ends. The 5′-RACE was carried out with a modified lock-docking oligo(dT) primer using a "SMART" RACE cDNA Amplification Kit (Clontech Laboratories, Inc., Palo Alto, CA).[23] Then, nested PCR was carried out with Mongolian gerbil-specific primers and nested primer in the RACE kit, and the amplified products were subjected to sequence analysis. The 3′-RACE was carried out with Mongolian gerbil-specific primers and oligo(dT) primers (Clontech, TaKaRa Bio and Invitrogen, Carlsbad, CA).

Subsequent analysis of sequence data was performed with the GENETYX software package (Genetyx Co., Tokyo).

***H. pylori* infection of Mongolian gerbils.** Specific pathogen-free male Mongolian gerbils (Seac Yoshitomi, Ltd.), 6 weeks old, were housed in an air-conditioned biohazard room with a 12 h light-dark cycle. The animals were handled according to the guidelines of the Committee for Ethics of Animal Experimentation in the National Cancer Center, Tokyo. They were fed a normal diet (CE-2; Clea Japan, Inc., Tokyo) and water *ad libitum* throughout the experimental period. At 7 weeks of age, the animals were divided into control and *H. pylori* infection groups, and each animal was fasted for 24 h. Then, *H. pylori* (0.5 ml, 2.4×10^8 CFU/ml) was orally inoculated by gavage to the animals in the *H. pylori* infection group. Control animals received sterilized broth alone. After inoculation, each animal was kept without food and drinking water for 4 h. Five animals of each group were sacrificed under ether anesthesia at 2 days, 1, 2, 4 and 8 weeks after bacterial inoculation and their stomachs were resected, opened along the greater curvature, and washed with saline twice to remove the gastric content and mucus. Then, the wet weights of the stomach and features of macroscopic gastritis were recorded. The stomachs were divided into right and left parts, and the right part was subjected to *H. pylori* detection. Pieces of the pyloric and fundic regions in the left part were subjected to RNA extraction for RT-PCR (n=3). The residue of the left part was formalin-fixed and embedded in paraffin for histological observation. Pathological diagnosis of gastritis was made according to the criteria described previously,[24, 25] with a microscopic score varying from 0 to 7.

Detection of *H. pylori* colonization in the gastric mucosa. To detect *H. pylori* colonization, the glandular stomach was separated into fundic and pyloric regions, and mucosa of each region was scraped off and homogenized with 3 ml of phosphate-buffered saline. An aliquot (100 μl) of serially diluted homogenate was inoculated onto segregating agar plates for *H. pylori* (Nissui Pharmaceutical Co., Ltd., Tokyo) and incubated at 37°C under microaerobic conditions. After 5 days, the colonies were counted to determine the level of *H. pylori* colonization in each region.

mRNA expression of inflammatory factors in gastric tissue. Tissue samples from fundic and pyloric regions were immediately submerged in RNA protective solution ("RNA Later," Ambion, Austin, TX) and kept for 18 h at 4°C. Then, the tissue samples were homogenized with ISOGEN (Nippon Gene), followed by total RNA extraction. The expression levels of IL-1β, TNF-α, COX-2, iNOS and β-actin were examined by RT-PCR using Mongolian gerbil-specific primers designed from identified sequences (see Table 1 for primers and PCR conditions). The PCR products were electrophoresed on 2% agarose gels and visualized by staining with ethidium bromide.

Results

cDNA cloning of inflammatory factors in Mongolian gerbils. Cross-species RT-PCR was applied to obtain Mongolian gerbil cDNA fragments for IL-1β, TNF-α, COX-2 and β-actin from LPS-stimulated macrophages. The iNOS fragment was obtained from the glandular stomach of *H. pylori*-infected Mongolian gerbils, since no iNOS expression could be detected in LPS-stimulated macrophages. We then determined the cDNA sequences of IL-1β, TNF-α, COX-2, iNOS and β-actin of Mongolian gerbils (DDBJ/EMBL/GenBank accession number, AB177840-4). As shown in Table 2, the IL-1β, TNF-α, COX-2 and iNOS cDNAs shared more than 85% sequence homology with the mouse and rat genes, and 76.8–82.9% sequence homology to the human genes. β-Actin cDNA showed more than 90% sequence homology to mouse, rat and human β-actin. The deduced amino acid homologies to the mouse and rat proteins were in the range of 83.6–91.9%, and those to human proteins were in the range of 67.8–84.8%. The amino acid sequences of β-actin could be shown maintained more than 99% homology among Mongolian gerbil, mouse, rat and human.

