In Vitro Cytotoxic Effects of Vacuolating Cytotoxin Produced by Clinical Isolates of *Helicobacter pylori*

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Abstract

The vacuolating cytotoxin produced by *Helicobacter pylori* is considered to be one virulence factor causing peptic ulceration. In this study, we examined the activity of vacuolating cytotoxin in induction of intracellular vacuolation of rabbit gastric epithelial cells (RGECs). We used culture supernatants of *H. pylori* as a source of vacuolating cytotoxin and quantitated cytotoxic activity by the MTT method. Intracellular vacuolation of RGECs was observed in the presence of 36 of 57 (63%) clinically isolated *H. pylori* strains. However, there were no differences in the incidence of *H. pylori* strains with positive vacuolating cytotoxin (Tox^+) among patients with gastritis, gastric ulcers or duodenal ulcers. The MTT assay showed that the cytotoxic activity of *H. pylori* supernatants obtained from patients with gastric ulcers was significantly higher than in patients with gastritis ($p < 0.01$), but was not different to duodenal ulcer patient supernatants. Similar results were also observed in Tox^+ isolates, however, there were no significant differences between patients with regard to the incidence of vacuolating cytotoxin-negative isolates. Although our data may not indicate a clear correlation between prevalance of vacuolating cytotoxin and clinical manifestations, they suggest that *H. pylori* harboring vacuolating cytotoxin may particularly induce damage to the gastric epithelium in patients with gastric ulcers.

Introduction

Helicobacter pylori infection is closely associated with gastroduodenal diseaes including gastric and peptic ulcers[1)~3)]. Several virulence factors that may enhance inflammation and damage in mucosal epithelium have been investigated and identified[4)~9)]. Vacuolating cytotoxin, which is encoded by the *vacA* gene, induces intracellular vacuolation of eukaryotic cells *in vitro* and is closely associated with the development of peptic ulcers. While most *H. pylori* isolates contain a gene that hybridizes with *vacA* probes, approximately 50% of *H. pylori* isolates fail to produce detectable vacuolating cytotoxin activity *in vitro*[10)~12)]. Differences in cytotoxic activity have been ascribed to sequence divergence in *vacA* gene. In order to clarify the relationship beteeen the *in vitro* activity of vacuolating cytotoxin and development of peptic ulcers, we investigated the role of vacuolating cytotoxin by MTT assay using cultured rabbit gastric epithelial cells.

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Methods

Strains

Strain types NCTC11673, 11916 and 11639 were used in this study. NCTC11637 is a known vacuolating cytotoxin-producing strain (Tox^+). The clinically isolated strains were obtained from 57 consecutive dyspeptic patients [41 males and 16 females; mean age, 50.9 ± 14.8 years (± SD), range 14-78 years] who were examined by gastroduodenoscopy and antral biopsy in our hospital between April 1988 to March 1995. The patients were endoscopically classified into three groups; those with gastric ulcers, duodenal ulcers or no evidence of gastric or duodenal ulcers. Patients who had received non-steroid anti-inflammatory agents for a long period of time were excluded from the study. Patients with gastritis, in whom endoscopy showed no evidence of ulceration, including ulcer scar or deformity, only showed changes in the gastric mucosa on histological examination. To obtain *H. pylori* gastric biopsy specimens were smeared onto 7% sheep blood agar plates (basic medium: heart infusion agar, BBL Microbiology Systems, Cockeyville, MD) and Belo-Horizonte medium, and cultured under microaerobic conditions in aerobic jars (Campypak System; BBL Microbiology Systems) at high humidity and 37°C for 4 days. All strains of *H. pylori* used in this study were confirmed by bacteriological identification using Gram stain and the presence of urease, catalase and oxidase. All strains were stored in sterile 10% skim milk solution at −80°C.

Preparation of H. pylori culture supernatants

Stored *H. pylori* isolates were thawed at a density of 10^7 CFU/ml and cultured in brucella broth (BBL Microbiology Systems) supplemented with 10% fetal bovine serum (FBS; Gibco, Grand Island, NY) at 37°C for 24 hours on a rotary shaker under microaerophilic conditions. The density of bacterial cells was determined by measuring the optical density of each culture medium. When the number of cultured *H. pylori* grew to more than 10^{11} CFU/ml, cells were centrifuged at 1,000 ×g, for 15 minutes and the cell-free supernatants were sterilized with a 0.45-um-pore-size filter (Millipore Products Division, Bedford, UK). These supernatants were stored at −80°C until use.

