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Oxidative-stress-related proteome changes in *Helicobacter pylori*-infected human gastric mucosa

Hye Yeon BAEK*, Joo Weon LIM*, Hyeyoung KIM*[1], Jung Mogg KIM†, Joo Sung KIM‡, Hyun Chae JUNG‡ and Kyung Hwan KIM*

*Department of Pharmacology and Brain Korea 21 Project for Medical Science, Yonsei University College of Medicine, Seoul 120-752, South Korea, †Department of Microbiology, Hanyang University College of Medicine, Seoul, South Korea, and ‡Department of Internal Medicine, Seoul National University College of Medicine, Seoul, South Korea

Helicobacter pylori infection leads to gastroduodenal inflammation, peptic ulceration and gastric carcinoma. Proteomic analysis of the human gastric mucosa from the patients with erosive gastritis, peptic ulcer or gastric cancer, which were either infected or not with *H. pylori*, was used to determine the differentially expressed proteins by *H. pylori* in the human gastric mucosa in order to investigate the pathogenic mechanism of *H. pylori*-induced gastric diseases. Prior to the experiment, the expression of the main 18 proteins were identified in the gastric mucosa and used for a proteome map of the human gastric mucosa. Using two-dimensional electrophoresis of the protein isolated from the *H. pylori*-infected tissues, Coomassie Brilliant Blue staining and computerized analysis of the stained gel, the expression of eight proteins were altered in the *H. pylori*-infected tissues compared with the non-infected tissues. MS analysis (matrix-assisted laser desorption/ionization–time of flight MS) of the tryptic fragment and a data search allowed the the identification of the four increased proteins (78 kDa glucose-regulated protein precursor, endoplasmin precursor, aldehyde dehydrogenase 2 and L-lactate dehydrogenase B chain) and the four decreased proteins (intracellular chloride channel protein 1, glutathione S-transferase, heat-shock protein 60 and cytokeratin 8) caused by *H. pylori* infection in the gastric mucosa. These proteins are related to cell proliferation, carcinogenesis, cytoskeletal function and cellular defence mechanism. The common feature is that these proteins are related to oxidative-stress-mediated cell damage. In conclusion, the established gastric mucosal proteome map might be useful for detecting the disease-related protein changes. The *H. pylori*-induced alterations in protein expression demonstrate the involvement of oxidative stress in the pathogenesis of *H. pylori*-induced gastric diseases, including inflammation, ulceration and carcinogenesis.

Key words: gastric mucosa, *Helicobacter pylori*, proteome, reactive oxygen species, two-dimensional electrophoresis.

INTRODUCTION

Helicobacter pylori infection leads to gastroduodenal inflammation, peptic ulceration and gastric carcinoma [1,2]. *H. pylori* is believed to be a major aetiological agent that causes chronic gastritis, along with the other features, including the lymphoid follicles or lymphoid aggregates, surface epithelial degradation with mucous depletion, and intestinal metaplasia. One characteristic event in gastritis is an infiltration of the sub-epithelial gastric lamina propria by phagocytes, mainly neutrophils and macrophages, which produce large amounts of ROS (reactive oxygen species) in the host defence reaction.

ROS are believed to be involved in inflammation, the expression of oncogenes and cell proliferation [3]. *H. pylori* stimulated gastric hyperproliferation [4], which is a necessary step in the preliminary stages of the development of a gastric carcinoma. *H. pylori* infection induces the expression of proto-oncogenes such as c-*fos* and c-*jun*, and cyclo-oxygenase-2 in the gastric epithelial cells [5]. The *H. pylori*-induced expression of the inflammatory genes, oncogenes and cell-cycle regulators may be mediated by the ROS-induced activation of oxidant-sensitive transcription factors in the gastric epithelial cells. It was previously demonstrated that *H. pylori*-induced gastric mucosal injury and inflammation might be caused by the oxidant-mediated expression of inflammatory cytokine interleukin-8, and inflammatory enzymes such as cyclo-oxygenase-2 and inducible nitric oxide synthase, which are mediated by the oxidant-sensitive transcription factors NF-κB (nuclear factor κB) and AP-1 (activator protein 1), and by MAPK (mitogen-activated protein kinase) [6–10].

Molecular genetic analysis of *H. pylori* has shown that approx. 50–60 % of the strains have a 40 kb DNA segment called the cytotoxin-associated gene (*cagA*) pathogenicity island [11]. Some of the proteins encoded by the *cagA* pathogenicity island genes are responsible for the oxidant-sensitive transcription factor NF-κB and MAPK activation in gastric epithelial cells [12]. Infection by the *cagA* strain is more likely to result in peptic ulceration, atrophic gastritis and gastric carcinoma [13,14]. Therefore, the expression of *cagA* in the *H. pylori* strain may be important in signal transduction in the *H. pylori*-induced gene expression, regulating inflammation, proliferation and carcinogenesis. The presence of *cagA* in the *H. pylori* strain showed a different expression pattern of the genes compared with the *cagA*-negative *H. pylori* in gastric epithelial AGS cells [15]. Therefore, the protein-expression profile among the *H. pylori*-infected gastric mucosal tissues may be different, depending on the presence of the *cagA* in *H. pylori* strain. A recent microarray study showed that the *cagA*-positive *H. pylori* strain induced the expression of the cell-adhesion-related genes in the AGS cells, which may be related to *H. pylori*-associated gastric carcinogenesis [16]. Since the

Abbreviations used: GRP78, 78 kDa glucose-regulated protein precursor; ALDH, aldehyde dehydrogenase; LDH, L-lactate dehydrogenase; GST, glutathione S-transferase; Hsp, heat-shock protein; CK, cytokeratin; ROS, reactive oxygen species; 2-DE, two-dimensional electrophoresis; CBB, Coomassie Brilliant Blue; MALDI-TOF MS, matrix-assisted laser-desorption/ionization–time-of-flight MS; *cagA*, cytotoxin-associated gene; NF-κB, nuclear factor κB; AP-1, activator protein 1; MAPK, mitogen-activated protein kinase; IPG, immobilized pH gradient; TFA, trifluoroacetic acid; ER, endoplasmic reticulum.

