RESEARCH COMMUNICATION

Changes in Biological and Virulent Characteristics of Helicobacter pylori Exposed to High Salt

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Abstract

The effect of high salt environments on biological characteristics of Helicobacter pylori is still unclear. In the present study, we therefore investigated biological characteristics of the bacterium exposed to high salt concentrations. H. pylori strain, L301, was cultured in media supplemented with different concentrations (3%, 15% and 30%) of sodium chloride (NaCl) under microaerophilic conditions for 48 h. Morphology was assessed by light microscopy, the ATP content was quantitated by single-tube fluorescent light-emission and the levels of CagA and UreB proteins were determined by Western blotting. After exposure to NaCl, H. pylori transformed from common spiral shape to U or even coccoid shapes. The ATP content was significantly higher in 30% NaCl group than in 15% and 3% NaCl group and the level of CagA protein increased with the salt concentration. The urease reaction was all strongly positive in H. pylori exposed to different salt concentrations. The level of 8-OHdG expression was significantly increased in GES-1 cells co-cultured with H. pylori exposed to high salt, compared with the level in uninfected cells. H. pylori survives under exposure to high salt concentrations up to 30%, exhibiting changes in mobility, morphology and CagA expression, associated with increased 8-OHdG in the gastric epithelial cells, indicative of DNA damage.

Keywords: H. pylori - gastric epithelial cells - high salt - DNA damage

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Introduction

It is acknowledged that H. pylori infection is closely associated with diseases such as chronic gastritis, peptic ulcer, gastric cancer, and B-cell lymphoma (Nilsson et al., 2002; Sachs, Wen, and Scott, 2009). Chaput et al. found that changes in the biological characteristics of H. pylori were accompanied by changes in polysaccharide in the cell wall, which may help the bacterium escape host immune response (Chaput et al., 2006). These findings indicate that H. pylori can adapt to the changing environment by self-regulation in order to adhere to and colonize the gastric mucosa, and thus cause consequent gastroduodenal diseases.

H. pylori infection is thought to be an initial etiological factor for the development of gastric cancer, which is a multifactorial and multi-step process (Szabo and Ohshima, 1997). It has been suggested that sodium chloride (NaCl) concentration is an environmental factor closely related to H. pylori infection and gastric diseases (Ishii et al., 2006; Yermilov et al., 1996). Epidemiological studies have shown that development of gastric atrophy in the presence of H. pylori infection is closely associated with high dietary salt intake (Szabo and Ohshima, 1997).

In human epidemiologic studies and also in animal models (Kuroiwa et al., 2007; Naito and Yoshikawa, 2002; Nozaki et al., 2002), a link has been shown between high salt intake and the development of gastric cancer. In the studies to detect the transmission routes of H. pylori, researchers observed that contaminated food or water, some of which with very high NaCl concentration, may serve as reservoir in the transmission of H. pylori (Carbone et al., 2005; Vale and Vitor, 2010). However, due to the strain-specific difference in salt resistance or the limitation of experimental methods, the studies evaluating the effect of high salt environments on biological characteristics of H. pylori have produced inconsistent results (Loh, Torres, and Cover, 2007; Yan et al., 2008). In addition, the interaction between H. pylori and very high salt environments (e.g. 30% NaCl) has never been reported.

Therefore, the present study was conducted to investigate the changes in biological characteristics on exposure to high salt concentrations (including 30% NaCl). In addition, the effect of high-salt exposed bacteria on 8-OHdG expression in gastric epithelial cells was assessed as an index of DNA damage.
**Materials and Methods**

**Culture and treatment of H. pylori with high-salt**

H. pylori strain, L301, which is positive for CagA and ureB, was kindly provided by the Third Laboratory, Cancer Institute, China Medical University, Shenyang, China. The bacteria were grown on brain heart infusion agar containing 7% sheep blood, 0.4% BBLTM IsoViteXTM Enrichment (Becton, Dickinson and Company, Franklin Lakes, NJ USA), 0.08% amphotericin B (Sigma), 0.2% vancomycin (Eli Lilly, Indianapolis, USA), and 0.5% trimethoprim (Sigma), and incubated under microaerophilic conditions (5% O2, 10% CO2, and 85% N2) at 37°C and 95% humidity. In the present study, the above media supplemented with different concentrations of NaCl (3%, 15%, and 30%, respectively) were prepared; 3% NaCl group was used as a control.

**Observation of motility and morphology and measurement of ATP content of H. pylori**

The morphology of H. pylori was identified by Warthin-Starry silver staining (Jhala et al., 2003). Moreover, ultrastructure was also observed under transmission and scanning electron microscope, respectively. For scanning electron microscopy (H-600, Olympus, Japan), fixation, dehydration, and coating were performed as previously described (Sato et al., 2003).

For the measurement of ATP content, H. pylori were harvested and then washed with phosphate-buffered saline (PBS, pH7.3-7.4) centrifugation 10 min, 5000×g, 3 times, and then detected with the Bactiter-Glo Microbial Cell Viability Assay (Zhou et al., 2008).

