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Changes in the Evolution of the Antigenic Profiles and Morphology during Coccoid Conversion of *Helicobacter pylori*

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Objectives : *The significance of the coccoid forms of H. pylori is still controversial and the questions of whether these forms are viable and infective or degenerative are still open. We induced conversion from rod to coccoid forms and studied morphological changes and antigenic evolutions during this conversion and, thereby, elucidated the viability of coccoid forms.*

Methods : *The H. pylori strain (C001) used for Western blotting was isolated from the patient with gastric cancer. The antigenic evolution during coccoid conversion of H. pylori was studied by Western blotting, using different sera from thirty patients known to be culture positive. These sera were used to reveal the total antigens of the strain cultured for 2 days (100% rod) and 15 days (>99% coccoid). After SDS-PAGE, with 10% separating gel of total antigens (rod and coccoid), transblotting (Trans-Blot electrophoretic cell, Bio-Rad) was taken onto a nitrocellulose membrane (Bio-Rad). Then, the blots, with human sera diluted at 1/100, were developed with color reaction by goat serum anti-human IgG with alkaline phosphatase and BCIP.*

Results : *The antigenic profiles were not changed in 46.7% (14/30 cases) and were changed in 53.3% (16/30 cases) during coccoid conversion. Antigenic fractions changed during coccoid conversion were protein band at 120 kDa and band at 35 kDa, and were not detected in coccus forms. The rest of the profiles were identical between rod and coccoid forms. The protein which disappeared include CagA (120 kDa) and porin, or adhesin (35 kDa). The morphological changes during coccoid conversion were U shaped at day 7, doughnut shaped at day 9 and full coccoid at day 15.*

Conclusions : *The results showed that coccoid forms of H. pylori retain cellular structures similar to rod form, and some of the antigens (CagA and porin) disappeared during coccoid conversion. Therefore, coccoid form might be viable and represent one of the stages of H. pylori biological cycle.*

Key Words : *Helicobacter pylori, Coccoid form, Antigenic Profiles, Morphologic Change*

INTRODUCTION

Helicobacter pylori infection is now recognized as the major cause of chronic gastritis throughout the world[1-3]. A fraction of infected persons developed peptic ulcer disease[4] or gastric cancer[2,5,6], accounting for its clinical significance. The pathophysiology of this infection can be better understood by several concepts, such as heterogeneity of strains[7], persistence of infection[8], immunological down regulation[9], physiological consequences and variability in outcome[10]. Microbial, host and environmental factors must contribute to the outcome

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variation, respectively. Especially, the relapse of *H. pylori* has been reported after antimicrobial therapy[11-13], and its transmission is still unknown. The fact that *H. pylori* can convert under unfavorable conditions into a metabolically active but non-culturable coccoid state has stimulated speculation about its role in transmission and reinfection[14, 15]. Some investigators[16-18] suggested that the coccoid forms are degenerative and have no potentiality of infection, like *Campylobacter jejuni*, and others[14, 19-21] supposed that they are dormant and one stage of the biological cycle of *H. pylori*, like *Vibrio vulnificus*. Some reported some studies[22, 23] have suggested that the coccoid forms of *H. pylori* could be potentially viable. However, regrowth of *H. pylori* from the coccoid forms could not be possible. In the present study, to elucidate the viability of coccoid forms, we induced conversion from bacillary to coccoid forms and studied the morphological changes and antigenic evolutions during coccoid conversion.

MATERIALS AND METHODS

1. Strain and culture conditions

The *H. pylori* strain used (C001) was isolated in the Research Institute for Gastroenterology of Dankook University (Chunan, Korea) from a gastric biopsy of a patient with gastric cancer. Cells were grown at Brucella blood agar with 5% horse serum and antimicrobial agents, at 37℃ under microaerophillic conditions with 10% CO_2 and 5% O_2. Added antimicrobial agents were vancomycin (10 mg/L), colistin (5 mg/L), trimethoprim (5 mg/L) and amphotericin B (5 mg/L). The cells of a 2day culture of *H. pylori* C001 were harvested from one plate, suspended in phosphate-bufferd saline (PBS, pH 7.4), and adjusted to a turbidity of 1 MacFarland unit. After Gram staining, it was microscopically observed that this suspension contained only bacillary forms. The suspension was then used to innoculate 10 plates of Brucella blood agar (0.1 ml/plate). The plates were incubated at 37℃ under microaerophillic conditions. The cells of one plate were harvested daily and suspended in 1 ml of PBS (pH 7.4). Enumeration of colony forming unit were performed by standard serial dilution and plate count procedures. Total cell (bacillary or coccoid forms) counts were assessed by turbidimetry at 540 nm. To each suspension, Gram-stained smears were used to assess the relative percentages of coccoid and bacillary forms. These measurements were made blindly by 30 different persons, and the averages of their determinations were considered as the final results. Also, the suspensions of day 2, 7, 9 and 15 were used for electron microscopic examinations.