Conditions of cell culture

The experimental protocol was approved by the Ethics Review Committee for Animal Experimentation of our institution. Cultured rabbit gastric epithelial cells (RGECs) prepared by the method of Matsuoka *et al*[13] were kindly provided by Research Laboratories, Nippon Shinyaku Co.[14]. Briefly, the fetuses of JW/NIBS white rabbits were removed on day 24 of gestation. The stomachs were isolated, everted and digested with 0.2% Pronase E. The dispersed epithelial cells were seeded in 75-cm^2 tissue culture flasks and subcultured in Dulbecco's modified Eagle Medium (DMEM; GIBCO) supplemented with 20% FBS, 100 μg kanamycin, 100 units penicilin G sodium (GIBCO) and 50 units Dispase-I (GODO SHUSEI, Tokyo) per ml, in a humidified atomosphere of 5% CO_2 in air. At population doubling level 5, the cells were frozen at −80°C and stored.

Assay of RGEC vacuolation

The frozen cells were first thawed and cultured. At confluence, RGECs were trypsinized and distributed into 24-well plates (Becton Dickinson, Lincoin Park, NJ) at concentrations of 1×10^5 cells per ml, and allowed to adhere for 24 hours. After adherence, media was removed and replaced with 1 ml of fresh DMEM supplemented with 10% FBS containing *H. pylori* cultured supernatants to give dilutions of 10%, 20%, 30%, 40% and 50%. Uninoculated brucella broth was used as a negative control. After 24 hours of incubation at 37°C with 5% CO_2, intracellular vacuolation of RGECs was scored visually under a light microscope at a magnification of ×200.

Colorimetric MTT (tetrazolium) assay of RGECs

RGECs were seeded into 96-well microtiter plates (Nunc A/S, Roskilde, Denmark) at a concentra-

tion of 5×10^3 cells per well and cultured at 37°C for 24 hours. After removal of the media, 100 μl of fresh DMEM-10% FBS containing 20 μl of *H. pylori* culture supernatant was added to each well. After 24 hours of incubation at 37°C, MTT [3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H tetrazolium bromide, Wako Junyaku Co., Osaka, Japan] assay was performed according to the method of Mossmann[15] with some modifications. Instead of acid-isopropanol, 150 μl of dimethyl sulfoxide (DMSO) was added to the wells to dissolve MTT formazan products. After 15 minutes of dissolution at room temperature, the optical density of each well was measured by multi-well scanning spectrophotometers at a test wavelength of 540 nm. Initial experiments confirmed that *H. pylori*-free brucella broth did not have any cytotoxic effects on RGECs at a concentration of 20% using MTT assay (data not shown). Therefore, this medium was used as a control in every plate. The net optical density (NOD) of each experimental well was determined by subtracting the background optical density of the cell-free well incubated in medium alone and measured in the same manner. All assays were performed in quadruplicate. The index of cytotoxicity of each experimental supernatant was expressed as a percentage calculated by the following equation:

Percent NOD (%)=[NOD sample/NOD control brucella broth]×100

Measurement of ammonia concentration in H. pylori supernatants

The concentrations of ammonia in the supernatants of three control strains and 20 clinical isolates were determined by a modification of the method of Okuda *et al*[16]. The concentration was expressed in mM.

Statistical analysis

The frequency of the expression of vacuolating cytotoxin activity in *H. pylori* clinical isolates was compared in patients with gastritis, gastric and duodenal ulcers by the chi-squared test, and the statistical significance was set at $p<0.05$. The results of MTT assay were expressed as mean ± SEM, and compared by Fisher's PLSD test.