**Urease activity**

H. pylori cultured on the agar plates with different concentrations of NaCl was collected 24, 48, 72, 96, and 120 hours after culture, and then incubated on the urease medium. A positive result was recorded when the color of the medium changed from yellow to pink.

**Expression of CagA and UreB proteins in H. pylori as detected by Western blotting**

Bacteria were lysed in radio-immunoprecipitation assay (RIPA) buffer (Beyotime, China). The protein levels were determined using a mouse anti-CagA monoclonal antibody (Santana, Bethel, USA) and a rabbit anti-ureB polyclonal antibody (Santana, Bethel, USA). A rabbit anti-GAPDH (Santana, Bethel, USA) was used to detect GAPDH as a control.

**Determination of 8-hydroxy-2’-deoxyguanosine in GES-1 cells after infection with H. pylori by immunofluorescence**

GES-1 cells were co-cultured with H. pylori as described above, and then washed twice with PBS and fixed on slides with acetone at 4°C. The slides were incubated in bovine serum albumin for 30 min and with mouse anti-human 8-hydroxy-2’-deoxyguanosine (anti-8-OHdG) antibody (1:20, JaICA, Shizuoka, Japan) overnight at 4°C. The slides were washed twice with PBS, and then incubated with goat anti mouse IgG (Santana) at 37°C for 1 h. The cells were observed under upright fluorescence microscope (Ke, Ning, and Wang, 1994). The optical density of 10 visions was detected by image analysis system and calculated the average optical density value.

**Statistical analysis**

Statistical analysis was performed by using the SPSS® version 11.5 (SPSS, Chicago, IL.). The results were expressed as the mean ± standard deviation (SD) and comparisons between groups were performed by using the Student-t test. A P value of less than 0.05 (two-sided) was considered as statistically significant.

**Results**

**Effect of high salt concentrations on mobility and ATP content of H. pylori**

H. pylori were collected from the agar plate into PBS, and the dynamic changes observed under the inverted microscope. In cultures with different concentrations of NaCl for 48 h, H. pylori represented weak drill-like movement.

The ATP contents in H. pylori exposed to 30% NaCl were significantly higher than those in H. pylori exposed to 3% or 15% NaCl at 24, 72, 96, and 120 h (P<0.05).

**Effect of high salt concentrations on morphology of H. pylori**

H. pylori exposed to different concentrations of salt was positive for Warthin-Starry silver staining, and exhibited following morphological changes: the bacterial cells were rod-shaped, short rod-shaped, or even coccoid-shaped and in various sizes (Figure 1).

Transmission electron microscopy showed that the majority of H. pylori cells exposed to 30% NaCl were in U form (Figure 2A), chain-shaped (Figure 2B), or coccoid (Figure 2C) with or without homogenous cytoplasm. Under the scanning electron microscope, H. pylori cells exposed to 30% NaCl lost the spiral shape, and a substantial proportion of the cells were coccoid-shaped, and had a rough and irregular surface with single polar

| Table 1. ATP Concentration in H. Pylori Exposed to Different Concentrations of NaCl at Different Time Points (10⁶ relative light units) |
|---|---|---|---|---|---|---|
| Group | 0 h | 24 h | 48 h | 72 h | 96 h | 120 h |
| 3% NaCl | 1.0±0.10 | 0.05±0.01 | 0.3±0.07 | 0.3±0.01 | 1.1±0.40 | 0.7±0.46 |
| 15% NaCl | 1.0±0.10 | 0.05±0.5* | 0.5±0.03 | 0.9±0.25** | 2.0±0.14* | 0.07±0.04* |
| 30% NaCl | 1.0±0.10 | 20.2±4.00** | 9.6±0.15** | 5.7±1.87*** | 4.5±0.57** | 3.8±0.42** |

*P<0.05 vs. 3% NaCl; **P<0.05 vs. 15% NaCl.
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Effect of high salt concentrations on urease activity and the expression of CagA and UreB of *H. pylori*

The urease reaction was strongly positive (change within 5 min) for the bacteria exposed to different concentrations of NaCl for 48 h.

In *H. pylori* exposed to different concentrations of salt, CagA and UreB proteins were still expressed. The expression of CagA protein increased with salt concentration, and the difference was significant among *H. pylori* exposed to 3%, 15%, and 30% NaCl (P<0.05). There was no significant difference in the level of UreB protein at 3%, 15%, and 30% NaCl (P>0.05).

Western blotting illustrating CagA (A) and UreB (B) proteins were expressed at lower levels in *H. pylori* cells exposed to different concentrations of NaCl (Figure 3). Moreover, CagA expression was NaCl concentration dependent (P<0.05), the level of CagA increased with NaCl concentration elevation. There was no significant difference in UreB expression levels among *H. pylori* control group and after exposure to 3%, 15%, and 30% NaCl (Figure 3, Table 2).

