L-Lactic Acid Secreted from Gastric Mucosal Cells Enhances Growth of Helicobacter pylori

Tetsufumi Takahashi,* Tsukasa Matsumoto,† Masahiko Nakamura,* Hidenori Matsui,* Kanji Tsuchimoto* and Haruki Yamada*†‡

*Graduate School of Pharmaceutical Sciences, Kitasato University, Minato-ku, Tokyo, Japan; †Kitasato Institute for Life Sciences and Graduate School of Infection Control Sciences, Kitasato University, Minato-ku, Tokyo, Japan; ‡Oriental Medicine Research Center, The Kitasato Institute, Minato-ku, Tokyo, Japan

Abstract

Background: Helicobacter pylori mainly inhabit the mucus layer in the gastric mucosa. However, mechanisms involving H. pylori colonization and proliferation in gastric mucosa are not well established. This study focuses on elucidating the role of gastric mucosal cells on growth of H. pylori.

Materials and methods: H. pylori was co-cultured with the murine gastric surface mucosal cells (GSM06), and the growth of H. pylori on the cells was assessed by enumerating the colony-forming units (CFU). The H. pylori growth factor in the culture media conditioned by GSM06 cell was purified by HPLC, and the chemical structure of the growth factor was identified by analyses of 1H- and 13C-NMR spectra.

Results: A marked increase in the number of CFU of H. pylori was observed in the GSM06 cells. The enhanced H. pylori growth was also observed when indirectly incubated with GSM06 cells through semi-permeable membrane. In addition, culture media conditioned by GSM06 cell stimulated H. pylori growth approximately one thousand-fold. By bioassay-guided purification, the H. pylori growth factor was isolated from the conditioned medium of GSM06 cells and identified as L-lactic acid. The H. pylori growth-enhancing activity under microaerobic condition was well correlated with L-lactic acid concentrations in the conditioned media.

Conclusions: This study demonstrates that L-lactic acid secreted by gastric mucosal cells enhances the growth of H. pylori, and this L-lactic acid-dependent growth of H. pylori may be important to the long-term colonization of H. pylori in the stomach.

Keywords
H. pylori, L-lactic acid, gastric mucosal cells, Alamar blue.

Chronic infection of H. pylori causes gastritis, peptic ulcers, gastric adenocarcinoma, and mucosa-associated lymphoid tissue lymphoma [1]. In more than half of the human population, the human pathogen Helicobacter pylori persistently colonizes the gastric mucosa [2] despite the attacks by acid, reactive oxygen and phagocytic cells in its severe environment [3]. It has been considered that some H. pylori factors such as urease [4] and flagella [5] are required for lifelong colonization of H. pylori in the gastric mucosa.

The gastric mucous layer is constantly degraded and solubilized from the luminal surface by the actions of gastric acid and digestive enzymes. To sustain the mucous layer, gastric mucous cells continuously synthesize mucin. Thus, H. pylori proliferation is dependent on gastric mucous cell functions.

It has been reported that medium supplements such as whole blood [6,7], lysed human erythrocytes [8], serum [7–9], yeast extract [7,10], Iso VitaleX [11,12], hemin [11], cyclodextrin [7,13,14], and cholesterol [15] are able to enhance the growth of H. pylori. However, the intragastric substances that are responsible for inducing and/or enhancing the growth of H. pylori in the stomach have not been clearly identified.

In patients who are positively diagnosed with H. pylori infection, most of the H. pylori are associated with the gastric surface mucous layer containing extracellular MUC5AC mucin or the apical domain of MUC5AC mucin-producing cells [16]. Therefore, the interaction of H. pylori with the MUC5AC mucin and the gastric surface mucous cells may be important for growth of H. pylori in the stomach. However, the detailed colonization mechanism...
of *H. pylori* in the stomach, especially the contribution of the interaction between host gastric mucous cells and *H. pylori*, has not been fully understood.

Previously, we reported a novel in vitro infection model of *H. pylori* using normal murine gastric surface mucous cells (GSM06) [17] as the host, and studied this interaction [18].