Results

In vitro activity of vacuolating cytotoxin against various cultured cells

Since the activity of vacuolating cytotoxin varies between different cell lines[17], we tested the sensitivity of cells to cytotoxin activity using three different cell lines, HeLa cells, RGM1 rat gastric mucosal cells, and RGECs, in a series of preliminary experiments. The supernatant of NCTC11637 (Tox^+ strain) was centrifuged at 3,000 ×g for 50 min to a 10-fold concentration by using Centriplus-100 concentrator (Amicon, Inc., Beverly, MA). This process induced vacuolation of HeLa cells in the presence of a half volume of concentrated supernatant in the culture medium. However, there was no visible vacuolation of RGM1 cells under the same conditions. Unconcentrated culture supernatants of strain NCTC11637 induced intracellular vacuolation of the RGECs even in the presence of a 10% volume of unconcentrated supernatant in the culture medium. Another *H. pylori* strain, NCTC11916, induced vacuolation of RGECs under the same conditions. However, RGECs did not exhibit vacuolation even in the presence of a half volume of concentrated NCTC11639 culture supernatant. Thus, RGECs were considered to be suitable for the assay of cell vacuolation induced by cytotoxin. Figure 1 shows the percentages of RGECs with vacuole formation in the presence of different *H. pylori* isolate supernatants. Tox^- strains did not show apparent intracellular vacuolation of RGECs even in the presence of a half volume of 10-fold concentrated supernaton in the medium. Therefore, intracellular vacuolation was considered positive when more than 20% of cells exhibited vacuolation in the presence of a hlaf volume of unconcentrated *H. pylori* culture supernatant in RGECs culture medium.

Table 1 shows the frequency of *H. pylori* harboring vacuolating cytotoxin in 57 clinical isolates. Although 36 of these clinical isolates (63%) produced intracellular vacuolation of RGECs, there were

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Fig. 1 Percentage of RGECs with intracellular vacuolation produced by *H. pylori* isolate supernatants.
After 24 hours of incubation with the *H. pylori* isolate supernatants at a concentration of 50%, intracellular vacuolation of RGECs was scored visually under a light microscope. A positive cytotoxic effect was considered when wells contained more than 20% of vacuolated cells. Twenty-one isolates had no vacuolating cytotoxic activity (Tox^-) while 36 isolates were considered Tox^+ isolates.

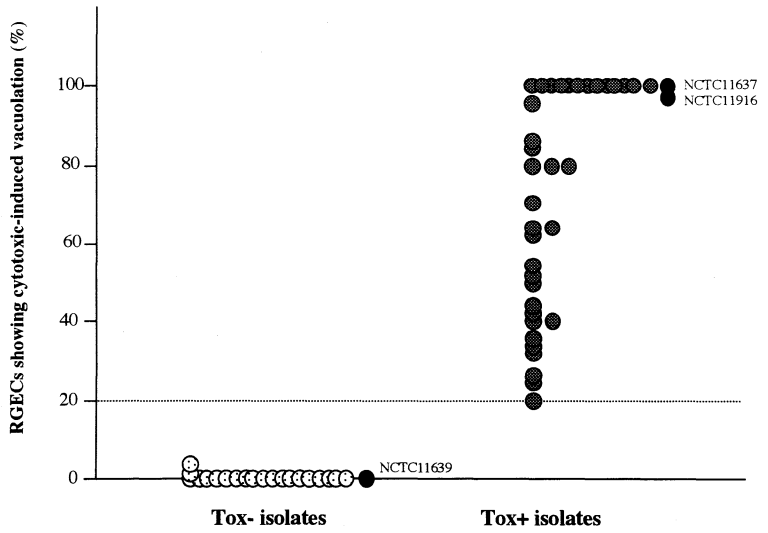


Table 1 Number (percentage) of *H. pylori* isolates producing vacuolating cytotoxin among 57 clinical isolates

