Cytotoxic Effects of *Helicobacter pylori* on Guinea Pig Gastric Glands

Toshihiro KUBOTA, Toshio FUJIOKA and Masaru NASU
Department of Internal Medicine, Oita Medical University
(Received: August 11, 1994)
(Accepted: September 12, 1994)

Key words: *Helicobacter pylori*, cytotoxicity, guinea pig gastric glands, ammonia, acetohydroxamic acid

**Abstract**

To study the cytotoxic effect of *Helicobacter pylori* on the gastric mucosa, gastric glands harvested from guinea pigs were incubated with clinical isolates of *H. pylori*. *H. pylori* alone (H group), urea alone (U group), *H. pylori* plus urea (HU group), and *H. pylori* plus urea and the urease inhibitor acetohydroxamic acid (HUA group) were incubated with isolated gastric glands. The controls were incubated without additives. Incubation was for 30, 60 and 180 min at 37°C in a microaerophilic atmosphere. The HU group showed an increase in the ammonia concentration and pH of the culture supernatant; release of lactate dehydrogenase (LDH) and glutamic oxaloacetic transaminase (GOT) into the supernatant owing to cell disruption was also increased. In the HUA group, since urease activity was inhibited, the ammonia concentration and pH were also significantly lower (p < 0.001), and LDH and GOT release into the supernatant was significantly reduced (p < 0.001-0.01). Observation by light and electron microscopy showed clear intracellular vacuolization of the gastric glands and adherence of *H. pylori* to the cell surfaces. These results suggest that ammonia, a metabolite of urea released by *H. pylori* urease, is one of the important factors in cytotoxicity in isolated gastric glands.

**Introduction**

Recent studies on the relation between *Helicobacter pylori* and gastritis or gastroduodenal ulcers have indicated that this organism can induce chronic active gastritis with neutrophil infiltration. In addition, atrophic gastritis has been found in association with persistent *H. pylori* colonization, and this may progress to gastric cancer via metaplastic gastritis.

The ammonia produced by *H. pylori*, which has strong urease activity, has been reported to have a cytotoxic effect. In addition, the gastric mucosal protective mechanism is disrupted by the protease, lipase, and phospholipase-A produced by *H. pylori* and it has been shown that the amount of periodic acid-Schiff-positive substance is decreased in gastric mucosa infected with this organism owing to the production of cytotoxins. However, the mechanism of onset of gastroduodenal lesions remains poorly understood.

Recently, many studies have demonstrated a cytotoxic effect of *H. pylori* on various kinds of cultured cells. However, the present study assessed the cytotoxic effect of *H. pylori* on gastric glands isolated from guinea pigs. To investigate damage to the gastric mucosa, it was considered desirable to use fresh gastric glands rather than cultured cells.

---

Correspondence to: Toshihiro KUBOTA, Department of Internal Medicine, Oita Medical University, Hasama-machi, Oita, 879-55, Japan

平成7年1月20日
The study revealed cytotoxicity of *H. pylori* due to the release of ammonia by urease activity.

**Materials and Methods**

**Animals**

Female Hartley guinea pigs weighing about 300 g were used in this study. They were given food (Oriental Yeast Co., Ltd., Tokyo, Japan) and tap water ad libitum until sacrificed.

**H. pylori strains**

Sixteen clinical strains of *H. pylori* were used in the study. They were isolated from the gastric biopsy specimens of eight patients with peptic ulcers (four gastric and four duodenal ulcers) and eight patients with gastritis treated in this department.

**Isolation and identification of *H. pylori***

*H. pylori* isolates were stored at −80°C, thawed at room temperature, and cultured on 7% sheep blood agar plates based on Mueller-Hinton II agar (BBL Microbiology Systems, Cockeysville, Md., U.S.A.), and Belo Horizonte medium (BHM) based on brain-heart infusion agar (Difco Laboratories, Detroit, Mich., U.S.A.)