Table 1. Mongolian gerbil-specific primers[1] for RT-PCR

	Forward primer (5′-3′)	Reverse primer (5′-3′)	Annealing temperature (°C)	Cycle No.[2]	Product size (bp)
IL-1β	GGCAGGTGGTATCGCTCATC	CACCTTGGATTTGACTTCTA	58	35	493
TNF-α	GCTCCCCCAGAAGTCGGCG	CTTGGTGGTTGGGTACGACA	57	40	274
COX-2	CATGGAGTGGACTTAAATCA	ATCTCTCTGCTCTGGTCAAT	53	40	699
iNOS	TCACACAGGCTGCTCCCGGC	CCATAGGAAAAGACTGCCCCG	60	35	282
β-Actin	TCCTCCCTGGAGAAGAGCTA	CCAGACAGCACTGTGTTGGC	60	30	203

1) Primers were designed from the cDNA sequences identified in this study.
2) Each cycle consisted of denaturation at 94°C for 30 s, annealing at the given temperature for 30 s and extension at 72°C for 1 min.

Table 2. The homologies of cDNA and deduced amino acid sequences of cloned Mongolian gerbil genes compared with mouse, rat and human sequences

	ORF size[1] (bp)	cDNA homology (%)			Deduced amino acid homology (%)		
		Mouse	Rat	Human	Mouse	Rat	Human
IL-1β[2]	801	88.0	87.6	76.8	86.2	83.6	67.8
TNF-α	705	86.2	85.3	81.2	87.2	85.1	78.4
COX-2	1812	89.3	89.0	82.9	91.9	90.2	84.8
iNOS	3453	89.7	89.4	82.2	90.8	89.1	80.1
β-Actin	1125	95.2	95.7	92.6	100	99.7	100

1) ORF: open reading frame.
2) cDNA sequences are registered on DDBJ/EMBL/GenBank. The accession numbers are AB177840 (IL-1β), AB177841 (TNF-α), AB177842 (COX-2), AB177843 (iNOS) and AB177844 (β-Actin).

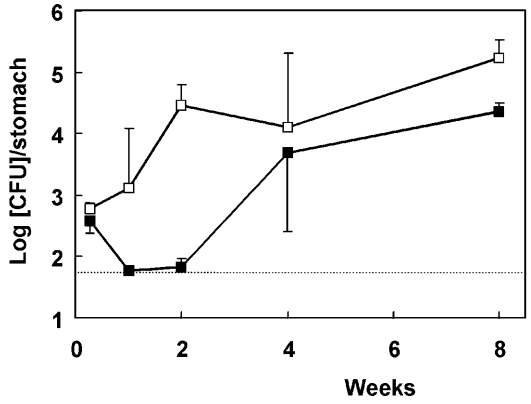

Fig. 1. The changes of *H. pylori* colonization in *H. pylori*-inoculated Mongolian gerbils. Data are mean±SD. The open squares (□) represent the values in the pyloric region and closed squares (■) represent the values in the fundic region (n=3 at 0 week and n=5 at the other weeks). The viable counts in the animal in which *H. pylori* colonization was not detected were calculated as $10^{1.78}$ CFU/stomach. The dotted line represents the detection limit (<$10^{1.78}$ CFU/stomach).

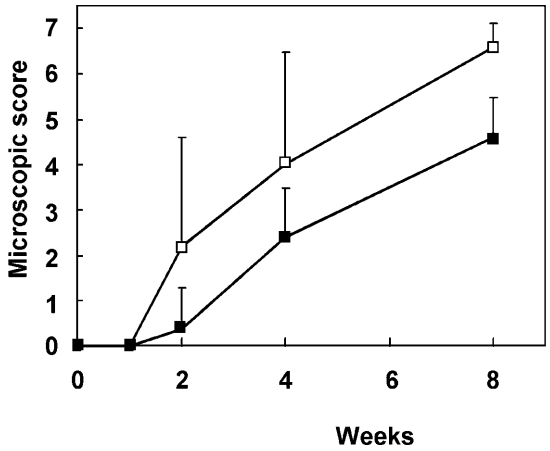

Fig. 2. The changes of pathological scores in *H. pylori*-inoculated Mongolian gerbils. Data are mean±SD. The open squares (□) represent the values in the pyloric region and closed squares (■) represent the values in the fundic region (n=3 at 0 week and n=5 at the other weeks).