2. Preparation for electron microscopic examination

Bacteria were harvested from plates, fixed for microscopy with 2.5% glutaraldehyde in 100mM sodium cacodylate buffer. Cells were concentrated by centrifugation and samples were removed for examination by differential interference contrast light microscopy. To assist in identification, selected samples were also negatively stained with 1% uranyl acetate for transmission electron microscopy (TEM).

3. Western blotting

The evolution of the antigenic profiles during coccoid conversion of *H. pylori* C001 was studied by Western blotting, using sera from thirty patients known to be colonized by *H. pylori*. Total antigens of *H. pylori* C001 were obtained by sonification of bacterial cells harvested at different stages of rod and coccoid, respectively, and suspended in PBS (pH 7.4). After sonification, the unlyzed bacteria were eliminated by centrifugation (3000 rpm, 65 min), and the supernatants were saved, adjusted to 1 ㎖ of protein per ㎖, and frozen at -70℃ until use. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to the method of Laemmeli[24] with a 4% stacking gel and a 10% separating gel. Prior to electrophoresis, the protein solutions were heated at 100℃ for 5 min in a sample buffer containing 5% (wt/vol) SDS and 0.42% (wt/vol) 2-mercaptoethanol. Electrophoresis was conducted for 1 hour under a constant voltage (15 v/um) using the minigel system (Bio-Rad). Proteins were blotted onto a pre-wetted nitrocellulose membrane (Bio-Rad) by using a Mini Trans-Blot electrophoretic transfer cell (Bio-Rad) under a constant current of 200 mA for 1 hour. The blots were incubated for 1 h with human sera diluted at 1/100; they were then rinsed and incubated in goat serum anti-human IgG conjugated with alkaline phosphatase (Dakopatts, Copenhagen, Denmark). After a final wash, the nitrocellulose filters were developed with 5-bromo-4-chloro-3-indolylphosphosphate (BCIP) as a substrate, and nitroblue tetrazolium as a chromogenic

indicator. The reactions were stopped after 20 min by washing the filters extensively with distilled water.

RESULTS

1. Examination of morphological changes

As assessed by turbidimetry, the bacterial mass increased from day 1 to day 3. The maximal active growth was observed on day 2-3 (36 hours). The stationary phase was day 4-6. The proportion of coccoid forms increased from 0 to 100% from day 2 to day 15 (Fig. 1).

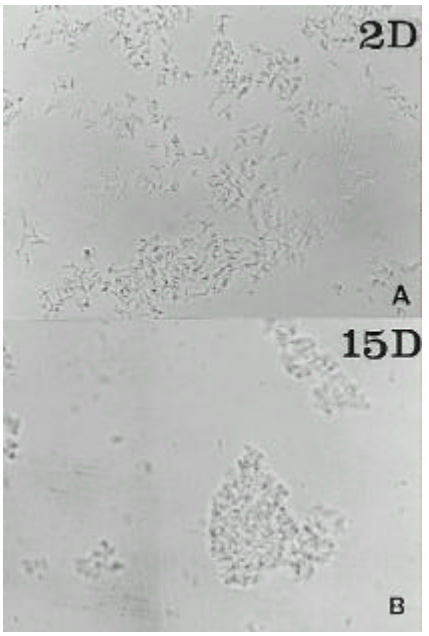

Fig. 1. Phase contrast micrograph showed (A) active spiral rod forms at day 2, and (B) coccoid form at day 15.

Between day 5 and day 9, the fraction of coccoid forms on the plates was increased. Transmission electron micrographs at day 2 showed spiral rod forms. At day 7, the relative number of bacillary forms decreased and U-shaped forms became predominant, and they looked like invaginated bacilli. At day 9, U-shaped forms were converted to doughnut-shaped forms. At day 15, only globular full coccoid forms were observed (Fig. 2). The ultrastructure of the inner cell side was not observed.