The cultures were incubated for 4 days at 37°C in a microaerophilic atmosphere (5% O₂, 15% CO₂, 80% N₂). The organisms were morphologically identified as Gram-negative curved rods, and their identification as *H. pylori* was confirmed by various biochemical characteristics such as a positive reaction to oxidase, catalase, urease, and alkaline phosphatase, as negative reaction to nitrate reductase, nonfermentation of glucose, resistance to nalidixic acid, and sensitivity to cephalothin.

**Liquid culture medium**

Bovine serum albumin (0.2%, Sigma Chemical Co., St. Louis, Mo., U.S.A.) and 10 mM HEPES (Sigma) were added to Hanks’ solution (JRH Biosciences, Lenexa, Kans., U.S.A.) and the mixture was adjusted to pH 7.4.

**Isolation of gastric glands**

About 1 ml of Nembutal Sodium Solution (Abbott Laboratories, North Chicago, Ill., U.S.A.) was injected intraperitoneally into the guinea pigs to be killed, and a total gastrectomy was carried out immediately after sacrifice. The greater curvature of the stomach was excised, and the mucosa was everted. After being washed three times with sterile cold saline, the stomachs were incubated in 50 ml of liquid culture medium with 0.08% type IV collagenase (Sigma) and 0.08% pronase (Kaken Pharmaceutical Co., Ltd., Tokyo) for 30 min at 37°C with pure oxygen in a shaking water bath. They were further incubated twice in 30 ml of liquid culture medium with 1.5 mM Ethylene Glyco-O,O'-bis (2-aminoethyl)-N,N,N',N'-tetraacetic Acid (EGTA) (Sigma) for 10 min at 37°C under pure oxygen with shaking. Both of the resultant solutions were filtered through 30-μm nylon mesh (Lobster Tool Co., Ltd., Tokyo) to yield a suspension of isolated gastric glands.

The viability of the isolated gastric glands was found to be 90% or higher by the trypan blue dye exclusion test.

**Experimental design**

One-milliliter quantities of the gastric gland suspension were transferred to tissue culture dishes (60 mm in diameter and 15 mm in depth, Becton Dickinson and Co., Paramus, N.J., U.S.A.).

In addition, 1 ml of a 10⁹ CFU/ml *H. pylori* suspension in liquid culture medium (H group), 1 ml of a 0.5 mg/ml solution of urea in liquid culture medium (final concentration: 0.25 mg/ml) (Wako Pure Chemical Industries, Ltd., Osaka) (U group) and 1 ml of *H. pylori* plus urea (HU group) were added to the gastric gland suspension and the mixture was incubated for 30, 60 or 180 min at 37°C in a microaerophilic atmosphere (5% O₂, 15% CO₂, and 80% N₂). Furthermore, 0.5 mg of a urease inhibitor, acetoxyhydroxamic acid (AHA) (Sigma) per ml was added to a suspension of *H. pylori* and
Cytotoxic Effects of *Helicobacter pylori*

urea (final urea conditions: 0.25% mg/ml) and the mixture was incubated under the same conditions (HUA group). All five groups of cultures including controls were studied for each strain of *H. pylori* (Fig. 1).

**Cytotoxic activity against isolated gastric glands**

As an indicator of the viability of isolated gastric glands, lactate dehydrogenase (LDH) and glutamic oxaloacetic transaminase (GOT) release into the culture supernatant as well as the ammonia concentration and pH of the supernatant were measured. The suspensions of isolated gastric glands were centrifuged at 46,440 × g for 5 min, after which the LDH level was measured by the method of Wroblewski and the GOT level by the method of Karmen. The cytotoxic activity against isolated gastric glands was assessed by comparing the LDH and GOT concentrations in the experimental and control groups at each designated time, and the results were expressed as percentages of the concentration in the control group. The ammonia concentration was quantified by a modification of the method of Okuda and Fuji, and the pH was measured with a pH meter (HORIBA, Ltd., Kyoto).

