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RAPID COMMUNICATION

Effects of *H pylori* infection on gap-junctional intercellular communication and proliferation of gastric epithelial cells *in vitro*

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Abstract

AIM: To explore the effects of *H pylori* infection on gap-junctional intercellular communication (GJIC) and proliferation of gastric epithelial cells *in vitro*.

METHODS: A human gastric epithelial cell line (SGC-7901) cultured on coverslips was exposed overnight to intact *H pylori* ($CagA^+$ or $CagA^-$ strains) and sonicated extracts, respectively. GJIC between the cells was detected by fluorescence redistribution after photobleaching (FRAP) technique. Proliferation of SGC cells was determined by methylthiazolyl tetrazolium (MTT) assay.

RESULTS: When compared with control in which cells were cultured with simple medium alone, both $CagA^+$ and $CagA^-$ *H pylori* isolates could inhibit GJIC ($CagA^+$: $F = 57.98$, $P < 0.01$; $CagA^-$: $F = 29.59$, $P < 0.01$) and proliferation ($CagA^+$: $F = 42.65$, $P < 0.01$; $CagA^-$: $F = 58.14$, $P < 0.01$) of SGC-7901 cells. Compared with $CagA^-$ strains, $CagA^+$ *H pylori* more significantly down-regulated GJIC of gastric cells (intact *H pylori*: $t = 13.86$, $P < 0.01$; sonicated extracts: $t = 11.87$, $P < 0.01$) and inhibited proliferation gastric cells to a lesser extent *in vitro* (intact *H pylori*: $t = 3.06$, $P < 0.05$; sonicated extracts: $t = 3.94$, $P < 0.01$).

CONCLUSION: Compared with $CagA^-$ *H pylori* strains, $CagA^+$ strains down-regulate GJIC of gastric epithelial cells more significantly and inhibit proliferation of gastric cells to a lesser extent *in vitro*. *H pylori*, especially $CagA^+$ strains, may play an important role in gastric carcinogenesis.

Key words: *H pylori*; Gap-junctional intercellular communication; Gastric epithelial cell; CagA; Fluorescence redistribution after photobleaching; Methylthiazolyl tetrazolium assay

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INTRODUCTION

Epidemiological and animal studies have demonstrated a strong causal relationship between gastric cancer and chronic infection with *H pylori*, especially cytotoxin-associated gene A (*cagA*)-positive strains[1,2]. The cagA gene product CagA is directly delivered into gastric epithelial cells *via* type Ⅳ secretion system. Following membrane localization and subsequent tyrosine phosphorylation, CagA interacts with a variety of host cell proteins that are involved in the regulation of cell growth and motility[3]. However, the exact mechanism responsible for the development of gastric cancer in *H pylori*-infected patients still remains unclear.

Gap-junctional intercellular communication (GJIC) is an important mechanism controlling cellular homeostasis, proliferation and differentiation. Inhibition of GJIC between adjacent cells has been postulated to be one of the important events occurring during the promotional stage of cancer[4]. The vast majority of neoplastic cells reduce GJIC compared to their nonneoplastic counterparts[5]. A number of tumor promoters, such as 12-O-tetradecanoylphorbol-13-acetate (TPA), have been known as potent inhibitors of GJIC[6].

So far, changes of GJIC in *H pylori*-associated gastric carcinoma have not been extensively exploited. In the present study, we attempted to explore the molecular mechanisms of *H pylori* infection in gastric carcinogenesis by studying its effects on GJIC of gastric epithelial cells *in vitro*.

MATERIALS AND METHODS

H pylori strains

H pylori strains 97002 and 97004 were identified by and stored in Department of Medical Microbiology and

Table 1 Effects of intact *H pylori* and sonicated extracts on GJIC of SGC-7901 cells (*n*, mean ± SE)

Group	CagA$^+$ strain[b]	CagA$^-$ strain[b]
Intact *H pylori*[d]	26.05 ± 3.39 (40)[a]	36.95 ± 3.78 (44)[a]
Sonicated extracts[d]	15.92 ± 2.53 (40)[a]	22.69 ± 2.60 (41)[a]
Negetive control	66.39 ± 9.95 (24)	
Positive control (TPA)	8.47 ± 0.95 (22)	

[b]$P < 0.01$ one-way ANOVA *vs* negative control, [a]$P < 0.05$ ANOVA/Dunnett *vs* negative control, [d]$P < 0.01$ *vs* *t*-test of CagA$^+$ and CagA$^-$ *H pylori* strains.