Changes of gastritis parameters in *H. pylori*-infected Mongolian gerbils. Data for numbers of viable bacteria obtained from *H. pylori*-inoculated stomach samples are shown in Fig. 1. Two days after inoculation, *H. pylori* was detected in the pyloric and fundic regions of every animal, and viable counts were $10^{2.8\pm0.1}$ CFU/stomach in pyloric region and $10^{2.6\pm0.2}$ CFU/stomach in fundic region. At 1 and 2 weeks, *H. pylori* was detected in the pyloric region of every animal, and viable counts were $10^{3.1\pm0.1}$ and $10^{4.5\pm0.3}$ CFU/stomach, respectively. On the other hand, colonization was low in the fundic region, at <$10^{1.78}$ CFU/stomach in some animals. At 8 weeks, counts for *H. pylori* colonization were $10^{5.2\pm0.3}$ CFU/stomach in the pyloric region, and $10^{4.4\pm0.13}$ CFU/stomach in the fundic region.

The average stomach weights of Mongolian gerbils inoculated with *H. pylori* increased in a time-dependent manner, being 0.53±0.0, 0.62±0.1, 0.77±0.2 and 1.02±0.1 g, respectively, at 1, 2, 4 and 8 weeks after inoculation. The average stomach weights of control animals were from 0.51±0.0 to 0.52±0.0 g at 1, 2, 4 and 8 weeks. Macroscopically, gastritis with edema was observed in two of five *H. pylori*-inoculated animals at 2 weeks after inoculation, and severe gastritis with edema and hemorrhage was seen in every inoculated animal at 4 and 8 weeks. The presence of active gastritis was determined by scoring the following parameters: lymphocyte infiltration (0 to 3), polymorphonuclear leukocyte infiltration (0 to 3), and superficial erosions (0 to 1). Microscopically, erosion with infiltration, featuring many polymorphonuclear leukocytes and lymphocytes, was observed in infected animals. Fig. 2 shows the changes in pathological scores. Gastric changes were severe in the pyloric region, but moderate in the fundic region. The pathological scores at 8 weeks were 6.6±0.5 in the pyloric region and 4.6±0.9 in the fundic region. The score for animals without inoculation was 0 throughout the experimental period.

Expression of inflammatory proteins in the stomach of *H. pylori*-infected Mongolian gerbils. Total RNA was prepared from stomach tissue samples of control and *H. pylori*-infected Mongolian gerbils (n=3) at 1, 2, 4 and 8 weeks after inoculation and mRNA expression levels were assessed by RT-PCR. In the pyloric region, expression of IL-1β and TNF-α was unclear in control animals, while two of three *H. pylori*-infected animals were positive at 2 weeks, and all of the *H. pylori*-infected animals at 4 and 8 weeks (Fig. 3). In the fundic region, IL-1β and TNF-α expression levels were clearly increased in *H. pylori*-infected animals at 4 and 8 weeks. The COX-2 expression levels in the fundic region were clearly increased in infected as compared with control animals at 4 and 8 weeks, but in the pyloric region, similar expression was observed in both infected and control animals (Fig. 4). In contrast to the lack of iNOS expression in control animals, two of three *H. pylori*-infected animals were positive at 2 weeks, and the expression was markedly increased at 4 and 8 weeks in the pyloric region (Fig. 4). In the fundic region, iNOS expression was also increased in *H. pylori*-infected animals at 4 and 8 weeks. Expression levels of β-actin, evaluated as a house-keeping gene, were similar in *H. pylori*-inoculated and control animals at all time points examined (Fig. 3).