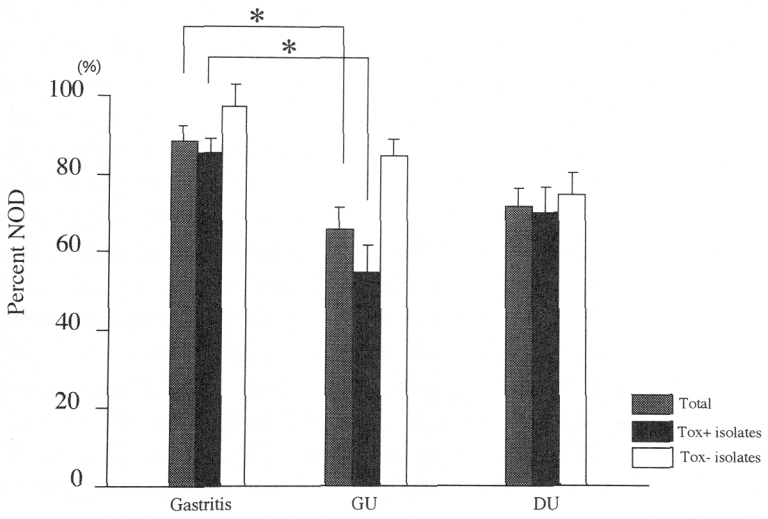
Diagnosis	No. of patients (M : F)	Age (mean±SD)	Number of Tox^+ isolates (M : F) : %
Gastritis	15(7 : 8)	52.2±14.3	11(6 : 5) : 73
Gastric ulcer	21(16 : 5)	52.6±12.0	13(10 : 3) : 62
Duodenal ulcer	21(18 : 3)	47.7±18.0	12(10 : 2) : 57
Total	57(41 : 16)	50.9±14.8	36(26 : 10) : 63

no significant differences among the three groups in the percentage of Tox^+ isolates. There was also no significant difference in the frequency of Tox^+ isolates between male and female. The percentages of RGECs showing vacuolation induced by the supernatants of Tox^+ isolates obtained from gastritis, GU and DU, were 60 ± 29%, 75 ± 23% and 73 ± 30%, respectively. Although Tox^+ isolates obtained from patients with peptic ulceration tended to be more cytotoxic than those with gastritis, there was no significant difference in vacuolyzing activity among the three groups (Fisher's PLSD test).

Cytotoxic activity of H. pylori supernatants against RGECs

Figure 2 shows the cytotoxic activities of supernatants of *H. pylori* against RGECs determined by MTT assay. The mean percent NOD of supernatants from patients with gastric ulcer (66 ± 6.6) was significantly lower than in patients with gastritis (90 ± 3.8; $p<0.01$), but there was no significant difference between isolates from patients with duodenal ulcer (72 ± 5.0) and isolats from gastritis

Fig. 2 Cytotoxic effect of supernatants of *H. pylori* on RGECs (MTT assay). The cytotoxic effect of each supernatant on RGECs was evaluated as the percent net optical density (NOD) of control brucella broth at a concentration of 20% in the medium. Clinical isolates were classified into three groups according to endoscopic diagnosis and were further divided into two groups by the expression of the vacuolating cytotoxic activity. *Solid bars*: Tox^+ isolates, *open bars*: Tox^- isolates. Each value represents mean ± SEM (%), *P<0.01.



patients. In Tox^+ isolates, similar results were also seen among the three disease groups (GU 55 ± 8.7, Gastritis 87 ± 4.3, p<0.01, DU 70 ± 6.8), whereas in Tox^- isolates, a similar trend among the three disease groups was seen, but with no significant differences (GU 85 ± 5.8, DU 75 ± 7.7, Gastritis 98 ± 7.5).

Effects of ammonia concentration produced by H. pylori on cellular cytotoxicity

Urease produced by *H. pylori* may play an important role in survival of the organism in acidic pH. Ammonia is catalytically synthesized by urease at low urea concentrations and may be cytotoxic to gastric epithelial cells and/or enhance neutrophillic-dependent gastric mucosal cell injury[7]. Therefore, we evaluated the effect of ammonia produced by *H. pylori* on cellular injury *in vitro*.

The concentration of ammonia in *H. pylori*-free brucella broth was 32×10^{-2} mM. the concentrations of ammonia in supernatants of NCTC11637, 11639 and 11916 cells were 1.30 mM, 1.25 mM, and 1.49 mM, respectively. The mean ammonia concentration of supernatants obtained from 10 isolates with vacuolating cytotoxin activity (1.15 ± 0.24 mM) was not different from that of 10 isolates without vacuolating cytotoxin activity (1.12 ± 0.26 mM, p=0.79, Student's *t*-test). To examine the cytotoxic effect of ammonia on RGECs, the cells were incubated for 24 hours in a medium containing various concentrations of ammonium sulfate (range, 10 to 100 mM). The minimal concentration of ammonum sulfate that caused visually-evident vaculoes in RGECs was 20 mM while a cytotoxic effect against RGECs was noted at concentrations exceeding 30 mM. Since the concentration of ammonia in conventional culture supernatants were too low to induce direct cytotoxicity, the vacuolating cytotoxin rather than ammonia might play an important role in mediating bacterial cytotoxicity against mucosal cell injury.