The present study shows that *H. pylori* growth is enhanced by co-cultivation with GSM06 cells and in the presence of media conditioned by GSM06 cells. The enhanced growth of *H. pylori* is due to a soluble factor produced by GSM06 cells, and L-lactic acid was identified as one of active substances.

**Methods**

**Cell Culture**

Murine gastric surface mucosal cell line, GSM06, was kindly provided by Dr T. Horiuchi (Daichii Pharmaceutical, Tokyo, Japan). GSM06 cells were established from transgenic mice harboring the temperature-sensitive simian virus 40 large T-antigen gene [19]. GSM06 cells were cultured in Dulbecco's modified Eagle's medium/Ham's F-12 medium (DMEM/F-12, Sigma, St. Louis, MO, USA) containing 1% SITE (Sigma, 10 mg/mL insulin, 5.5 mg/mL transferrin, 5 mg/mL selenium, 2 mg/mL ethanolamine) and 10 ng/mL epidermal growth factor (EGF, Sigma) (DMEM/F-12/SE) supplemented with 10% fetal bovine serum (FBS) and 1 mg/mL glucose, and incubated at 37 °C in a humidified atmosphere of 5% CO₂, 5% O₂ and 80% N₂. The cultures were harvested from the plates, suspended in 20 mL BHI broth containing 10% FBS and 1 mg/mL glucose, and incubated at 37 °C for overnight with agitation on a rotary shaker at 100 r.p.m. in a microaerobic condition. Two milliliter of the seed culture was inoculated into 200 mL of the same medium, and incubated further for 48 hours at 37 °C. The resulting *H. pylori* culture was used for the experiments.

**Preparation of the Conditioned Medium of GSM06 Cells**

GSM06 cells were cultured in DMEM/F-12/SE for 24 hours, and the conditioned medium from the cell culture was collected by aspiration and filtered through sterile cellulose acetate membrane (pore size 0.2 μm, Toyo Roshi, Tokyo, Japan) and stored at −80 °C until use.

**Transwell Assay**

The 24-transwell with a 0.45-μm pore-size polycarbonate membrane (Intercell®, Kurabo, Tokyo, Japan) was used. Aliquots (0.5 mL) of *H. pylori* were added to the inner chamber, which was inserted into the outer chamber containing 1 mL of DMEM/F-12/SE. *H. pylori* was cultured for 24 hours with or without GSM06 cells, which were cultured in the outer chamber.

**Determination of CFU of *H. pylori***

A suspension of *H. pylori* was diluted with phosphate-buffered saline containing 0.01% gelatin and spread onto BHI agar plate. The plate was incubated for 4 days under microaerobic conditions at 37 °C, and then the number of the colony-forming unit (CFU) was determined.

**Growth of *H. pylori* in the Conditioned Medium of GSM06 Cells**

*H. pylori* (1 × 10⁴ CFU/mL) was suspended in the conditioned medium of GSM06 cells, and then the suspension was incubated under aerobic (15% CO₂ in air) and microaerobic condition (15% CO₂, 5% O₂ and 80% N₂) for 24 or 48 hours. After the incubation, the number of the CFU was determined.
Isolation of the *H. pylori* Growth-Promoting Factor from the Conditioned Medium of GSM06 Cells

The lyophilizate of the conditioned medium of cultured GSM06 cells was partitioned with the solvent mixture (chloroform/methanol/water = 2:1:1; v/v). The aqueous layer as an active fraction was concentrated by evaporation and then subjected to Sephadex G-15 (Amersham Biosciences, Uppsala, Sweden) column chromatography using water as an eluant. The active fraction was loaded onto a Sep-Pak C<sub>18</sub> cartridge (Waters, Millford, MA, USA), and the unabsorbed fraction was loaded onto an Oasis MAX cartridge (Waters). The cartridge was eluted with trifluoroacetic acid (TFA) in water (0, 0.01 and 0.1%), and three fractions were obtained by stepwise manner. The 0.01% TFA eluate fraction was further subjected to an anion-exchange HPLC column chromatography (Cosmogel DEAE, 7.5 mm i.d. × 75 mm, Nacalai Tesque, Kyoto, Japan) equilibrated and eluted with 10 mmol/L NaCl at a flow rate of 0.8 mL/minute. Each fraction obtained during the purification process was evaporated to dryness in each well of 96-well plate, and then subjected to fluorometric bioassay for measurement of *H. pylori* growth.

