Gene-expression profiles in gastric epithelial cells stimulated with spiral and coccoid *Helicobacter pylori*

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Human gastric epithelial immortalized GES-1 cells were infected with spiral and coccoid *Helicobacter pylori*. Scanning electron microscopy was used to determine the ability of the two forms of *H. pylori* to adhere to GES-1 cells. GES-1 cell apoptosis induced by coccoid and spiral *H. pylori* was analysed using flow cytometry. A cDNA microarray for 22 000 human genes was used to identify the gene-expression differences in GES-1 cells infected with the two forms of *H. pylori*, and the gene expression identified by the cDNA microarray was confirmed by RT-PCR. Scanning electron microscope observation showed that both coccoid and spiral bacteria can adhere to GES-1 cells. After 4 h infection, apoptosis induction was 27.4 % for spiral-form infection and 10.2 % for coccoid-form infection. Of 268 differentially expressed genes identified by cDNA microarray, 166 showed higher expression with the spiral *H. pylori* infection than with the coccoid *H. pylori* infection. To the best of the authors' knowledge, this is the first report that GES-1 cells infected with spiral *H. pylori* have higher expression of *cxcl10*, *ccl11*, *ccl5*, *groα*, *TLR5*, *ATF3*, *fos*, *fosl2*, *gadd45a* and *myc*. The cells infected with coccoid *H. pylori* had higher expression of *survivin*. The global profile of gene expression in GES-1 cells infected with coccoid and spiral *H. pylori* is described for the first time.

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INTRODUCTION

Helicobacter pylori is a prevalent Gram-negative microaerophilic bacterium that infects human gastric mucosa and causes many digestive diseases, such as chronic gastritis, peptic ulcers and gastric cancer (Nomura *et al.*, 1991; Parsonnet *et al.*, 1991; Graham *et al.*, 1992). *H. pylori* exists in two morphological forms: an actively dividing spiral form and a non-culturable but viable coccoid form (Bode *et al.*, 1993). Spiral *H. pylori* converts to the coccoid form under various unfavourable conditions, such as extended incubation (Catrenich & Maki, 1991; Reynolds & Penn, 1994), antibiotic treatment (Bode *et al.*, 1993), nutrient deprivation (Mizoguchi *et al.*, 1999), increased oxygen tension (Catrenich & Maki, 1991; Cellini *et al.*, 1994), increased temperature (Shahamat *et al.*, 1993), aerobic culture and alkaline pH (Cellini *et al.*, 1994). Many researchers think that this morphological change may be a transitory

adaptation to a particular environment as a means of species preservation, and that it may play an important role in antibiotic resistance and make the bacterium more difficult to eradicate. Also, the coccoid form might lead to difficult recovery, easy relapse and epidemic transmission (Mizoguchi *et al.*, 1998). The spiral bacteria are the most common form found in the human stomach, but the coccoid bacteria are observed in more severely damaged regions of the gastric mucosa (Saito *et al.*, 2003; Chan *et al.*, 1994; Janas *et al.*, 1995). Several reports have shown that although the coccoid form is non-culturable, it may be viable, as transcription and translation may actively take place in coccoid cells (Sisto *et al.*, 2000; Monstein & Jonasson, 2001; Ng *et al.*, 2003). Vijayakumari *et al.* (1995) have shown that the adhesion of the coccoid form to Kato III cells *in vitro* is similar to the interaction detected in the gastric epithelium infected with the spiral form *in vivo* (Hessey *et al.*, 1990). Wang *et al.* (1997) have shown that both coccoid and spiral *H. pylori* can infect BALB/c A mice, and both forms can cause acute gastritis in BALB/c A mice. Furthermore, Nilsson *et al.* (2002) have reported that ATP is

Abbreviations: IL, interleukin; RGS, regulator of G protein signalling.

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detectable for at least 25 days after the morphological change from the spiral form to the coccoid form. It is therefore probable that the coccoid form, like the spiral form, is viable and infective, even though it is non-culturable *in vitro*.