**Observation of isolated gastric glands**

Morphological changes in the isolated gastric glands were observed by light microscopy (Optiphot; Nikon Corp., Tokyo), transmission electron microscopy (HEM-100CX; JEOL Ltd., Tokyo), and scanning electron microscopy (S-800, Hitachi Ltd., Tokyo). Warthin-Starry silver staining was used for light microscopy. In preparation for transmission electron microscopy, isolated gastric glands were fixed in 2.5% glutaraldehyde, dehydrated with ethanol, and embedded in Epon 812. Ultrathin sections were cut and double stained with uranyl acetate and lead citrate.

---

**Fig. 1 Experimental design**

- **isolated guinea pig gastric glands**
  - HP (10^6 cfu/ml)
  - +urea 0.5mg/ml (AHA 0.5mg/ml)
  - incubated under microaerophilic conditions at 37°C (30, 60, or 180 min)

**Supernatant**
- LDH, GOT, ammonia, and pH
- AHA : acetohydroxamic acid

**gastric epithelial cells**

---

**Fig. 2 LDH concentration in the culture supernatant**

Data are shown as a percentage of the LDH concentration in the control group (n = 16) at the corresponding time. The LDH concentration in the supernatant of the HU group (759 ± 110% at 30 min, 1,258 ± 357% at 60 min, and 839 ± 257% at 180 min) was significantly higher than in the H and U groups. The LDH level in the HUA group (309 ± 53% at 30 min, 386 ± 99% at 60 min, and 462 ± 161% at 180 min) was similar to that in the H group, and was significantly lower than that in the HU group at 30 min (p < 0.05) and 60 min (p < 0.01).
Statistical methods

All results are presented as the mean ± standard error (SE), and Student's t-test was used to assess the significance of differences. The level of significance was set at p<0.05.

Results

LDH release into the culture supernatant

As shown in Fig. 2, the LDH concentration did not increase in the H and U groups. The LDH concentration in the HU group was significantly higher than in the H and U groups (p<0.05-0.001) after incubation for 30 and 60 min. The LDH concentration in the HUA group was similar to that in the H group, and significantly lower than that in the HU group (p<0.05-0.01).

GOT release into the culture supernatant

The GOT concentration showed trends similar to those of the LDH concentration (Fig. 3). After incubation for 30, 60 and 180 min the GOT concentration was significantly higher in the HU group than in the H and U groups (p<0.01-0.001). In contrast, the GOT concentration in the HUA group was significantly lower than in the HU group (p<0.05-0.001).

Ammonia concentration and pH in the culture supernatant

No ammonia was detected in the control and U groups. In the H group, the concentration was below 300 μg/dl at 180 min. After incubation for 30 min, the ammonia concentration increased to 12, 305 ± 383 μg/dl in the HU group (p<0.001), and then levelled off. In the HUA group, the concentration was significantly lower than in the HU group because the urease activity of H. pylori was

---

Table 1 Ammonia concentration and pH of the culture medium

<table>
<thead>
<tr>
<th></th>
<th>30 min</th>
<th>60 min</th>
<th>180 min</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Ammonia</strong> μg/dl</td>
<td>26±48</td>
<td>36±67</td>
<td>53±70</td>
</tr>
<tr>
<td>control</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>urea only</td>
<td>24±65</td>
<td>216±53</td>
<td>0</td>
</tr>
<tr>
<td>HP only</td>
<td>73±19</td>
<td>117±32</td>
<td>260±38</td>
</tr>
<tr>
<td>HP + urea</td>
<td>12,305±383</td>
<td>12,236±349</td>
<td>11,622±408</td>
</tr>
<tr>
<td>HP + urea+ AHA</td>
<td>3,675±677</td>
<td>4,114±621</td>
<td>6,534±923</td>
</tr>
<tr>
<td><strong>pH</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>control</td>
<td>7.12±0.07</td>
<td>7.13±0.14</td>
<td>7.24±0.10</td>
</tr>
<tr>
<td>urea only</td>
<td>7.22±0.07</td>
<td>7.13±0.14</td>
<td>7.24±0.10</td>
</tr>
<tr>
<td>HP only</td>
<td>7.07±0.02</td>
<td>7.10±0.03</td>
<td>7.11±0.04</td>
</tr>
<tr>
<td>HP + urea</td>
<td>7.53±0.02</td>
<td>7.55±0.02</td>
<td>7.52±0.02</td>
</tr>
<tr>
<td>HP + urea+ AHA</td>
<td>7.53±0.04</td>
<td>7.28±0.04</td>
<td>7.44±0.04</td>
</tr>
</tbody>
</table>