[1] To whom correspondence should be addressed (e-mail Kim626@yumc.yonsei.ac.kr).

predominant genotype of *H. pylori* in South Korea has been reported to be the *cagA*-positive genotype [17,18], the high incidence of gastric cancer in South Korea may be related to the *cagA*-positive *H. pylori* infection.

In an attempt to characterize the pathogenic mechanism of gastric diseases associated with *H. pylori* infection, a strategy using proteomics was used to characterize the proteins induced by the *H. pylori* infection in the human gastric mucosa. Human gastric mucosa from the patients with erosive gastritis, peptic ulcer or gastric cancer, which were either infected or not with *H. pylori*, was used to determine the differentially expressed proteins by *H. pylori*. The altered protein patterns separated by 2-DE (two-dimensional electrophoresis) using pH gradients of 5–8 were conclusively identified by MALDI-TOF MS (matrix-assisted laser-desorption/ionization–time-of-flight MS) analysis of the peptide digests. Prior to this study, the expression of the dominant proteins was determined in all 60 clinical gastric mucosal isolates in order to establish a proteome map of the human gastric mucosa. The established gastric mucosal proteome map was used for the differentially expressed proteins by *H. pylori* infection in the human gastric mucosa. For a comparison between the non-infected tissues and the *H. pylori*-infected tissues, 15 samples from each group were subjected to proteomic analysis.

EXPERIMENTAL

General materials

The electrophoresis reagents including acrylamide solution (25 %), *N,N′*-methylenebisacrylamide, *N,N,N′,N′*-tetramethylethylenediamine, Tris base, glycine, SDS, ammonium persulphate, dithiothreitol, CHAPS, urea, thiourea, Bio-lyte, SB3-10 (sulphobetaine 3-10), tributyl phosphine, Immobiline Dry Strips, IPG (immobilized pH gradient) buffer, IPG cover mineral oil, iodoacetamide and TFA (trifluoroacetic acid) were purchased from Bio-Rad Laboratories (Hercules, CA, U.S.A). CBB (Coomassie Brilliant Blue) G-250 was purchased from Amersham Biosciences (Piscataway, NJ, U.S.A.). The trypsin (modified) was obtained from Promega (Madison, WI, U.S.A.). The ZipTipC$_{18}$ microcolumn was acquired from Millipore (Bedford, MA, U.S.A.). α-Cyano-4-hydroxy-*trans*-cinnamic acid was purchased from Sigma (St. Louis, MO, U.S.A.). All other analytically pure reagents were obtained domestically.

Gastric mucosal collection

The human gastric mucosa from the patients with erosive gastritis, peptic ulcer or gastric cancer, which were either infected or not with *H. pylori*, was used to determine the differentially expressed proteins of *H. pylori*. The human gastric biopsy samples were obtained from Seoul National University Hospital, Seoul, South Korea. Some 60 patients with erosive gastritis, peptic ulcer or gastric cancer undergoing diagnostic gastroduodenoscopy were enrolled in this study. None of the subjects had taken antibiotics, proton-pump inhibitors or non-steroidal anti-inflammatory drugs during the preceding 3 months. Informed consent was obtained from all the subjects, according to the World Medical Association Helsinki Declaration. At the time of endoscopy, five biopsy specimens were taken from the antrum of each subject; one was used to measure the urease activity (CLO test; Delta West, Perth, Australia), one was used for a histological examination using haematoxylin–eosin stain to determine whether or not it was a *H. pylori*-positive or -negative sample, and three were used for proteomic analysis. For the protein map of the human gastric mucosa, all 60 samples were used regardless of the *H. pylori* infection. For comparison between the non-infected tissues and the *H. pylori*-infected tissues, 15 samples from each group were subjected to proteomic analysis.

Protein extraction, isoelectric focusing and 2-DE separation

The tissues were washed with PBS and homogenized with 40 mM Tris buffer (pH 9.5). The particulates were removed by centrifugation (15 000 ***g***, 15 min) and the supernatant was collected. The protein concentration was determined using a Bradford assay [19]. The cells were diluted with extraction buffer [5 M urea, 2 M thiourea, 2 % CHAPS, 40 mM Tris, 2 % SB3-10, 0.2 % Bio-lyte (5/8; working pH range 5.5–7.5), 0.2 % Bio-lyte (8/10; working pH range 8.5–9.5), 10 μl tributyl phosphine] to 100 μg/μl. The protein (1 mg in 350 μl) was adsorbed on to a 17 cm IPG strip (pH 3–10 and pH 5–8), and then electrophoresed on an isoelectric focusing cell (Bio-Rad) for 70 000 V · h at 20 °C. Following isoelectric focusing, the IPG strips were subjected to equilibration for 15 min in an equilibration buffer [375 mM Tris/HCl, pH 8.8, containing 6 M urea, 2 % w/v SDS, 20 % (v/v) glycerol and 2 % (w/v) dithiothreitol]. The strips were then re-equilibrated for 15 min in the same buffer containing 2.5 % (w/v) iodoacetamide in place of dithiothreitol. In all cases, molecular-mass separation was achieved using a Protean II xi cell gel SDS/PAGE system (Bio-Rad). Duplicate samples were separated by linear SDS/polyacrylamide gel (11 %).