In man, the gene-expression changes in gastric epithelial cells induced by the spiral form of *H. pylori* infection have been well reported (Chiou *et al.*, 2001; Cox *et al.*, 2001; Susanne *et al.*, 2002). However, a comparison of the differences in gene expression between gastric epithelial cells infected with coccoid and those infected with spiral *H. pylori* has not hitherto been reported. The aim of this study was to assess by cDNA microarray the differences in gene expression in the gastric epithelial immortalized cell line GES-1 infected with coccoid and spiral *H. pylori*.

METHODS

Cell culture. The human gastric epithelial immortalized GES-1 cells were maintained in our laboratory. The cells were routinely cultured in RPMI 1640 medium supplemented with 10% fetal calf serum, 100 U penicillin ml⁻¹ and 100 U streptomycin ml⁻¹ in air with 5% CO₂ at 37 °C.

Bacterial culture and induction of the coccoid form. The wild-type *H. pylori* 26695 strain was kindly provided by Dr Zhang Jianzhong of the Chinese Disease Control and Prevention Center, Changping District, Beijing. The bacterium was cultured on Columbia agar with 5% (v/v) sheep blood under microaerobic conditions (5% O₂, 10% CO₂, 85% N₂) at 37 °C. After the bacteria had been grown for 48 h, more than 90% of the bacteria were in the spiral form. To obtain coccoid *H. pylori*, the bacteria were maintained for 10 days; after this period, more than 90% of the bacteria were coccoid.

Co-cultivation of gastric epithelial cell line GES-1 with *H. pylori*. GES-1 cells were prepared by seeding 2 × 10⁶ cells on plates. After overnight incubation, the medium was replaced with fresh medium without antibiotics. The spiral and coccoid *H. pylori* were harvested from plates by suspension in 2 ml PBS and washing twice with PBS. After centrifugation at 4000 r.p.m. for 5 min, the bacteria were resuspended in 2 ml PBS and added immediately to cell culture plates. The ratio of the number of bacteria to the number of cells was 100:1. Gastric epithelial cells infected with spiral or coccoid *H. pylori* were cultured for 4 h in air with 5% CO₂ at 37 °C.

Scanning electron microscope observation. GES-1 cells were grown to confluence on cover glasses in six-well plates, and spiral or coccoid bacteria were added to each cover glass at a ratio of about 100:1. The cultures were placed in air with 5% CO₂ at 37 °C for 4 h in a total volume of 2 ml. Then, the cover glasses were removed and washed twice with PBS. The cells were fixed first with 2.5% glutaraldehyde for >2 h, and then with 2% osmic acid. Adherence of coccoid and spiral *H. pylori* to GES-1 cells was observed with a Hitachi S2520 scanning electron microscope. The number of adherent bacteria on 25 GES-1 cells was counted. The *t* test was used for statistical analysis.

Apoptosis assay by flow cytometry. About 1 × 10⁶ cells infected with coccoid or spiral *H. pylori* were harvested by trypsin digestion and centrifugation (at 1000 r.p.m. for 5 min), then washed twice with PBS. Then the cells were fixed with 70% ethanol. The apoptosis of GES-1 cells was determined by flow cytometry.

RNA extraction. Total RNA was extracted from the cells with the Trizol reagent (Invitrogen), according to the manufacturer's instructions. The amount of RNA was measured by A₂₆₀.

cDNA microarray. The cDNA microarray consisted of 22 000 distinct cDNAs, which were purchased from Qiagen. The array also included cDNAs of housekeeping genes, such as β -actin and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), to serve as internal controls. Cy3-dCTP or Cy5-dCTP were incorporated when 50 μ g total RNA was reverse-transcribed into cDNA using M-MLV reverse transcriptase and random hexamer primer (MBI). The cDNA probes from the cells co-cultured with spiral *H. pylori* were incorporated with Cy3, while the cDNA probes from the cells co-cultured with coccoid *H. pylori* were incorporated with Cy5. Different fluorescently labelled cDNA probes were mixed in 30 μ l hybridization buffer (3 × SSC, 0.2% SDS, 5 × Denhardt's solution and 25% formamide) and applied to the microarray following incubation at 42 °C for 16 h. After hybridization, the slide was washed with 0.2% SDS/2 × SSC at 42 °C for 5 min, and then was washed with 0.2 × SSC at room temperature for 5 min. The fluorescent images of the hybridized microarray were scanned with a ScanArray Express fluorescent laser confocal slide scanner (Packard Bioscience). Images and quantitative data of the gene-expression levels were analysed by GenePix Pro 4.0 image analysis software (Axon Instruments).