8-OHdG expression in GES-1 cells infected with *H. pylori* cultured with high salt

Overall, the expression levels of 8-OHdG was significantly greater in GES-1 cells infected with *H. pylori* exposed to different concentrations of NaCl than in those without *H. pylori* infection. (Figure 4, Table 3). However, the 8-OHdG level was significantly decreased in cells infected with *H. pylori* exposed to 30% NaCl, compared with the level in cells infected with *H. pylori* exposed to 3% NaCl (P=0.035<0.05). The 8-OHdG level was lower, albeit not statistically significant, in cells infected exposed...
to 15% NaCl than in those infected with \textit{H. pylori} exposed to 3% NaCl (P=0.06).

\section*{Discussion}

The present study demonstrated that \textit{H. pylori} survived in the present of salt at concentration of up to 30%. Bacteria exposed to high salt led to changes in the morphology, ATP contents, CagA expression. In addition, the expression of 8-OHdG was decreased in gastric epithelial cells co-cultured with \textit{H. pylori} exposed to high salt.

The reported epidemiological studies have provided evidence for a combined effect of salt or salted food consumption and \textit{H. pylori} infection in gastric carcinogenesis (Lee et al., 2003; Wang, Terry, and Yan, 2009). In our preliminary study, we found that individuals who liked high salt diet such as bacon were more likely to have \textit{H. pylori} infection than those who did not in a high risk population in Zhubhe region, an area with high incidence of gastric cancer (Yuan, 2005). Several studies have been conducted to identify the underlying effects of high salt on the biological behaviors of \textit{H. pylori} (Gancz, Jones, and Merrell, 2008; Loh, Torres, and Cover, 2007; Wang, 2007). However, those studies are all conducted under relative lower NaCl concentrations (<30%), and thus the adaptation to the very high salt environment is still unclear. Therefore, the present study investigated the tolerance ability of a local \textit{H. pylori} strain, L301, in very high salt environment (i.e. 30%), and subsequently the changes of the biological characteristics.

\textit{H. pylori} switched from the typical spiral form to long rod, U, or even coccoid form in the high salt environment in the present study, which indicates that the bacterium may switch from active proliferative phase into “non-culturable” phase, during which the bacteria are more likely to escape host immunity and to infect the new host (She et al., 2003; Wang et al., 1997). We further measured the ATP contents exposed to different concentrations of NaCl. It was found that the ATP contents were significantly higher in \textit{H. pylori} exposed to 30% NaCl than those exposed to 3% and 15% NaCl, suggesting that the ATP contents in \textit{H. pylori} increases with the concentrations of NaCl, and switches from active form to dormant form under high salt. In addition, although \textit{H. pylori} cultured in high salt environment (30%) did not grow to form colonies in the present study, previous studies have demonstrated that some \textit{H. pylori} bacterial cells that become coccoid form in a hostile environment are still viable and re-cultivable (Bumann et al., 2004; She et al., 2003; Sorberg et al., 1996; Wang et al., 1997).

Urease activity plays an essential role in \textit{H. pylori} colonization of the gastric mucosa (Sachs et al., 2005; van Vliet et al., 2002; Zaidi et al., 2009). In this study, high salt did not affect the urease activity and the expression of the urease subunit, UreB, of \textit{H. pylori}. These findings indicate that urease activity is not abolished by the high concentration of salt, and thus may still facilitate the development of gastric cancer; however, the underlying mechanism is required to be further investigated. CagA is accepted as the most important virulence biomarker that is associated with vacuolating cytotoxin A (VacA) of \textit{H. pylori} (Tuo et al., 2004). In the present study, the CagA expression levels of the \textit{H. pylori} strain, L301, were increased proportionally with NaCl concentrations. Thus, \textit{H. pylori} not only tolerates to very high salt conditions but also up-regulates CagA protein in response to high salt conditions accordingly.

Furthermore, we found that the expression level of 8-OHdG was significantly increased in GES-1 cells that were co-cultured with \textit{H. pylori} exposed to different concentrations of NaCl, compared with the level in cells without \textit{H. pylori} infection (Cooke et al., 2003). Therefore, a high salt environment may lead to gastric carcinogenesis through up-regulating 8-OHdG expression in individuals infected with \textit{H. pylori}.

In conclusion, \textit{H. pylori} survives in the exposure of high salt at concentration of up to 30%. Exposure to high salt results in changes in mobility, morphology and CagA expression. \textit{H. pylori} exposed to a very high salt increases the expression of 8-OHdG of gastric epithelial cells, indicating DNA damage of the cells. These findings indicate that \textit{H. pylori} can adapt to the very high salt environment, such as high-salt food or water, and importantly, high dietary salt and \textit{H. pylori} infection are synergistic in the development of gastric cancer. Further investigation is required to explore the underlying mechanisms on how high salt affects the biological changes of \textit{H. pylori}, and how these two “carcinogens” interact in gastric carcinogenesis.

\section*{Acknowledgements}

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