CBB G-250 staining

The proteins in one gel were CBB stained using a modification of a method described previously [20]. After overnight fixation [50 % ethanol/2 % (w/v) phosphoric acid], the gels were washed three times for 20 min in double-distilled water and incubated for at least 48 h in a solution containing 34 % methanol, 17 % $(NH_4)_2SO_4$, 3 % (w/v) phosphoric acid and 0.1 % CBB G-250 powder. The stained gels were digitalized using a GS 690 Imaging densitometer (Bio-Rad) at a resolution of 400 × 400 d.p.i.

Image analyses and statistical analysis

The digitalized images from both the CBB-stained gels (from non-infected and *H. pylori*-infected mucosa) were analysed using the 2-DE gel analysis program PDQuest (Bio-Rad). A comparison report of the qualitative and quantitative differences of the samples for each set of data was then generated. For one set of comparisons between the non-infected and *H. pylori*-infected tissues, replicate gels were simultaneously run three times. The expression level was determined by the relative spot volume of the proteins compared with the total amount of the protein in the gel, and is expressed as a percentage of volume. The differentially expressed proteins whose expression level was more than two times higher or lower in the *H. pylori*-infected tissues than the non-infected tissues were selected for MALDI-TOF MS analysis. For each spot, the percentage volume was averaged and expressed as a mean ± S.E.M. from 15 samples. Student's *t* test analysis was performed ($P < 0.05$ was considered significant).

In-gel digestion

The in-gel digestion of the proteins from the CBB-stained gels was performed as follows. Spots were excised to 1–2 mm^2 slices using a blade, macerated, destained and incubated three times with 30 % methanol, washed with 100 % acetonitrile, and dried in a SpeedVac Plus SC100A (Savant, Holbook, NY, U.S.A.) vacuum concentrator. The dried gel pieces were rehydrated with 3–10 μl of a 0.1 μg/μl trypsin solution (Promega) and 50 mM ammonium

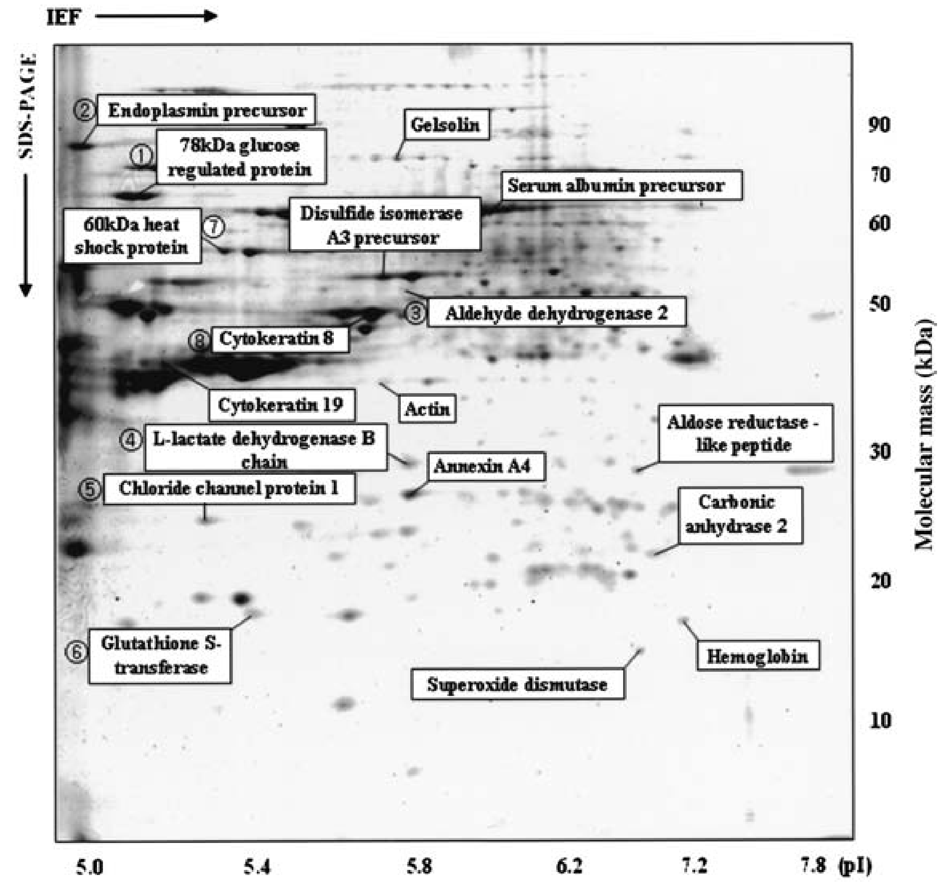

Figure 1 2-DE gel map derived from the human gastric mucosa

Sixty samples, regardless of *H. pylori* infection, were used. The protein (1 mg in 350 μl) was applied to pH 5–8 linear IPG strips (17 cm), with 11 % linear vertical SDS/PAGE as the second dimension. The gel was visualized by CBB staining. The main 18 proteins were determined by MALDI-TOF MS. Details of the proteins are given in Table 1. For a comparison between the non-infected and *H. pylori*-infected tissues, 15 samples from each group were subjected to proteomic analysis. The numbers 1–4 indicate increased protein levels while the numbers 5–8 denote decreased protein levels in the *H. pylori*-infected tissues. IEF, isoelectric focusing.

bicarbonate (pH 8.0). The solution volume was sufficient for the dried gel to be swollen. The digestion was continued at 37 °C for 14–18 h. The tryptic peptides were first extracted using 5 % TFA for 40 °C for 1 h, then 2.5 % TFA/50 % acetonitrile at 30 °C for 1 h. The extracted solutions were mixed in an Eppendorf tube, and dried using a vacuum concentrator.