Semi-quantitative RT-PCR. We used RT-PCR to verify the microarray data. The primers for RT-PCR are listed in Table 1. The internal standard β -actin was co-amplified with the specific genes. The total RNA was reverse-transcribed into cDNA using

Table 1. Primer pairs for RT-PCR

Gene	Primer sequence (5'–3')		Size of PCR product (bp)
	Sense	Antisense	
IL-8	ATGACTTCCAAGCTGGCCG	CTCAGCCCTCTTCAAAACTT	290
ccl5	GCTGTCATCCTCATTGCTAC	TCCATCCTAGCTCATCTCCA	257
ccl11	GGACCTTTACAAGATTCCAGG	TGGGTGCAGGATCCATGAAG	381
cxcl10	TCTGACTCTAAGTGGCATTTC	TGGCCTTCGATTCTGGATTTC	205
Fos	AGGAGAATCCGAAGGGAAG	CAAGGGAAGCCACAGACATC	247
gadd45a	GTTGATGTCGTTCTCGCAGC	TGAGTGAGTGAGAAAGCAG	357
Survivin	ACCACCGCATCTCTACATTC	GCACTTTCTTCGCAGTTTCC	349
gro α	ATGGCCGCGCTGCTCTCT	AGCTTTCCGCCCATCTTTC	252
β -Actin	GTGGGGCGCCCCAGGCACCA	CTCCTTAATGTCACGCACGATTTC	540

M-MLV reverse transcriptase and random hexamer primer (MBI). The cDNAs were amplified by 30 PCR cycles of denaturation at 94 °C for 30 s, annealing at 52 °C for 30 s, with a further period at 72 °C for 1 min. As a final step, extension was at 72 °C for 10 min. PCR products were separated on 1.5% agarose gels containing 0.5 g ethidium bromide ml⁻¹ and visualized by UV transillumination.

RESULTS AND DISCUSSION

Gastric adenocarcinoma cell lines, such as AGS, MKN28, Kato III and SGC7901, are often used to study the interaction between *H. pylori* and gastric mucosal epithelial cells. These cells may have mutated in the process of evolution, and as a consequence, their genetic backgrounds may display many differences from that of normal gastric mucosal epithelial cells. Thus, it is far from certain that the interactions between normal gastric mucosal epithelial cells and *H. pylori* are accurately reflected in these malignant cell lines. In this study, we used an immortalized fetal gastric mucosal cell line transformed with SV40. The cell line was established by Ke *et al.* (1994). It has a phenotype similar to that of normal stomach mucosal cells, and does not grow into tumour masses in nude mice. Therefore, it was hoped that our results could reflect more accurately the interaction of *H. pylori* with normal gastric mucosa. Asahi *et al.* (2000) and Odenbreit *et al.* (2001) have shown that CagA translocation can occur 30 min after infection, and that it is at a maximum in the time range of about 4–5 h (Susanne *et al.*, 2002). Cole *et al.* (1997) have observed that the adhesion of coccoid and spiral *H. pylori* at a constant bacteria:cells ratio does not increase significantly after 2 h. Accordingly, the time point of 4 h chosen in this study seemed to be appropriate.

Adhesion of spiral and coccoid *H. pylori* to GES-1 cells

A scanning electron microscope was used to observe the adhesion of spiral and coccoid *H. pylori* to GES-1 cells. After GES-1 cells had been infected with spiral *H. pylori* for 4 h, we found that a number of *H. pylori* can adhere to the GES-1 cell surfaces (Fig. 1A). After coccoid *H. pylori* had infected GES-1 cells for 4 h, adhesion to GES-1 cell surface was observed with this form also (Fig. 1B). The mean adhesion index of spiral *H. pylori* was 28.08 ± 9.82 , while the mean adhesion index of coccoid *H. pylori* was 22.84 ± 9.88 . There were no significant differences in the adhesion index between the coccoid and spiral *H. pylori* ($P > 0.05$).