*p<0.05  **p<0.01  ***p<0.001  (n=16) (mean±SE)
inhibited by AHA (p<0.001).

The pH differed little among the control, U, and H groups. In the HU group, the pH increased as the ammonia concentration increased (p<0.001). In the HUA group, the pH was significantly lower after 30 and 60 min of incubation than in the HU group (p<0.05-0.001), and it was slightly higher than in the H and U groups (Table 1).

**Histological findings**

We examined the cultures after incubation for 60 min. In the HU group, Warthin-Starry silver staining showed that *H. pylori* organisms were adherent to the cells and had caused disruption of the isolated gastric glands (Fig. 4). In the HU group, scanning electron micrographs showed *H. pylori* adherent to the cells (Fig. 5), and intracellular vacuolation was observed by transmission electron microscopy (Fig. 6).

![Microscopic appearance of gastric glands](Fig. 4)

**Fig. 4** Microscopic appearance of gastric glands from the HU group after incubation for 60 min. *H. pylori* organisms are adherent to the cells and disruption of cells in the gastric glands can be seen (Warthin-Starry silver staining, ×1000).

![Scanning electron micrographs of glands from the HU group](Fig. 5)

**Fig. 5** Scanning electron micrographs of glands from the HU group. *H. pylori* organisms are adherent to the surface of gastric mucosal cells (A, ×2000, B, ×10,000).
Fig. 6 Transmission electron micrographs of glands from the HU group, showing intracellular vacuolation (A, ×2600, B, ×3300).

(A) (B)

H. pylori count in the cultures

H. pylori were collected from suspensions after incubation for 180 min. The H. pylori count ranged from $3.3 \times 10^7$ to $6.5 \times 10^7$ CFU/ml, and no significant differences were noted between the groups.

Discussion

Quantification of LDH and GOT release into the culture supernatant of isolated gastric glands provides a measure of enzyme flux from the cells and thus can be used as an indicator of cytotoxicity. The LDH and GOT concentrations were significantly higher in the HU group than in the control and H groups, suggesting that the release of ammonia from urea by the strong urease activity of H. pylori results in a cytotoxic effect. In the HUA group, the LDH and GOT levels decreased in proportion to the ammonia concentration. However, the LDH, GOT, and ammonia concentrations were increased in the HUA group after incubation for 180 min, since the activity of AHA decreased over time. In our previous clinical study, the gastric juice urea concentration was 5-25 mg/dl, and the gastric juice ammonia concentration in patients with H. pylori infection was significantly higher than in H. pylori-negative patients, the average level in the positive patients being $14,473 \pm 4,345$ μg/dl. This was similar to the ammonia concentration detected in the HU group in the present in vitro study, suggesting that ammonia produced by H. pylori could have a cytotoxic effect on the gastric mucosa.