ZipTipC$_{18}$ purification for MS analysis

The dried extract was reconstituted in 10 μl of 0.1 % TFA. A reverse-phase ZipTipC$_{18}$ microcolumn (15 μm, 200 Å spherical silica; a tip coated with spherical silica-based C$_{18}$ resin for the peptide concentration, desalting and fractionation) was pre-equilibrated with 50 % acetonitrile and washed with TFA. The reconstituted sample was drawn into the tip in order to allow peptide binding, and was washed three times with 10 μl of 0.1 % TFA to remove any contaminants that might interfere with matrix-peptide co-crystallization and/or peptide ionization. The peptides were eluted with 1–2 μl of 50 % acetonitrile that contained 10 mg/ml α-cyano-4-hydroxy-*trans*-cinnamic acid as the matrix, spotted on to the MALDI sample plate and air-dried.

MALDI-TOF MS identification of peptide mixtures

The peptide mixture was dissolved in 0.5 % TFA for MS analysis. MS was performed on a Micromass M@LDI™-TOF (Manchester, U.K.) with saturated α-cyano-4-hydroxy-*trans*-cinnamic acid solution in 0.1 % TFA/50 % acetonitrile as the matrix. The mass spectra were externally calibrated with the auto-digest peaks of trypsin (MH$^+$, 906.505, 1020.504, 1153.574, 2163.057 and 2273.160 Da). The peptide mass maps produced by the MALDI-TOF MS were compared with the published databases using the MS-Fit module in Protein Prospector (http://prospector.ucsf.edu/ucsfhtml4.0/msfit.htm) and Mascot (Marix Science; http://www.matrixscience.com). A mass tolerance of 50 p.p.m. was used for the peptide search.

RESULTS AND DISCUSSION

2-DE separation of the proteins extracted from gastric mucosa was carried out to identify the proteins differentially expressed by *H. pylori* infection. For the protein-expression profile, 2-DE separation was first performed in the pH range of 3–10 (results not shown). Since major protein changes were shown in the range of pH 5–8, a further 2-DE separation was repeatedly performed from the protein extracts of the gastric mucosa between pH 5 and 8. Figure 1 shows a protein map of the human gastric mucosa. After spot detection, background subtraction and volume normalization, 18 dominantly expressed proteins were detected from the 60 human gastric mucosal samples. MALDI-TOF MS analysis of the tryptic fragment and a data search allowed for the

Table 1 Proteins in the gastric mucosa that were analysed with MALDI-TOF MS

Protein modification: *, oxidation of methionine; †, acetylation of protein N-terminus; ‡, conversion of N-terminal glutamine into pyroglutamic acid. Details about the MOWSE program can be found at http://www.matrixscience.com/help/history.html and http://srs.hgmp.mrc.ac.uk/cgi-bin/mowse. Masses matched expresses the number of peptides identically matched between identified peptides of in-gel-digested samples by MALDI-TOF and the peptides of the known protein; the numbers in parentheses indicate the percentage of peptides identically matched compared with the total numbers of the identified peptide of the sample by MALDI-TOF (http://prospector.ucsf.edu/ucsfhtml4.0/instruct/fitman.htm#min_matches).

No.	MOWSE score	Masses matched	Molecular mass (Da), pI	Accession no.	Description	Sequence coverage (%)
1	2.591e + 014	21 (58)	72 334, 5.1	P11021	GRP78*	40
2	1.329e + 009	17 (48)	92 470, 4.8	P14625	Endoplasmin precursor*	29
3	7.595e + 005	9 (45)	56 382, 6.6	P05091	ALDH2*	17
4	1.161e + 005	9 (34)	36 639, 5.7	P07195	LDH B chain*	24
5	2.730e + 006	10 (31)	26 923, 5.1	O00299	Chloride channel protein 1*†	47
6	9.152e + 005	7 (21)	23 356, 5.4	P09211	GST*	44
7	1.415e + 007	9 (37)	61 055, 5.7	P10809	Hsp60*	23
8	1.890e + 005	10 (31)	53 675, 5.5	P05787	CK8*‡	24
9	6.854e + 04	7 (33)	85 698, 5.9	P06396	Gelsolin	11
10	4.069e + 005	15 (53)	69 367, 5.9	P02768	Serum albumin precursor	26
11	8.409e + 10	19 (54)	56 783, 6.0	P30101	Disulphide isomerase A3 precursor	41
12	1.292e + 05	11 (40)	44 106, 5.0	P08727	Cytokeratin 19	37
13	1.128e + 04	9 (42)	41 737, 5.3	P02570	Actin	32
14	7.850e + 05	7 (36)	36 021, 7.1	O60218	Aldose reductase-like peptide	30
15	6.859e + 06	12 (63)	35 883, 5.8	P05925	Annexin A4	40
16	6.949e + 06	9 (36)	29 246, 6.9	P00918	Carbonic anhydrase 2	48
17	4.648e + 04	8 (26)	24 722, 8.3	P04179	Superoxide dismutase	42
18	7.304e + 04	8 (22)	15 999, 6.7	P02023	Haemoglobin	62

identification of these proteins, as shown in Table 1. Based on the protein map established, the differentially expressed proteins, whose expression level was more than twice as high or low in the *H. pylori*-infected tissues than the non-infected tissues, was selected for further analysis, and are indicated by the numbers 1–8. The increased protein levels as a result of *H. pylori* were numbered 1–4, whereas the proteins with decreased levels were numbered 5–8 in Figure 1. A Mascot search using the peptide mass fingerprinting data indicated an increase in the levels of four proteins [GRP78 (78 kDa glucose-regulated protein precursor), endoplasmin precursor, ALDH (aldehyde dehydrogenase) 2 and LDH (L-lactate dehydrogenase) B chain] and a decrease in the levels of four proteins [intracellular chloride channel protein 1, GST (glutathione S-transferase), Hsp (heat-shock protein) 60 and CK (cytokeratin) 8] caused by the *H. pylori* infection in gastric mucosa (Figures 2 and 3).