**Fluorometric Bioassay for Measurement of *H. pylori* Growth**

*H. pylori* growth-enhancing activity was also determined by the Alamar blue assay. This assay is based on reduction of the dye by cellular respiration as an indicator of cell growth and/or viability [21]. *H. pylori* suspensions (100 µL; 10<sup>5</sup> CFU/mL in DMEM/F12/SE) were transferred into each well containing tested substance on a 96-well plate, and the plate was incubated for 24 hours under microaerobic conditions at 37 °C. After incubation, 10 µL of Alamar Blue reagent (Alamar Bio-Siences, Sacramento, CA, USA) was added to each well. The plate was incubated further for 2 hours and then the fluorescence intensity in each well was read on a microplate fluorescence reader, Fluoroskan II (Labsystems, Helsinki, Finland), using an excitation wavelength of 544 nm and an emission wavelength of 590 nm, respectively. *H. pylori* growth-enhancing activity of each substance was expressed as the relative fluorescence intensity.

**1<sup>H</sup>- and 13<sup>C</sup>-NMR Spectroscopy**

1<sup>H</sup>- and 13<sup>C</sup>-NMR spectra were recorded using D<sub>2</sub>O as solvent on a Varian XL-400 spectrometer (Varian Inc. Palo Alto, CA, USA) operating at 400 and 100 MHz, respectively.

**Determination of the Concentrations of Lactic Acid and Hypoxanthine**

The content of lactic acid was determined by the use of HPLC, and analytical conditions were as follows: column: Cosmosgel DEAE (7.5 mm i.d. × 75 mm); carrier: 10 mmol/L NaCl; flow rate: 0.8 mL/minute; and detection: UV 205 nm. The HPLC conditions of hypoxanthine analysis were as follows: column: Senshu pak ODS (4.6 mm i.d. × 250 mm); carrier: 20% acetonitrile; flow rate: 1.0 mL/min; and detection: UV 254 nm.

**Determination of the Absolute Configuration of Lactic Acid from the Conditioned Medium of GSM06 Cells**

Each sample was dissolved in water and chiral HPLC analysis was performed using a SUMICHIRAL OA-5000 column (4.6 mm i.d. × 150 mm, Sumitomo, Osaka, Japan). Analytical conditions for HPLC were as follows: carrier: 1 mmol/L CuSO<sub>4</sub>; flow rate: 1 mL/minute; and detection: 254 nm.

**Determination of the Concentration of Amino Acids**

One milliliter of the medium was lyophilized and suspended into 30 µL of coupling buffer (water-ethanol-triethylamine, 2:2:1). The amino acids in the suspension were derivatized in the presence of 6.25% phenylisothiocyanate at room temperature. The derivatized PTC-amino acids (phenylthiocarbamyl derivatives) were determined by the use of HPLC, and analytical conditions were as follows: Waters PicoTag® column (3.9 mm i.d. × 150 mm) using a gradient of two eluents (PicoTag® solution A and B) with absorption detection at a wavelength of 254 nm.

**Statistical Analysis**

To define statistical significant difference, data were subjected to Welch’s t-test or Student’s t-test. The analysis was performed using a personal computer with the DA Stats (version 1.0, freeware soft, copyright® 1993 by Dr O. Nagata) after the variances of data were examined using the F-test.