Many studies have been performed to assess the effects of ammonia, a metabolite of urea produced by H. pylori, on the gastric mucosa. Harzell and Lee suggested that ammonium ions produced by H. pylori caused hydrogen ion back-diffusion in the epithelial cells of the gastric mucosa and thus had a cytotoxic effect. Murakami et al. found that HOCl, produced by a reaction involving neutrophil myeloperoxidase, reacted with ammonia to form monochloramine (NH₂Cl), which was toxic to the gastric mucosa. However, the present in vitro study detected cytotoxicity in isolated gastric glands without neutrophils, suggesting that ammonia was a causative factor in the cytotoxicity. Kawano et al. administered ammonia orally to rats in their drinking water (10,000 and 100, 000 μg/dl) and found a decrease in mucosal thickness and in the number of parietal cells and oxyntic glands which was dose- and time dependent, suggesting that the ammonia produced by H. pylori may have an etiologic role in chronic atrophic gastritis. The ammonia concentration administered to rats was similar to the concentration detected in our study.
Cytotoxic Effects of Helicobacter pylori

Many in vitro studies of the mechanisms of H. pylori toxicity for cultured cells have also been reported\(^1\),\(^1\),\(^2\),\(^3\),\(^4\). Leunk et al.\(^3\) found that broth culture filtrates of H. pylori induced a cytopathic effect in mammalian cells and Intestine 407 cells, and the intracellular vacuolation that they noted was also found in the present study. In addition, Figura et al.\(^1\) reported that broth culture filtrates of H. pylori isolated from patients induced intracytoplasmic vacuolation in CHO cells, Vero cells, and Hela cells. Jia-Ke et al.\(^1\) detected intracellular vacuolation when H. pylori culture supernatant and urea were added to suspensions of Vero cells and Intestine 407 cells. The addition of the urease inhibitor AHA caused a decrease in the number of cells showing vacuolization, suggesting that ammonia produced by H. pylori caused the vacuolization. To investigate the cytotoxicity of H. pylori, culture supernatants have often been used, but we cultured the organism directly with gastric glands isolated from guinea pigs and observed adherence of H. pylori to the cell surface.

In the present study, LDH and GOT concentrations were increased even when the ammonia concentration showed only a slight rise (50-300 \(\mu\)g/dl). This may imply that different toxins were produced by H. pylori. It has recently been reported that the cagA\(^2\) and vacA\(^2\) genes of H. pylori produce a vacuolating cytotoxin. But ammonia produced by H. pylori is one of the important factors which are cytotoxic to the gastric mucosa. Our results suggest that H. pylori can induce cytotoxicity for these cells, but further studies are required to confirm out findings.

References

モルモット遊離胃腺を用いた Helicobacter pylori の
細胞障害性についての検討
久保田利博 藤岡利生 那須勝

要 旨
Helicobacter pylori の胃粘膜細胞障害性を検討
するために、モルモット遊離胃腺に H. pylori 臨床分離株を反応させた。
遊離胃腺に H. pylori を加えた群（H 群）、尿素
を加えた群（U 群）、H. pylori と尿素を同時に加
えた群（HU 群）、H. pylori と尿素にさらに urease
活性阻害剤である acetohydroxamic acid (AHA)
を加えた群（HUA 群）、何も加えず遊離胃腺のみ
の群（コントロール）の 5 群に分け、それぞれを
微好気条件下で 37℃, 30 分, 60 分, 180 分反応させ
た。HU 群は培養上清中のアンモニア濃度の上昇,
pH の上昇と、細胞崩壊し細胞内部より流出し
た培養上清中の LDH と GOT 濃度の上昇が認め
られた。AHA 群は urease 活性の阻害により、アン
モニア濃度及び pH の上昇値に有意に抑制され
(p<0.001), LDH, GOT 濃度の上昇が有意に抑制
された (p<0.001〜0.01)。光顕・電顕による形態
的な観察により、HU 群では有意な細胞の空胞化
と細胞表面への菌の付着を認めた。これらの結果
は、H. pylori の持つ urease 活性によって尿素よ
り産生されたアンモニアが、遊離胃腺の細胞障害
性を引き起こす重要な要素であることを示唆す
る。