Segments of the 2-DE gel map derived from the non-infected and *H. pylori*-infected gastric mucosa are shown in Figures 2 and 3. As shown in Table 1, all eight proteins showed a modification, such as the oxidation of methionine (GRP78, endoplasmin precursor, ALDH2, LDH B chain, intracellular chloride channel protein 1, GST, Hsp60, CK8), N-terminal acetylation (intracellular chloride channel 1) and the conversion of N-terminal glutamine into pyroglutamic acid (CK8). These modifications slightly altered the original pI values and molecular masses of the intact protein in the 2-DE gel map. One advantage of proteomic analysis is the identification of the modified amino acids in the proteins, which provides a clue when investigating the pathogenesis of *H. pylori*-induced gastric diseases. The modified proteins may suppress the beneficial effects or essential roles of the proteins [21]. These proteins are related to cell proliferation, carcinogenesis, cytoskeletal function and the cellular defensive mechanism. The common feature of these proteins is that their expression has been reported to be affected by oxidative stress.

The GRP78 is a molecular chaperone and a Hsp70 homologue, which is constitutively expressed. It facilitates the import, glycosylation, folding and assembly of the proteins as well as the recognition and removal of the denatured/unfolded proteins [22]. GRP78 expression is dramatically enhanced under a variety of stressful conditions, including glucose deprivation, treatment with Ca^{2+} ionophores, the blockage of glycosylation, oxidative stress and hypoxia [23]. The induction of GRP78 is essential for maintaining the viability of cells that are subjected to such stresses [24]. The transcriptional activation of the GRP78 gene is regulated by a complex interplay of several *cis*-elements and transcriptional factors that bind to the GRP78 promoter. This promoter contains the important motifs such as CRE (cAMP-response element) and TRE (PMA-response element) motifs [25]. The AP-1 transcriptional factor complex is a major target of the MAPK signalling pathway and consists of c-Jun/c-Jun, c-Jun/c-Fos or c-Jun/ATF-2 dimers. Song et al. [26] reported that enhancement of the AP-1 DNA-binding activity involves the transcriptional induction of the GRP78 gene through a TRE-like motif in human gastric tumour cells. The endoplasmic resident chaperones, including GRP78, are involved in cellular survival during chronic hypoxia [27]. In addition, the induction of GRP78 was reported to protect the cells by suppressing oxidative damage and stabilizing calcium homoeostasis [28]. The sustained induction of GRP78 by chronic hypoxia is mainly attributable to the transcriptional activation rather than the mRNA stability and cancer cell survival [26]. The *cagA* pathogenicity island genes present in the *H. pylori* strain are responsible for the oxidant-sensitive transcription factor NF-κB as well as MAPK activation in human gastric epithelial cells [11,12] and increase the intracellular Ca^{2+} levels in human gastric mucous epithelial cells [29]. Chen et al. [30] demonstrated that the activation of MAPK and mitochondrial Ca^{2+}-mediated oxidative stress are essential for the enhanced expression of GRP78.

The ER (endoplasmic reticulum) plays a key role in the synthesis and distribution of many cellular proteins. Before the proteins can be transported towards their final destination, the disulphide bonds essential for their proper folding need to be formed. A requirement for this oxidative protein folding is a high redox state [31]. In the ER lumen, the relative abundance of the oxidized (GSSG) compared with the reduced (GSH) form of glutathione has led to the suggestion that GSSG serves as the