**Results**

**Effect of Co-culture with Gastric Mucosal Cells on *H. pylori* Growth**

Because host cell culture must be done under aerobic condition, the effect of co-culture with gastric mucosal
cells on *H. pylori* growth was examined under aerobic condition. When *H. pylori* SS-1 was incubated in DMEM/F-12/SE medium under aerobic conditions, the number of CFU was declined with incubation time (Fig. 1A) as opposed to a marked increase in CFU when co-cultured with the murine gastric mucosal cell line, GSM06 (Fig. 1A). Enhancement of *H. pylori* SS-1 was also observed when co-cultured with the human gastric cell line, AGS. However, *H. pylori* growth was less enhanced when co-cultured with AGS versus GSM06 cells (Fig. 1B). These results suggest that *H. pylori* growth is enhanced when co-cultured with both murine and human gastric mucosal cells.

**Effect of Indirect Co-culture with GSM06 Cells on *H. pylori* Growth**

To assess whether enhanced *H. pylori* growth occurred by direct adhesion to GSM06 cells or through soluble factor(s) produced by gastric mucosal cells, *H. pylori* was co-cultured with GSM06 cells in the transwell chambers (Fig. 2A). Even when *H. pylori* was incubated indirectly with GSM06 cells through a semi-permeable membrane, enhanced growth of *H. pylori* was observed at the same level as when in direct contact with GSM06 cells (Fig. 2B). These results suggest that *H. pylori* growth was mainly induced or enhanced by a soluble factor(s) from GSM06 cells.
L-Lactic Acid Enhances Growth of H. pylori

Mammalian cells are normally cultured under aerobic conditions in vitro. However, the gastric mucous layer where *H. pylori* colonizes is considered to be microaerobic in vivo. In this study, *H. pylori* was suspended in fresh DMEM/F-12/SE or medium conditioned by GSM06 cells, and the suspensions were further incubated under aerobic and microaerobic conditions for 24 or 48 hours. After the incubation, the number of CFU of *H. pylori* was measured. The results showed that the growth of *H. pylori* was enhanced in the presence of conditioned medium of GSM06 cells under both aerobic and microaerobic conditions (Fig. 3A,B). On the contrary, when *H. pylori* was incubated in fresh DMEM/F-12/SE, poor growth was observed under microaerobic condition (Fig. 3B), but no growth was observed in the same medium under aerobic condition (Fig. 3A). These results strongly suggest that a soluble factor produced by gastric cells enhances the growth of *H. pylori* when cultured in medium conditioned by GSM06 cells.

**Identification of the H. pylori Growth-Enhancing Factor from GSM06 Cells**

When the lyophilizate of the conditioned medium of cultured GSM06 cells was partitioned with the solvent mixture (chloroform/methanol/water = 2:1:1; v/v), only aqueous phase showed the activity under microaerobic condition (data not shown). The factor that enhanced *H. pylori* growth from the aqueous phase was purified by gel filtration, reversed phase cartridge, and anion-exchange HPLC using a bioassay-guided method, and an active substance was obtained. During the purification process, dispersion of the *H. pylori* growth enhancing activity was not observed (data not shown). By analyses of 1H- and 13C-NMR spectra, and the comparisons with standard samples (data not shown), the active substance was identified as lactic acid.

The identified lactic acid was subjected to chiral HPLC to determine its absolute configuration. The retention time of the lactic acid obtained from the conditioned medium coincided with that of standard l-lactic acid, but not d-lactic acid (data not shown). From these results, the *H. pylori* growth-enhancing factor from GSM06 cells was identified as l-lactic acid. The enhancement of *H. pylori* growth by the condition medium of GSM06 cells or l-lactic acid was also observed using fresh isolate of *H. pylori* from biopsy specimens under microaerobic condition (Fig. 4A,B).