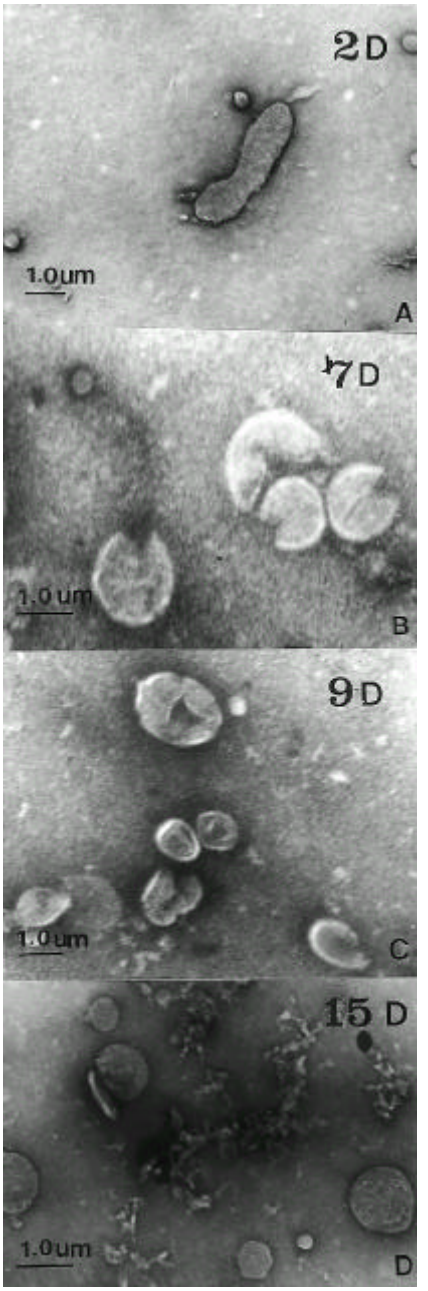

Fig. 2. Transmission electron micrograph showed morphological changes of coccoid conversion from the bacillary forms. (A), bacillary form of *H. pylori* at day 2; (B), U-shaped form at day 7; (C), doughnut-shaped form at day 9; (D), full coccoid form at day 15 (— ; 1.0um).

2. Evolution of antigens of H. pylori during coccoid conversion

The evolution of the antigenic profiles during coccoid conversion of *H. pylori* was studied by Western blotting, using different sera from culture positive patients. These sera were used to reveal the total antigens of the strain cultured for 2 day (0% coccoids) and 15 days (>99% coccoids). SDS-PAGE analysis of whole cell preparations of *H. pylori* showed numerous bands between 30 and 125 kDa. These proteins included CagA at 125 kDa, VacA (vacuolating cytotoxin) at 88 kDa, an adhesin and porin at 35 kDa and urease subunit at 30 kDa[25, 26]. The antigenic profiles were not changed in 46.7% (14/30 cases) (Fig. 3) and were changed in 53.3% (16/30 cases) during coccoid conversion. Antigenic fractions changed during coccoid conversion were protein band at 125 kDa and band at 35 kDa, which were intensively detected in bacillary forms. Those proteins which disappeared included CagA and porin, outer membrane (Fig. 4). Disappearance of CagA protein (125 kDa) during coccoid conversion was observed in 68.8% (11/16 cases) and disappearance of porin (35 kDa) in 62.5% (10/16 cases). The rest of the profiles were identical between rod and coccoid forms.

DISCUSSION

Helicobacter pylori, a gram negative spiral bacterium, can survive in such diverse environments as the human stomach of low pH[23], gut of high osmolarity[27] and water[28]. In order to adapt to such crucial conditions, the bacteria should carry systems that respond to changes in nutrient, osmolarity, temperature and other external factors. The organisms exist in two forms, an actively dividing spiral forms and a coccoid form of arrested growth under various stress, including the starvation for nutrients[29], extended incubation[28], accumulation of metabolic products[14], pH alteration[14] and exposure to antimicrobial agents[30]. The viability of the coccoid form of *H. pylori*, the possible role of this form in transmission and as a cause of reinfection is controversial[14, 15]. Although the mode of transmission still remains unclear, oral-oral and oral-fecal transmission have been suggested[31, 32]. If *H. pylori* follows the latter route, they must pass through the anaerobic atmosphere of the alimentary tract which is an adverse situation for *H. pylori*. Under these stressed conditions, infected bacillary forms might be changed to coccoid form. Shirai et al.[20] have reported that almost 100% cells changed to coccoid-like bodies within 24 hr of anaerobic incubation, and 0.1-1% produced colonies on Brucella agar. Also, they revealed that the colonies appeared from coccoid bodies which remained viable under anaerobic conditions, although some of them appeared from a few spiral bodies, thereby they assisted *H. pylori* in passing the adverse anaerobic

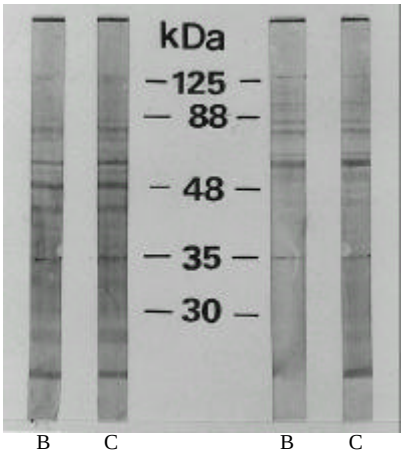

Fig. 3. Western blot analysis showed several antigenic proteins between 30 and 125 kDa. The same antigenic profiles in both the bacillary and coccoid forms. B, bacillary form; C, coccoid form.