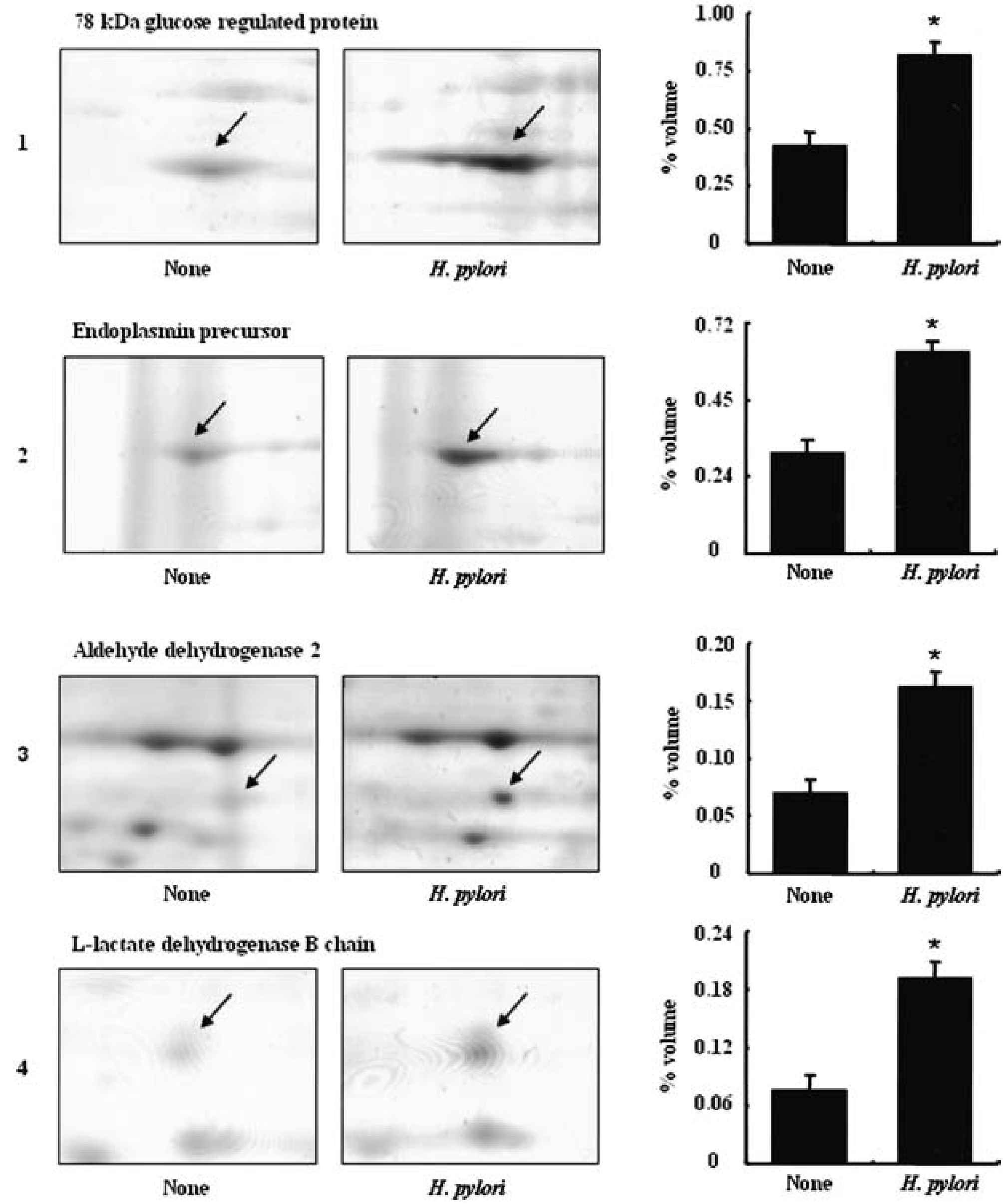

Figure 2 Segments of the 2-DE gel map derived from the non-infected (None) and *H. pylori*-infected (*H. pylori*) gastric mucosa: increased proteins

The arrows indicate the four proteins whose expression levels were more than twice as high in the *H. pylori*-infected tissues compared with the non-infected tissues (left-hand panel). The expression level was determined by the relative spot volume of the proteins compared with the total amount of the protein in the gel, and is expressed as the percentage volume (right-hand panel). A representative gel image and expression level (percentage volume) for each spot is shown. For each spot, the percentage volume was averaged and expressed as a mean ± S.E.M. from 15 samples. *$P < 0.05$ versus the non-infected treatment. The proteins identified with MALDI-TOF MS were (1) GRP78, (2) endoplasmin precursor, (3) ALDH2 and (4) LDH B chain.

oxidizing equivalent during protein folding. van der Vlies et al. [32] monitored the oxidized proteins in the intact human dermal fibroblasts exposed to hydrogen peroxide. They found that all the oxidized proteins (protein disulphide isomerase, GRP78, calnexin, endoplasmin) reside in the ER and form part of the protein-folding machinery. The oxidation of the protein-folding machinery may lead to the improper folding and/or accumulation of the proteins to be secreted because only correctly folded proteins exit the ER [33]. Incorrectly folded proteins are retained and are degraded. Since *H. pylori* induces oxidative stress to the gastric epithelial cells, this may lead to damage to the ER-resident protein endoplasmin as well as the ER-resident chaperone, GRP78. A *H. pylori*-induced increase in the endoplasmin precursor and ER chaperone GRP78 may be a defence mechanism of the cells against oxidative stress. The improper function of the oxidized ER proteins may contribute to *H. pylori*-associated gastric epithelial dysfunction.

The ALDH family is a family of several isoenzymes that are important in cellular defence against exogenous toxic aldehydes and endogenous aldehydes such as those derived from lipid peroxidation [34]. The latter appear to influence cell growth and differentiation in some tumour cell lines. The up-regulation of ALDH was reported in five gastric cancer cell lines [34]. The ALDH family is widely expressed in the tissues and subcellular components, but with some differences in the individual isoenzymes. Class 2 ALDH is expressed in a large number of