**Comparison of H. pylori Growth-Enhancing Activity of l-lactic Acid and Its Related Compounds**

When *H. pylori* growth-enhancing activity by l-lactic acid was compared to d-lactic acid in the DMEM/F-12/SE, which was void of any lactic acid, the activity of l-lactic acid was higher than that of d-lactic acid under microaerobic condition (Fig. 4C). Both d- and l-lactic acid enhanced the growth of *H. pylori* in a dose-dependent manner, and *H. pylori* growth reached a maximum level at 8 mmol/L of l-lactic acid with a rapid decline in activity above this concentration (Fig. 4C). When lactic acid-related compounds, such as pyruvic acid, DL-2-hydroxybutyric acid and DL-3-hydroxybutyric acid, were tested for *H. pylori* growth-enhancing activity under microaerobic condition, only pyruvic acid showed a weak activity compared with that of l-lactic acid (Fig. 4D).

**Relationship Between the Concentration of Lactic Acid and the Growth of H. pylori in the Conditioned Medium of Cultured GSM06 Cells**

To analyze the kinetics of l-lactic acid production by GSM06 cells, conditioned medium was harvested at identified times and the concentrations of l-lactic acid were...
determined. GSM06 cells were secreted l-lactic acid by time-dependent manner, and reached maximum after 24 hours of the cultivation (Fig. 5A). When each conditioned medium obtained at indicated time was tested for H. pylori growth-enhancing activity, it was dependent on the concentration of L-lactic acid in the medium under microaerobic condition (Fig. 5A). The results showed a good correlation between the content of L-lactic acid and the degree of growth-enhancing activity with a calculated correlation coefficient of 0.89 (Fig. 5B). These results indicate that L-lactic acid is the one of soluble factors produced by GSM06 cells that enhances H. pylori growth under microaerobic condition.

The Concentration of Alanine, Proline, and Hypoxanthine in the Conditioned Medium of Cultured GSM06 Cells

The concentrations of alanine, proline, and hypoxanthine were analyzed by HPLC using ODS column. The alanine and proline concentrations increased significantly in DMEM/F-12/SE cultured with GSM06 cells, whereas hypoxanthine concentration decreased (Table 1).}

**Discussions**

This study demonstrates that H. pylori growth is enhanced when co-cultured with host GSM06 gastric mucus cells, and a soluble active factor produced by GSM06 cells is responsible for this enhanced growth of H. pylori. van Amsterdam and van der Ende have reported that co-culturing of H. pylori with human carcinoma HM02 cells weakly enhances H. pylori growth, and that the supplementation of DMEM with L-alanine, L-proline, and hypoxanthine was sufficient for H. pylori growth. Because L-alanine, L-proline, and hypoxanthine were absent from DMEM, the mechanisms of the amino acids and hypoxanthine on H. pylori growth were attributed to an energy source and a substrate of purine biosynthesis, respectively [22]. H. pylori has the gene ald (encoding enzyme that produces pyruvate and ammonia from L-alanine) [23]. We found the supplementation of pyruvic...
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acid slightly enhanced the growth of *H. pylori*. Therefore, it is assumed that pyruvic acid converted from L-alanine may partly contribute to growth of *H. pylori* in DMEM. In this study, using HPLC analysis, alanine and proline concentrations increased markedly in DMEM/F-12/SE cultured with GSM06 cells, whereas hypoxanthine concentration decreased (Table 1). When *H. pylori* was incubated in fresh DMEM/F-12/SE under microaerobic condition, poor growth was observed (Fig. 3B). The DMEM/F-12/SE basically contains hypoxanthine, L-alanine, and L-proline as a medium composition, therefore the growth of *H. pylori* in DMEM/F-12/SE might be due to these nutrients. However, addition of L-alanine and L-proline to fresh DMEM/F-12/SE media did not enhance *H. pylori* growth (data not shown). These results suggest that increase in the concentrations of alanine and proline in the conditioned medium is not involved in the enhancement of *H. pylori* growth.