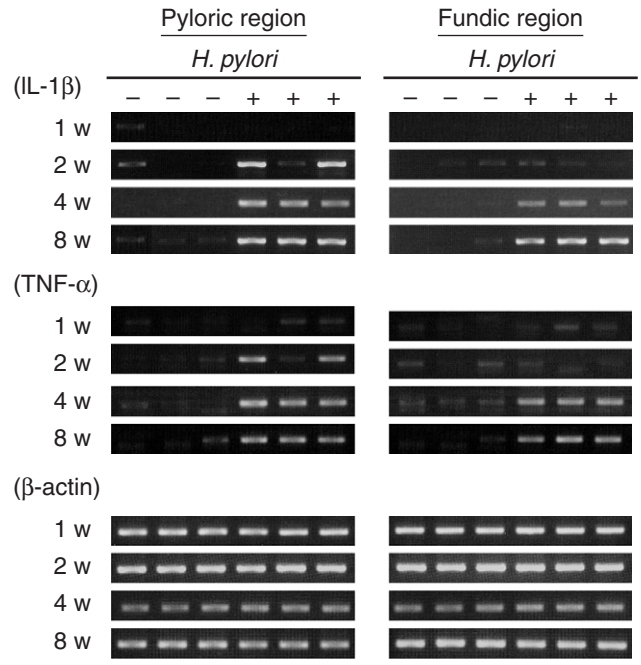

Fig. 3. Increase of IL-1β and TNF-α mRNAs in the glandular stomach of *H. pylori*-infected Mongolian gerbils. RT-PCR analysis was performed with samples from glandular stomach of control and *H. pylori*-infected animals at 1, 2, 4 and 8 weeks after inoculation. Note the similarity of expression levels of β-actin, assayed as a house-keeping gene, between *H. pylori*-inoculated and control animals. The sizes of the amplified products were determined to be 493 bp for IL-1β, 274 bp for TNF-α and 203 bp for β-actin.

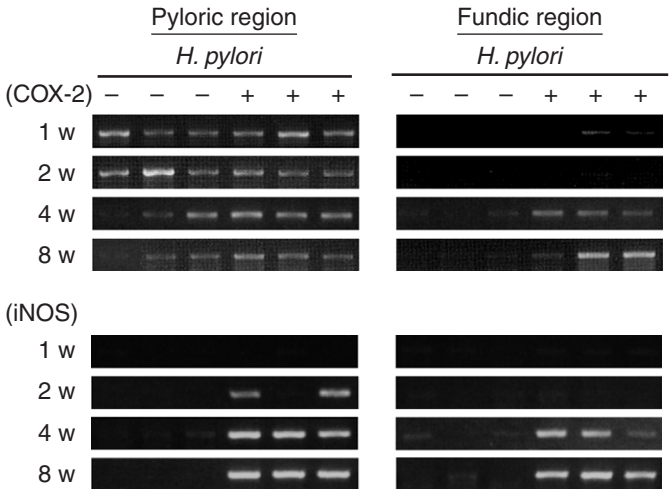

Fig. 4. Expression levels of COX-2 and iNOS mRNAs in the stomach of control and *H. pylori*-infected Mongolian gerbils. RT-PCR analysis was performed with samples from glandular stomach of control and *H. pylori*-infected animals at 1, 2, 4 and 8 weeks after inoculation. The sizes of the amplified products were 699 bp for COX-2 and 282 bp for iNOS.

Discussion

In the present study, we determined the cDNA sequences of IL-1β, TNF-α, COX-2, iNOS and β-actin in Mongolian gerbils. Partial sequences of Mongolian gerbil IL-1β, β-actin[26] and COX-2[27] have been reported earlier, and the homologies with our sequences were 99.7%, 97.6% and 99.9%, respectively. The deduced amino acid homologies of these inflammatory proteins were more than 80% among the Mongolian gerbils, mouse and rat forms. Human IL-1β consists of 269 amino acids containing a mature protein (153 amino acids), the homology of IL-1β with the Mongolian gerbils protein being higher in the mature protein (75.0%) than in the residual peptides (59.0%). TNF-α is also synthesized as a premature protein, but the homologies appear similar in the mature protein and residual peptides. It has been reported that seven amino acids (Leu105, Arg108, Leu112, Ala160, Ser162, Val167 and Glu222 in human premature TNF-α) are crucial for TNF-α activities,[28] and these amino acids were here found to be conserved in Mongolian gerbils, as in the mouse, rat and human cases. COX-2 catalyzes the synthesis of prostaglandins from arachidonic acid, and has an arachidonic acid binding domain, which consists of 24 amino acids.[29] All these were perfectly conserved in Mongolian gerbils, as in the mouse and human (rat; Ser339→Trp).