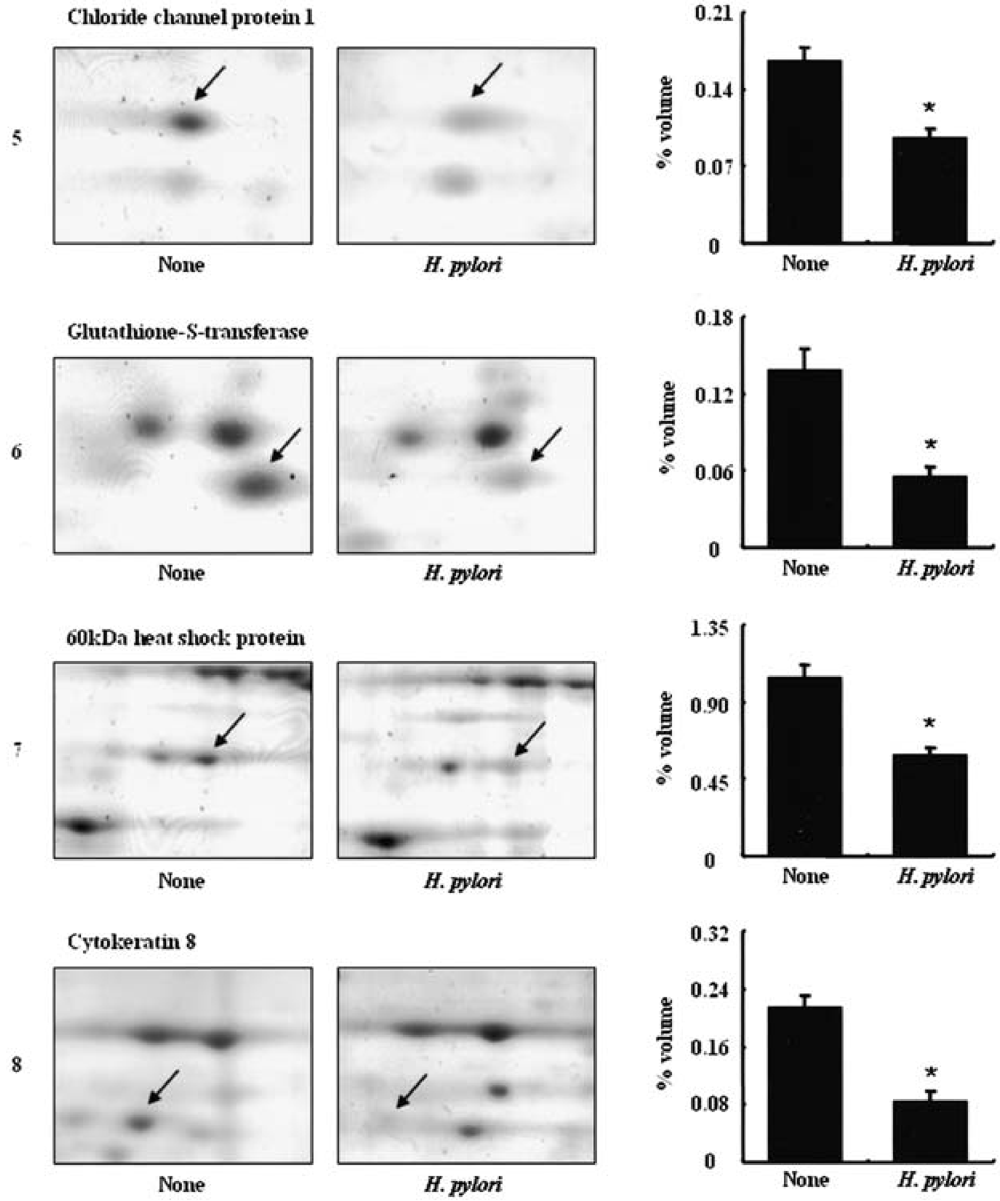

Figure 3 Segments of the 2-DE gel map derived from the non-infected (None) and *H. pylori*-infected (*H. pylori*) gastric mucosa: decreased proteins

The arrows indicate the four proteins whose expression levels in the *H. pylori*-infected tissues were less than half those in the non-infected tissues (left-hand panel). The expression level was determined by the relative spot volume of the proteins compared with the total amount of the protein in the gel, and is expressed as the percentage volume (right-hand panel). A representative gel image and expression level (percentage volume) for each spot is shown. For each spot, the percentage volume was averaged and expressed as a mean ± S.E.M. from 15 samples. *$P < 0.05$ versus the non-infected treatment. The proteins identified with MALDI-TOF MS were (5) chloride channel protein 1, (6) GST, (7) Hsp60 and (8) CK8.

tissues, with the highest levels occurring in the liver, kidney, muscle and heart [35]. It is synthesized as a high-molecular-mass precursor in the cytosol and is transported into the mitochondrial matrix space, where it is processed into the mature enzyme. It is believed that class 2 ALDH is mainly responsible for the oxidation of the acetaldehyde generated during ethanol oxidation *in vivo* [36]. Therefore, it might be possible that *H. pylori*-induced oxidative stress induces ALDH 2 expression to detoxify the lipid-peroxidation-derived aldehydes as a defence mechanism of the *H. pylori*-infected gastric mucosa in this study.

LDH is a terminal enzyme of anaerobic glycolysis. Under hypoxic conditions, one strategy for survival of the cells is to introduce glycolytic enzymes, facilitating ATP production by glycolysis rather than mitochondrial oxidative phosphorylation [37]. It appears that the hypoxia-stimulated transcription of the specific genes through hypoxia-inducible factor-1 activation is a highly conserved and a widely operative mechanism responding to a cellular oxygen deficiency [38]. The genes encoding the glycolytic enzymes, including LDH, enolase 1, aldolase 1, phosphoglycerate kinase 1 and phosphofructokinase L, are inducible by hypoxia [39]. Recent studies of the *cis*-acting DNA sequences for the genes encoding enolase 1 and LDH indicate that they have multiple sites for hypoxia-inducible factor-1 binding in the 5′-flanking region and that binding of a single specific site is essential for the hypoxic activation of transcription [39]. The increase in the LDH B chain in the *H. pylori*-infected tissues may be a survival mechanism of the cells exposed to oxidative stress from the *H. pylori* infection because the hypoxic condition generates large amounts of ROS in the cells.

One of the decreased proteins as a result of the *H. pylori* infection in this study is the intracellular chloride channel protein 1. The chloride channels are essential for the transepithelial fluid and ion transport. In general, the Ca^{2+}-dependent chloride channel, cAMP-dependent CFTR (cystic fibrosis transmembrane conductance regulator) and membrane chloride-conductance properties contribute to chloride secretion in the cells. There are two Ca^{2+}-dependent chloride channels, 1 and 2. Both channels 1 and 2 were down-regulated in approx. 80 % of colorectal carcinomas compared with the normal colon epithelium [40]. The Ca^{2+}-dependent chloride channels 1 and 2 are believed to be tumour suppressors in various types of cancer, including breast and colorectal cancers. ROS, particularly superoxide, induce the closure of the chloride channel in rabbit gastric parietal cells [41]. Hydrogen peroxide inhibits the chloride current in the retinal pigment epithelium [42]. This suggests the possible redox modulation of the chloride channel function in the cells. Even though there is no information as to whether the present intracellular chloride channel 1 is Ca^{2+}-dependent or not, it is possible that the decrease in the intracellular chloride channel 1 by *H. pylori* in the gastric mucosa in this study may induce the loss of tumour-suppressor function, which might result in cell proliferation and carcinogenesis of the gastric epithelium associated with *H. pylori* infection. A further study of the type, translocation and function of identified chloride channel 1 would contribute to understanding the pathophysiological mechanism of *H. pylori*-induced gastric diseases.