Table 2 Effect of intact *H pylori* and sonicated extracts on proliferation of SGC-7901 cells (*n*, mean ± SE)

Group	CagA$^+$ strain[b]	CagA$^-$ strain[b]
Intact *H pylori*[d]	0.755 ± 0.048 (6)[a]	0.680 ± 0.036 (6)[a]
Sonicated extracts[d]	0.938 ± 0.037 (6)	0.830 ± 0.056 (6)[a]
Negetive control	0.955 ± 0.038 (6)	
Positive control (TPA)	0.986 ± 0.045 (6)	

[b]$P < 0.01$ one-way ANOVA *vs* negative control, [a]$P < 0.05$ ANOVA/Dunnett *vs* negative control, [d]$P < 0.01$ *vs* *t*-test of CagA$^+$ and CagA$^-$ *H pylori* strains.

Parasitology, Zhejiang University School of Medicine. The genotypes of vacuolating cytotoxin gene A (*vacA*) of the strains 97002 and 97004 were s1a/m1 and m2, respectively. The results of Western blot and cell vacuolation test demonstrated that the strain 97002 was CagA$^+$/VacA$^+$ and 97004 CagA$^-$/VacA$^-$.

H pylori culture

H pylori strains were cultured on ECY blood-free medium[7] at 37℃ for 5 d, under 100% humidity and microaerophilic conditions (50 mL/L O_2, 100 mL/L CO_2, and 850 mL/L N_2). The bacteria were harvested from the agar plates, washed twice with 0.01 mol/L PBS and stored at -20℃.

Preparation of intact H pylori and sonicated extract samples

The frozen bacteria were dissolved in RPMI1640 culture medium and adjusted to 1×10^{10} CFU/L in intact bacterial samples and 1×10^{12} CFU/L in sonicated extract samples, respectively. The preparation of sonicated extract samples additionally included *H pylori* pulverization with ultrasound, centrifugation at 10000 r/min for 20 min with the supernatant collected.

Cell culture

Human gastric epithelial cell line SGC-7901 was obtained from Department of Medical Microbiology and Parasitology, Zhejiang University School of Medicine and cultured in RPMI1640 medium (Gibco, USA) supplemented with 10% fetal bovine serum (FBS) (Sijiqing, China), 1×10^5 IU/L penicillin and 100 mg/L streptomycin. The cells were incubated at 37℃ in a humidified atmosphere containing 950 mL/L air and 50 mL/L CO_2. The cells were grown on 22 mm × 22 mm coverslips in tissue culture dishes (35 mm in diameter) and the culture medium was changed every other day. To determine cell proliferation, SGC-7901 cells were plated into 96-well microplates (0.5×10^5 cells/well) and cultured for 12 h.

Cell treatment with H pylori extracts

Twenty-four hours prior to GJIC measurement, cells of the test groups were treated overnight with intact *H pylori* or sonicated extracts. Negative and positive controls were treated with RPMI1640 with 2% NBS and 5 μg/L TPA was added to the positive control during the last 1 h.

Measurement of GJIC by FRAP technique

GJIC between SGC-7901 cells was measured by fluorescence redistribution after photobleaching (FRAP) technique first described in 1986[8]. 6-carboxyfluorescein diacetate (6-CFDA) was used as the dye that could be retained inside the cells due to its hydrolysis by cytoplamic esterases into 6-carboxyfluorescein (6-CF). 6-CF could permeate gap junction channels due to its low molecular weight. FRAP was achieved under a confocal laser scanning microscope (Leica TCS-SP, Germany) and the detailed protocol was performed as previously described[9].

Determination of cell proliferation by MTT assay

When SGC-7901 cells confluenced by 70% in the 96-well microplates, cell proliferation was assessed by methylthiazolyl tetrazolium (MTT) assay as previously described[10]. The absorbance value per well at 570 nm was read on an automatic multiwell spectrophotometer (Bio-Rad, USA).

Statistical analysis

All data were presented as mean ± SE. Statistical analysis was carried out by ANOVA followed by Dunnett's *t*-test. $P < 0.05$ was considered statistically significant.