cDNA microarray results

Fig. 2 shows a representative scatter plot for gene-expression levels, where cDNA probes, derived from the cells co-cultured with coccoid or spiral *H. pylori*, were hybridized and labelled with Cy5 and Cy3 fluorochromes, respectively. A Cy3: Cy5 ratio between 2 and 0.5 indicated only small mRNA expression differences and was of no significance. A ratio of more than 2 or less than 0.5 indicated significant mRNA expression differences. Thus, the upper spots in

Fig. 1. Adhesion of *H. pylori* to GES-1 cells observed by scanning electron microscopy. (A) Adhesion of spiral-form *H. pylori* to a GES-1 cell; (B) adhesion of coccoid-form *H. pylori* to a GES-1 cell. Bars, (A) 1.83 µm, (B) 2.22 µm.

Fig. 2 indicate that expression of the corresponding gene was greater in cells infected with coccoid *H. pylori* than in cells infected with spiral *H. pylori*, and the lower spots indicate the reverse. The intermediate spots indicate similar mRNA expression levels. From Fig. 2, most of the genes examined showed only small differences.

Two hundred and sixty-eight genes showed more than a twofold difference in expression. One hundred and sixty-six genes in a co-culture with spiral *H. pylori* had a more than twofold increase in expression compared with a co-culture

Fig. 2. Representative scatter plot of cDNA microarray analysis. The scatter plot indicates the relation between relative fluoro-intensities of Cy5-labelled cDNA (from coccoid-form-infected cells) and those of Cy3-labelled cDNA (from spiral-form-infected cells). For each gene, the RNA expression level in the spiral-infected cells is given on the *x* axis [$\log_{10}(\text{intensity of Cy3})$] and the expression level of the same gene in the coccoid-infected cells is plotted on the *y* axis [$\log_{10}(\text{intensity of Cy5})$].

of coccoid *H. pylori*. One hundred and two genes in a co-culture with coccoid *H. pylori* had a more than twofold increase in expression compared with a co-culture with spiral *H. pylori*. Some of the differences in gene expression are listed in Table 2. Among the genes showing differential expression, the regulator of G protein signalling (RGS)2 and PCDHB8 genes showed the highest elevation in spiral *H. pylori*-infected cells compared with coccoid *H. pylori*-infected cells. Since the function of the PCDHB8 gene is not clear at present, the significance of the increase in the expression of this gene needs to be further investigated. RGS2 belongs to a family of diverse and multifunctional proteins able not only to regulate G-protein-coupled receptor signalling by binding to active $G\alpha$ subunits and by acting as GTPase-activating proteins (GAPs), but also to act as effector antagonists by interfering with the binding of downstream effectors (Hollinger & Hepler, 2002). RGS2 is a potent regulator of $G\alpha_q$ signalling and is also a recently identified regulatory protein for adenylate cyclase signalling (Sinnarajah *et al.*, 2001). Recent research indicates that RGS2 is an important negative-regulatory factor in cardiac hypertrophy through complex mechanisms involving modulation of MAP kinase signalling pathways (Zou *et al.*, 2006). However, there are few reports of the effect of RGS2 in gastric epithelial cells; further investigation is needed to determine the significance of the differential expression of this gene in cells infected with the two bacterial forms.

Table 2. Differentially expressed genes in spiral and coccoid *H. pylori*-infected GES-1 cells