GST is an important detoxification enzyme. The GST activity in the mucosa of the gastrointestinal tract is inversely correlated with the development of gastrointestinal cancer. Since *H. pylori* infection has been associated with gastric cancer, the GST activity and the substrate, GSH, in the patients with *H. pylori*-associated gastritis have been studied [43]. The antral GST activity was lower before the eradication of *H. pylori* compared with afterwards. The GSH level was significantly higher after the eradication of *H. pylori*. This demonstrates the loss of a detoxification mechanism of GST by *H. pylori* infection in the gastric mucosa. This result supports the decrease in GST expression in *H. pylori*-infected mucosal tissues compared with the non-infected tissues. The absence of the GST enzyme may increase the risk of developing gastric carcinoma in these patients, since GST detoxifies the exogenous carcinogen [43].

The Hsps regulate the activity of multiple intracellular signalling intermediates, many of which are intimately involved in the control of the apoptotic signalling pathways. Hsps include anti-apoptotic and pro-apoptotic proteins that interact with a variety of cellular proteins. Their expression levels can determine the fate of the cell in response to a death stimulus, and apoptosis-inhibitory Hsps, in particular Hsp70 and Hsp27, may participate in carcinogenesis [44]. Hsp60, Hsc70 (the constitutive form of Hsp70) and Hsp90 are constitutively expressed in mammalian cells, while Hsp27 and Hsp70 are strongly induced by different stresses such as heat, oxidative stress or anticancer drugs. Hsp27 and Hsp70 are anti-apoptotic, while Hsp60 and Hsp10 are pro-apoptotic. This suggests that the balance of Hsp proteins can determine the fate of stressed cells. Hsp60 mainly refolds and prevents the aggregation of denatured proteins [45]. Therefore, a decrease in Hsp60 by *H. pylori* may result in improper folding, the accumulation of misfolded proteins and the prevention of apoptosis in gastric epithelial cells. Mitochondrial proteins such as Hsp60 are the major target of hydrogen peroxide, since the mitochondrion is a major source of ROS in the cells [46]. A decrease in Hsp60 might be related to the *H. pylori*-induced hyperproliferation and carcinogenesis, which are mediated by oxidative stress in gastric epithelial cells.

CKs are a family of cytoplasmic structural proteins that have been described in the normal human epithelium and demonstrate a variable expression pattern which is dependent on the type and differentiation of the epithelium [47]. Thus far 20 different CK subsets have been identified. Although some CK subsets have a broad range of expression patterns for the columnar epithelium, such as CK8 and CK10, other subsets, such as CK7 and CK20, have demonstrated a restricted immunoreactivity. The co-ordinate expression of CK7 and CK20 is very useful or diagnosing a specific carcinoma, such as breast, colorectal, pancreatic, bladder and ovarian carcinoma [48]. For gastric adenocarcinoma, *H. pylori* infection was related to the expression of CK7 and CK20 [49]. ROS affects the cytoskeletal function and the expression of the CKs [50]. Since the ROS are strongly produced in *H. pylori*-infected gastric epithelial cells [6–10], a decrease in the CK8 expression level as a result of *H. pylori* infection in the gastric mucosa may contribute to oxidative-stress-induced cytoskeletal damage.

In this study, the overexpressed proteins (GRP 78, endoplasmin precursor, ALDH 2, L-LDH B chain) and the underexpressed proteins (intracellular chloride channel protein 1, GST, Hsp 60, CK8) are involved in cell proliferation, carcinogenesis, cytoskeletal function, and cellular defensive mechanism. Most of the proteomic studies in relation to *H. pylori* have focused on *H. pylori* itself rather than the host cells. Backert et al. [51] reported that *H. pylori cagA* protein is translocated into the host cell membrane and the cytoplasm. As the surface of *H. pylori* provides an important interface for the pathogen–host interactions, Sabarth et al. [52] identified 18 surface proteins of *H. pylori*, including urease, γ-glutamyltranspeptidase and *cag*16, which is a member of the *cag* pathogenic island. Immunoproteomics of the sera from the patients with active *H. pylori* infection showed that 310 antigenic protein species were recognized by the *H. pylori* positive sera [53]. The newly identified antigens were the predicted coding region, HP0231, the serine protease, HtrA (HP1019) and *cag*3 (HP0522). These studies will be useful for diagnostic purposes and vaccine design. Besides these studies searching for the immunogenic proteins for *H. pylori* infection, these results might contribute to the ongoing investigation of the pathogenic mechanism of *H. pylori*-induced gastric diseases.

Conclusions

The increased levels of four proteins (GRP 78, endoplasmin precursor, ALDH 2, L-LDH B chain) and the decreased levels of another four proteins (intracellular chloride channel protein 1, GST, Hsp 60, CK8) were identified in *H. pylori*-infected gastric mucosa, separated by 2-DE, and identified by MALDI-TOF MS analysis of the peptide digests. These proteins are related to cellular stress such as ROS, cell proliferation, carcinogenesis, cytoskeletal function and cellular defence mechanisms. The *H. pylori*-induced alterations in protein expression demonstrate the involvement of oxidative stress in the pathogenesis of *H. pylori*-induced gastric diseases, including inflammation, ulceration and carcinogenesis. The differentially expressed proteins may be useful as prognostic indices for gastric diseases associated with *H. pylori* infection. The expression of the main 18 proteins was identified in the gastric mucosa and was used for a proteome map of human gastric mucosa. The established gastric mucosal proteome map might be useful for detecting disease-related protein changes.

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