RESULTS

H pylori down-regulated GJIC of SGC-7901 cells

The GJIC of SGC-7901 cells was measured by FRAP after treated with intact *H pylori* or sonicated extracts for 24 h and presented as fluorescence transfer rate (K, 10^{-3}/s) (Table 1). In the present study, both CagA$^+$ and CagA$^-$ *H pylori* isolates including intact *H pylori* and sonicated extracts down-regulated GJIC of SGC-7901 cells (CagA$^+$: $F = 57.98$, $P < 0.01$; CagA$^-$: $F = 29.59$, $P < 0.01$). Compared with CagA$^-$ strains, CagA$^+$ *H pylori* more significantly down-regulated GJIC of gastric cells (intact *H pylori*: $t = 13.86$, $P < 0.01$; sonicated extracts: $t = 11.87$, $P < 0.01$). In addition, our study demonstrated that TPA (5 μg/L for 1 h) had a significant inhibitory effect on GJIC of gastric cells.

Effect of H pylori on cell proliferation

The effects of intact *H pylori* and sonicated extracts on the proliferation of SGC-7901 cells were evaluated by MTT assay ($A_{570\ nm}$) (Table 2). The results suggest that both CagA$^+$ and CagA$^-$ *H pylori* isolates inhibited proliferation of SGC-7901 cells (CagA$^+$: $F = 42.65$, $P < 0.01$; CagA$^-$: $F = 58.14$, $P < 0.01$). However, CagA$^+$ *H pylori* strain inhibited proliferation of gastric cells to a lesser extent when compared with CagA$^-$ strain (intact *H pylori*: $t = 3.06$, $P < 0.05$; sonicated extracts: $t = 3.94$, $P < 0.01$).

DISCUSSION

Among various forms of intercellular communication systems in multicellular organisms, GJIC is the only form by which cells exchange signals directly from the inside of one cell to the neighboring cells. GJIC plays a crucial role in maintaining homeostasis by keeping growth control signals at equilibrium among GJIC-connected cells[11,12]. Most tumor cells have a reduced ability to communicate among themselves and/or with surrounding normal cells, confirming the importance of functional GJIC in growth control[13-15]. GJIC is mediated by gap junction channels composed of tetramembrane spanning proteins, known as connexins. At least 13 subtypes of connexin have been identified and four or five subtypes are detectable in the gastrointestinal tract[16].

It has been reported that connexin 32 in normal gastric mucosa is reduced significantly or absent in atrophic gastric mucosa and metaplastic epithelial cells, and no malignant cells from patients with gastric carcinoma contain detectable connexin 32[17,18]. These results suggest that loss of cell-cell communication through the gap junction may act as an early indicator of gastric carcinoma.

In this study, the effects of *H pylori* infection on GJIC of gastric epithelial cells were detected *in vitro*, suppressing interferences of various cytokines and immune factors *in vivo*, suggesting that both $CagA^+$ and $CagA^-$ *H pylori* isolates inhibit GJIC of SGC-7901 cells and the down-regulating effect of $CagA^+$ *H pylori* is more significant than that of $CagA^-$ strains. These findings emphasize the close relationship between *H pylori* especially $CagA^+$ strains and gastric carcinoma.

Increased cellular proliferation rates are characteristic in malignant tissue. Because of unstability of the genome of proliferating cells, hyperproliferation increases the possibility of DNA damage and aneuploidy. Dysplasia may evolve into carcinoma if damaged DNA cannot be repaired on time or fails in promoting the apoptosis system[19]. *H pylori* infection of the gastric mucosa is closely associated with changes in gastric epithelial cell proliferation. *In vivo* data show that gastric epithelial hyperproliferation is common in *H pylori*-infected persons and the degree of proliferation is directly associated with the severity of mucosal neutrophilic infiltration[20-22]. However, it was reported that an overall increase in gastric epithelial cell proliferation is not associated with *H pylori* gastritis[23]. It is not very clear whether the increased proliferation seen *in vivo* is a direct effect of *H pylori*, or a reflex increase in proliferation in response to increased cell damage, indirectly caused by *H pylori*. A recent report by Cabral *et al*[24] suggested that the increased cell proliferation rate in patients with *H pylori* infection might be related to the *H pylori*-induced inflammation rather than to a direct action of the pathogen.