Gene name	Fold change spiral/coccoid	Accession no.
Apoptosis-related genes		
Survivin	0.49	NM001168
Aven	0.40	NM033303
IER3	2.76	NM052815
Fos	4.34	NM005252
myc	2.89	NM002467
gadd45a	3.70	L24498
MX1	2.11	NM002462
Aplp1	2.31	NM005166
Stk17b(Drak2)	2.15	NM004226
TNFAIP3	2.20	NM006290
TNFRSF10D	2.78	NM003840
Chemokine and chemokine receptors		
cxcl10	2.75	NM001565
Ccl11	2.01	NM002986
Ccl5	2.85	NM002985
Ccl13	2.03	NM005408
IL-8	2.43	NM000584
Groz	2.78	NM001511
ILs and IL receptors		
IL-18	0.49	NM001562
IL-17	5.89	NM002190
IL-17E	2.07	NM022789
IL-26	2.09	NM018402
Transcription regulators		
MMP10	2.62	NM002425
ATF3	2.54	NM004024
fosl2	2.09	AK055579
TCF15	3.71	NM004609
HSFY	2.81	NM033108
SPI1	2.20	NM003120
RNF12	2.51	NM016120
C19ORF2	2.22	NM003796
EGR4	2.39	NM001965
EGR1	2.33	NM001964
TFE3	2.13	AK055522
NSBP1	2.26	NM030763
NFAT5	0.30	AF346509
MYBL1	0.44	X66087
TAF5	0.37	NM006951
IRX5	0.33	NM005853
MMP3	0.26	NM002422
HOXD3	0.48	BC005124
TEAD1	0.40	NM021961
GTF2H1	0.49	BC000365
Signal-transduction factors		
RAP2A	3.45	NM021033
ALK	2.52	NM004304
CD14	2.03	NM000591
PDE7A	2.10	L12052
EPHA2	2.34	NM004431
NR4A2	2.81	NM006186

Table 2. cont.

Gene name	Fold change spiral/coccoid	Accession no.
PLCD1	2.01	NM006225
RGS2	8.29	NM002923
RGS6	2.67	NM004296
HTR2B	0.40	NM000867
HTR2C	0.31	NM000868
TANK	0.38	NM004180
GABRB2	0.31	NM021911
DGKD	0.48	NM003648
SOCS1	0.47	NM003745
TNFSF15	0.37	NM005118
FGF16	0.35	NM003868
Response-to-stress genes		
KLKB1	2.79	NM000892
CD83	2.04	NM00065
TLR5	2.03	NM003268
C5	2.38	NM001735
Cell-adhesion molecules		
PCDHB2	2.89	NM018936
PCDHB5	3.81	NM015669
PCDHB8	9.70	NM019120
PCDH19	2.44	AB037734
ITGA5	2.08	NM002205
MUC4	3.62	NM004532
NLGN4	2.04	AB033086
REG1A	2.10	NM002909
ALDOC	0.49	NM005165
NRP2	0.47	NM003872
COL8A1	0.25	NM001850
NCAM2	0.23	NM004540
ITGAE	0.39	NM002208
ITGA9	0.48	NM002207
CDH11	0.41	NM033664
Others		
FGFR2	2.19	NM023028
VGF	2.03	NM003378
CCNE	2.64	NM057749
CD48	0.44	NM001778

GES-1 cell apoptosis induced by spiral and coccoid *H. pylori* infection

The results of flow cytometry analysis of the apoptosis of GES-1 cells are shown in Fig. 3. After 4 h infection, spiral *H. pylori* infection could induce 27.4% apoptosis of GES-1 cells (Fig. 3A), while coccoid *H. pylori* infection could cause only 10.2% apoptosis (Fig. 3B). Therefore, spiral *H. pylori* infection induced a significantly higher level of GES-1 cell apoptosis than coccoid *H. pylori* infection.

The cDNA microarray showed gene-expression differences in apoptosis-related genes, such as the increased expression of proapoptotic genes *gadd45a*, *myc* and *fos*, and the reduced expression of the antiapoptosis gene *survivin* in

Fig. 3. GES-1 cell apoptosis induced by coccoid and spiral *H. pylori* infection. The left peak represents the apoptosis rate, the middle peak G0/G1-phase cells. DNA content on the x axis represents the logarithm of fluorescence intensity. (A) Cell apoptosis induced by spiral *H. pylori* infection; the apoptosis rate was 27.4%. (B) Cell apoptosis induced by coccoid *H. pylori* infection; the apoptosis rate was 10.2%.

spiral *H. pylori*-infected GES-1 cells (Table 2). The RT-PCR confirmed the expression differences of *gadd45a*, *fos* and *survivin* (Fig. 3B). These gene-expression differences may contribute to the high apoptosis rate in cells infected with spiral *H. pylori*. Gastric mucosal integrity is maintained by a balance between the rate of cell loss and the rate of epithelial cell regeneration (Xia & Talley, 2001). Thus, under normal circumstances, the rate of cell loss by apoptosis is matched by the rate of new cell production by proliferation (Hall *et al.*, 1994). However, spiral *H. pylori* infection may induce apoptosis in gastric epithelial cells and destroy the balance, resulting in an increase in cell proliferation as a host response to apoptosis. This will result in increased proliferation of the gastric mucosa (Shirin *et al.*, 1999; Yamaguchi *et al.*, 2000; Xia & Talley, 2001). Once spiral *H. pylori* converts to the coccoid form, the gastric epithelial