There are many reports that iNOS is induced in murine macrophages by stimulation with *E. coli* LPS.[30] However, no iNOS expression was observed in macrophages of Mongolian gerbils after LPS (1 μg/ml) treatment. Therefore, we obtained an iNOS cDNA fragment from the stomach of *H. pylori*-infected Mongolian gerbils. The active center of the iNOS oxygenase domain is Glu371 (mouse), which binds to substrate L-arginine, and Trp366, which binds to a heme.[31] These two amino acids were conserved in Mongolian gerbils, as in the mouse, rat and human forms.

It has been reported that *H. pylori* infection increases the expression of IL-1β, TNF-α, COX-2 and iNOS in clinical biopsy samples. However, data on these inflammatory proteins in the early stage of *H. pylori* infection have hitherto not been available. In the present study, expression of IL-1β, TNF-α and iNOS was observed from 2 weeks after inoculation in the pyloric region, and from 4 weeks in the fundus. The delay presumably reflects differences in *H. pylori* colonization levels in the two sites. IL-1β and TNF-α stimulate the production of reactive oxygen species from leucocytes, and iNOS produces NO. Thus, DNA damage may occur through oxidative stress from an early stage of *H. pylori* infection. Although it has been reported that COX-2 protein is not detectable in the glandular stomach of *H. pylori*-uninfected Mongolian gerbils,[18,32] in the present study, mRNA expression was observed in both control animals and infected animals. Similar results were also obtained using other COX-2-specific primer pairs (data not shown). Since these primers were designed from sequences that shared low homology with COX-1 and COX-2, it is most likely that these amplified products were not derived from COX-1 mRNA. It is reported that COX-2 expression in human normal mucosa was detected by RT-PCR, but COX-2 protein was not observed.[33] Thus, trace amounts of COX-2 mRNA in uninfected animals might be amplified and explain the discrepancy. In the present study, COX-2 mRNA expression was clearly upregulated in the fundic region, but not in the pyloric region. It is not yet clear why the expression of COX-2 mRNA was observed without development of gastritis in the pyloric region. It has been reported that COX-2 expression was observed in fibroblasts in the glandular stomach of Mongolian gerbils.[34] Therefore, it is speculated that COX-2 expression in the pyloric region of uninfected Mongolian gerbils might be derived from fibroblasts. It is also not clear why the upregulation of COX-2 mRNA was not observed in the pyloric region of *H. pylori*-infected animals. Further study is required to understand the mechanism.

The fundic and pyloric regions have very different functions. Gastric acid is secreted from the fundic region, and gastrin, which stimulates acid secretion, is secreted from the pyloric region. *H. pylori* colonization may be more difficult in the fundus because of the acid secretion.[12,35] Acid hyposecretion because of H_2 blockers or vagotomy enhances gastritis and *H. pylori*

colonization in the fundic region. Consistent with the available data, we found in the present study that gastritis developed more severely and rapidly in the pyloric region, where *H. pylori* colonization was more pronounced, particularly at 2 weeks after inoculation. IL-1β and TNF-α inhibit the secretion of gastric acid,[10, 11] and *H. pylori* infection decreases acid secretion from 4 weeks after inoculation in Mongolian gerbils.[25, 36] Since IL-1β and TNF-α expressions were observed from 2 weeks in the pyloric region, their expression might influence *H. pylori* infection in the fundic region through the acid hyposecretion. Indeed, *H. pylori* numbers in the fundus increased from 4 weeks after inoculation as shown in Fig. 1 in the present study.

In conclusion, cDNAs encoding the inflammatory proteins IL-1β, TNF-α and iNOS of Mongolian gerbils were cloned, and their expression levels were found to be correlated with gastritis development. Upregulation of COX-2 mRNA expression was seen in the fundic region of infected animals, while COX-2 mRNA expression in the pyloric region was observed in both infected and control animals. The observed upregulation from 2 weeks after *H. pylori* infection suggests that oxidative stresses may occur from an early stage of colonization.

We thank Naoaki Uchiya, Mika Kawamura and Yurika Teramoto for their expert technical assistance. This work was supported in part by Grants-in-Aid for Cancer Research and for the 2nd Term Comprehensive 10-Year Strategy for Cancer Control from the Ministry of Health, Labour and Welfare, Japan, and also a grant from the Yakult Bio-science Foundation.

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