Several *in vitro* studies reported that *H pylori* can inhibit cell proliferation[25,26] , which is consistent with the results of this study. The possible reason for the contradiction between the findings *in vivo* and *in vitro* is that *in vivo* studies are representative of the effect of persistent *H pylori* infection whereas *in vitro* experimental studies are representative of an acute *H pylori*-mediated effect. Also, the increased cell proliferation in patients with *H pylori* infection might be due to the increased production of gastrin *in vivo*[25]. Moreover, *in vivo* increased epithelial cell injury is associated with a reflex increase in proliferation of uninjured cells, which would not be seen *in vitro* as each cultured gastric cell is in contact with bacteria[27]. Cell proliferation is an essential process for the integrity of gastric mucosa. Deceasing cell turnover may increase the chances of ulcer formation and delay ulcer healing. Therefore, our findings seem to be relevant to the pathogenesis of *H pylori*-associated peptic ulcer diseases.

$CagA^+$ *H pylori* is frequently isolated from patients with gastric cancer in Western countries and may be more virulent in its pathogenesis[28,29]. *In vivo* studies reported that infection with $CagA^+$ *H pylori* strains is linked with higher acute inflammatory scores than $CagA^-$ strains[30,31], suggesting that these strains preferentially induce epithelial cell proliferation by stimulating inflammatory mediators. Our results show that $CagA^+$ strains could inhibit proliferation of gastric epithelial cells to a lesser extent than $CagA^-$ ones. Thus gastric cells injured by exposure to $CagA^+$ *H pylori* strains may be more likely to progress through the cell cycle, which possibly results in the risk of replication of cells with DNA damage[27].

In conclusion, *H pylori* can directly inhibit GJIC and proliferation of gastric epithelial cells *in vitro*. Compared with $CagA^-$ *H pylori* strains, $CagA^+$ strains more significantly down-regulate GJIC and inhibit proliferation to a of gastric epithelial cells lesser extent. Accelerated proliferation increases the risk of DNA damage and gene mutation. Inhibited GJIC makes cancer-initiated cells escape from the control of neighboring cells. *H pylori*, especially $CagA^+$ strains, may play an important role in gastric carcinogenesis.

COMMENTS

Background

It has been widely accepted that there is a strong association between *H pylori* infection and gastric cancer, but the exact molecular mechanism of the pathogen in gastric carcinogenesis has not clarified yet. Nearly 40 years ago, loss of functional gap junctions was described in cancer cells and led to the hypothesis that such a type of intercellular communication is involved in the carcinogenesis process. Since then, a lot of data have been accumulated confirming that gap junctions are frequently decreased or absent in cancer cells. Gap junction deficiency has been defined in the literature either as the lack of gap-junction plaques or as the lack of gap-junctional intercellular communication (GJIC). It has been reported that connexin 32 in normal gastric mucosa as a mediator of GJIC is reduced significantly or absent in atrophic gastric mucosa and metaplastic epithelial cells. However, these reports have not revealed the relationship between changed GJIC and *H pylori* infection of the gastric mucosa.

Research frontiers

There has been a considerable interest over recent years in factors that predispose individuals to develop gastric carcinoma. Complex interactions between several *H pylori*, host genetics and environmental factors determine this predisposition. Understanding the molecular mechanism of the interaction between *H pylori* and gastric epithelial cells will provide us with a new strategy for effective prevention of the development of gastric cancer induced by *H pylori* infection.

Innovations and breakthroughs

In this article, the molecular mechanism of *H pylori* infection in gastric carcinogenesis was explored by studying its effects on GJIC of gastric epithelial cells *in vitro*. The results suggest that *H pylori* could inhibit GJIC of cultured gastric epithelial cells and the down-regulation effect on GJIC of $CagA^+$ strains was more significant than $CagA^-$ ones.

Applications

This article emphasizes the close relationship between *H pylori* especially CagA$^+$ strains and gastric carcinoma. It provides a new direction to illuminate the molecular mechanism of *H pylori* in gastric carcinogenesis. It also implies that compounds able to restore GJIC in junctional deficient cells or prevent its disruption in junctional proficient cells may be used in making new strategies for the prevention and/or treatment of human gastric malignancies.

Terminology

Gap junctions: membrane structures made of intercellular channels which permit the diffusion of small hydrophilic molecules from cytoplasm to cytoplasm.

Peer review

The paper seems innovative. Altered expressions of connexins have been observed in various pathological processes of the digestive tract, including gastric cancer. To our knowledge, it is the first study to explore the molecular mechanism of *H pylori* infection in gastric carcinogenesis by studying its effects on GJIC of gastric epithelial cells *in vitro*.

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