Fig. 4. RT-PCR analysis of mRNA expression in spiral or coccoid *H. pylori*-infected GES-1 cells. (A) RT-PCR analysis of the expression of the immune-reaction genes IL-8, ccl11, ccl5, cxcl10 and groα. (B) RT-PCR analysis of the expression of the apoptosis-related genes gadd45a, fos and survivin

cells maintain the high proliferation rate. However, the coccoid form can induce a lower degree of apoptosis and can thus lead to the augmentation of cells, which may contribute to gastric cancer after *H. pylori* infection.

Immune reaction gene-expression differences between spiral and coccoid *H. pylori*-infected GES-1 cells

Among these differences in gene expression, we found an increased expression of several cytokines and chemokines, such as interleukin (IL)-8, IL-17, IL-18, IL-26, IL-17E, ccl11, ccl5, cxcl10 and groα in spiral *H. pylori*-infected GES-1 cells (Table 2). RT-PCR analysis confirmed the cDNA microarray results for genes IL-8, ccl11, ccl5, cxcl10 and groα (Fig. 4A). Cole *et al.* (1997) and Osaki *et al.* (2002) have shown that coccoid *H. pylori* have a reduced ability to induce IL-8 secretion compared with spiral *H. pylori* in AGS and KATO cells. The present study has also shown that the gene-expression level of IL-8 in GES-1 cells infected with coccoid *H. pylori* is lower than that of cells infected with spiral *H. pylori*. This is in keeping with the results of Cole *et al.* (1997). IL-8, groα and cxcl10 belong to the C-X-C chemokines, which preferentially recruit neutrophils. On the other hand, ccl5 and ccl11 belong to the C-C chemokines, which mainly attract lymphocytes and monocytes (Miller & Krangel, 1992; Baggiolini, 1993). Thus, the cells infected with spiral *H. pylori* have significantly greater mRNA expression of both C-X-C and C-C chemokines than those infected with coccoid *H. pylori*. Shimoyama *et al.* (1998) have shown that enhanced chemokine responses can result in a histologically more intense gastritis. Therefore, infection with spiral *H. pylori* may cause a more pronounced inflammatory response and lead to a more severe gastritis. This may increase the chance of the conversion of the spiral to the coccoid form of *H. pylori*. The coccoid form may exist in the host in a 'silent' state (Cole *et al.*, 1997) and induce a weaker inflammatory response. This is probably a transitory adaptation to a particular environment and contributes to species preservation. Once the environment changes, the coccoid form can also convert to the spiral form (Bode *et al.*, 1993), which in turn can lead to chronic infection and difficulty in the eradication of the pathogen.

The cDNA microarray results also showed an increase in the expression of fos and fosL2 in spiral *H. pylori*-infected cells. The fos- and fosL2-encoded C-fos and Fra-2 transcription factors can combine with other proteins to form transcription-factor complexes that are involved in various processes, such as cell proliferation, apoptosis, survival of tumour cells, invasion, growth and angiogenesis (Eferl & Wagner, 2003). In the present study, we also found gene-expression differences for other transcription factors, signal transduction factors and cell adhesion molecules between infections with the two *H. pylori* morphological types (Table 2).

In conclusion, to the best of our knowledge, this is the first study to report the global assessment of gene-expression differences by cDNA microarray in gastric epithelial cells infected with spiral or coccoid forms of *H. pylori*. Of the 22 000 genes, 268 showed a twofold gene-expression difference in Cy5: Cy3 ratio in gastric epithelial cells induced by spiral and coccoid *H. pylori* infection, which was regarded as differential expression. These results reveal the differences between the two *H. pylori* morphological types in inducing changes in GES-1 cell gene expression. Also, these results provide basic data and a research direction for further investigating the interaction of coccoid and spiral *H. pylori* and gastric epithelial cells.

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