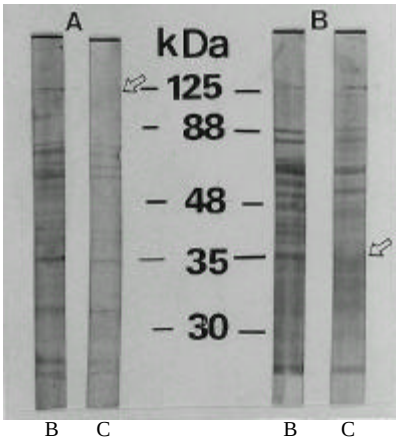

Fig. 4. Western blot analysis showed different antigenic profiles between bacillary and coccoid forms. (A) protein at 125 kDa (CagA) disappeared in coccoids(⇨); (B) protein at 35 kDa (porin) disappeared in coccoids(⇨). B, bacillary form;C, coccoid form

route of the human alimentary tract by changing their morphology. However, it is still unknown whether coccoid forms of *H. pylori* can revert to vital organism in vivo or if they are of any patho-physiological significance at all. According to some reports[16-18], the coccoid forms are degenerative and incapable to form complex adhesions and, hence, are of low pathogenic potential. But others[14, 19-21] suggested that coccoid forms are dormant cells and viable cells. Vijayakumari et al.[19] revealed that specialized attachment sites were seen in the interaction between coccoids and epithelial cells of KATO III cell, and these adherence patterns were similar to those observed with spiral forms in vivo, suggesting a possible pathogenic role for the coccoids of *H. pylori*. Also, with antigens prepared from both coccoid and spiral forms, immunoreactive protein bands of 128, 116, 110, 95, 91, 66, 60, 54, 50 and 33 kDa were conserved in both the coccoid and spiral forms by the results of Western blotting. Vijayakumari et al.[19] suggested that the coccoids could be differentiated infective form of *H. pylori*, and that they could evoke an immune response from the host after attachment to gastric epthelial cells. We found the morphological changes during coccoid conversion by the transmission electron microscopy. Spiral bacilli forms at day 2 converted to full coccoid forms at day 15, through the U-shaped forms at day 7, and doughnut-shaped forms at day 9. Although we could not detail the change of ultrastructure during coccoid conversion under our transmission electron microscopy, coccoid forms were considered via U-shaped and doughnut-shaped forms. Benaïssa et al.[21] revealed that initiation of conversion from bacillary was the formation of dense periplasmic material, followed by an inwardly curved formation of bacilli as an intermediate step between the bacillary and coccoid forms and, then, a change in the protoplasmic cylinder was evoked to full coccoid forms, strongly suggesting a transition of bacillary to full coccoid forms via U-shaped forms. The extracytoplasmic side of the invaginated membrane might serve as a site for the oxidation of toxic material, which has been described for *Ecthiorhodospira mobilis* and other spirilloid or vibroid gram negative organism[29, 33], just like *H. pylori*. Therfore, similar invagination of the membrane could consolidate the potential viability of *H. pylori* coccoids. On the antigenic evolution, we observed the identical antigenic profile between the bacillary and coccoid forms in 46.7%, and changed antigenic profile in 53.3%. Interestingly, disappeared protein bands during coccoid conversion were CagA and porin or adhesin, which were more intensively detected in bacillary forms. Those findings could suggest that virulent factors of *H. pylor*i might vanish in some coccoid forms. Vijayakumari et al.[19] and Benaïssa et al.[21] have reported that the protein bands were the same pattern in both the coccoid and bacillary forms, which were a little bit different from our results. We think the same protein bands between the bacillary and coccoids highlight the significance of these antigens. However, we cannot clarify the role of the antigenic components absent in the coccoid forms. In conclusion, these results showed that coccoid forms of *H. pylori* retain antigenic characteristics similar to bacillary forms, and some of the antigens disappeared in coccoid forms. Therefore, coccoid forms might be viable, and represent one of the stages of the *H. pylori* biological cycle. However, it is still unclear whether the coccoid forms can revert to infective bacillary forms in vivo, whether they represent a temporary adaptation to a particular environment, and whether they are actually involved in the transmission of the bacterium.

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