*H. pylori* could grow in the conditioned medium of GSM06 cells under the both aerobic and microaerobic conditions (Fig. 3A,B). As *H. pylori* inhabits under microaerobic environment in vivo, we performed the purification of *H. pylori* growth-enhancing factor from conditioned medium of host cell using bioassay under microaerobic condition, and active substance was identified as L-lactic acid. To our knowledge, this is the first report demonstrating that host gastric mucosal cell-derived L-lactic acid acts as a growth factor of *H. pylori*. Although L-lactic acid was isolated as an active ingredient from the conditioned medium, the growth-enhancing activity of L-lactic acid was observed only under the microaerobic condition (Fig. 4), but not under aerobic condition (data not shown). Because *H. pylori* is sensitive to oxygen stress, it is assumed that the enough amount of the anti-oxygen stress substance such as superoxide dismutase (SOD)-like substance is required for the *H. pylori* growth under aerobic condition. The combination effect of the L-lactic acid and SOD-like substance derived from GSM06 cells could be contributed to *H. pylori* growth by co-culture with GSM06 cells or the conditioned medium under aerobic conditions. However,
whether GSM06 cells secrete SOD-like substance into conditioned medium is still not known. At the moment, we cannot rule out the possibility of the L-lactic acid-independent mechanism for \textit{H. pylori} growth under the aerobic condition. To understand the detailed mechanism of the growth-enhancing activity of the conditioned medium, further experiments are required.

The high correlation ($r^2 = 0.89$) between L-lactic acid concentrations in media cultured with GSM06 cells and \textit{H. pylori} growth (Fig. 5B) suggests that L-lactic acid is the key soluble factor associated with enhanced \textit{H. pylori} growth under microaerobic condition. It was reported that the concentration of lactic acid in the human gastric juice was about 0.3–1 mmol/L [24,25]. This finding supports the concentrations of L-lactic acid produced by GSM06 cells (Fig. 5B) are not so different from the physiologic concentrations found in the gastric fluid of the stomach.

Alba et al. reported that when \textit{H. pylori} and \textit{Lactobacillus salivarius} were co-cultured, concentrations of lactic acid $> 10$ mmol/L produced from \textit{L. salivarius} in the incubation medium inhibited viability of \textit{H. pylori} [26]. Our findings also show that \textit{H. pylori} growth is inhibited at lactic acid concentrations of $10$ mmol/L. The findings that optimal \textit{H. pylori} growth occurs at approximately 5.3 mmol/L (Fig. 5B) support the in vivo concentrations of lactic acid in mouse gastric fluid.

Lactate dehydrogenase (LDH) converts pyruvic acid to lactic acid, which is considered to be the end-product of glycolysis. However, the LDH catalyzes the reversible interconversion of lactic acid and pyruvate [27]. Thus, under the presence of LDH, lactate can be oxidized generally to pyruvate and used as a substrate for other metabolic pathways such as the TCA cycle. However, studies on the genome of \textit{H. pylori} predict the absence of a gene that codes for L-LDH [23,28] and thus, suggest that \textit{H. pylori} cannot use L-lactic acid as an energy source. Because \textit{H. pylori} has also been predicted to code two lactic permease genes [23,29], \textit{H. pylori} incorporates L-lactic acid by the action of permease, and then it may be utilized through unknown metabolic pathways or act as a signal transducer of two-component system. Further investigations are required to elucidate how L-lactic acid enhances the growth of \textit{H. pylori}.

It has been reported that \textit{H. pylori} can bind to human gastric MUC5AC mucin and that the binding is mediated to the blood-group host and microbe factors, Lewis$^b$ and BabA adhesin, respectively [28]. Studies have shown that more than 99% of \textit{H. pylori} are associated with extracellular MUC5AC mucin or the apical domain of MUC5AC mucin-producing cells in patients positively diagnosed with \textit{H. pylori} infection [16]. This study shows that host mucosal cell-derived L-lactic acid acts as a \textit{H. pylori} growth-enhancing factor. Present results support the possibility that \textit{H. pylori} first binds to MUC5AC mucin on the gastric surface mucosa, and then continuously grows in the mucous by the growth stimuli due to L-lactic acid. Thus, the L-lactic acid-dependent growth promotion seems to be important for the long-term colonization of \textit{H. pylori} in the stomach.

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