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Discussion

The results of several studies examining the correlation between virulence factors and severity of *H. pylori* infection have shown that the vacuolating cytotoxin is one of the most likely factors causing peptic ulcer.

Several groups have reported the molecular cloning of the *vacA* gene[18)~20)], and all *H. pylori* isolates appear to possess the *vacA* gene. However, approximately 50% of *H. pylori* isolates fail to produce detectable vacuolating cytotoxin activity *in vitro*. Genetic analysis of the *vacA* gene showed a similarity between the vacuolating cytotoxin and the IgA protease system, and the mechanism of extracellular release of an 87 kDa protein into the supernatant was postulated by Schmitt and Haas[19)]. The diversity of vacuolating cytotoxin activity is related to sequences in the middle region of *vacA* genes[21)]. Telford *et al.*[20)] demonstrated ulceration of the gastric epithelium following oral administration of a purified vacuolating cytotoxin in mice, and showed that toxin-induced epithelial injury was due to a direct cytotoxic effect rather than an indirect inflammatory process.

In the present study, 63% of clinical isolates showed vacuolating cytotoxin activity, a similar to that found in other studies[8,12,17)]. Although the frequency of isolates with vacuolating cytotoxin activity was almost equal among patients with gastritis, duodenal and gastric ulcers, vacuolating cytotoxic activity was different in each *H. pylori* supernatant. The RGEC vacuolation assay was insufficient to quantify the activity of vacuolating cytotoxin or the cytotoxity of *H. pylori* supernatant. Therefore we used the MTT asay to quantify the cytotoxity of *H. pylori* supernatants. The results of the MTT assay showed that isolates obtained from patients with gastric ulceration were more cytotoxic than those from patients with gastritis. In addition, Tox^+ organisms had a higher cytotoxic activity than Tox^- isolates in MTT assay. As well as the vacuolating cytotoxin, it is possible that *H. pylori* culture supernatants may contain other cytotoxic compounds that cause *in vitro* cellular damage. Ammonia is one such compound that may cause epithelial irritation and damage. However, our results showed that the mean concentration of ammonia was low in the three disease, suggesting that ammonia is less likely to be an important virulence factor. On the other hand, three vacuolating cytotoxin non-producing isolates obtained from patients with duodenal ulcers showed low NOD values in the MTT assay, which influenced the mean percent of NOD in Tox^- isolates (DU 75 ± 7.7, GU 85 ± 5.8, Gastritis 98 ± 7.5). We postulate that other as yet unknown substances which may exert cytotoxic effects on RGECs might have contaminated these supernatants.

The activity of the vacuolating cytotoxin is neutralized by IgG antibodies which are detectable in sera of Tox^+ *H. pylori*-infected subjects. These results suggest that the vacuolating cytotoxin may play a direct role not only in cell damage but also in gastric acid secretion or in immunological response *in vivo*. Furthermore, the results also suggested that strains with a highly active vacuolating cytotoxin might cause severe damage to the gastric epithelium and ultimately result in ulceration. Further studies of molecular and biochemical functions of the vacuolating cytotoxin and host response are necessary to understand the pathogenic role of *H. pylori*.

In summary, we have demonstrated the usefulness of RGECs for cytotoxic assay due to their high sensitivity to the vacuolating cytotoxin of *H. pylori*. Our results also showed that the MTT assay is a simple and easy method for quantitation of the cytotoxity of *H. pylori* supernatants on cultured cells. Our data suggest that isolates obtained from patients with gastric ulcers are more cytotoxic than those from patiens with gastritis and that the vacuolating cytotoxin is an important virulence factor that may induce ulceration of gastric epithelium *in vivo*.

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Helicobacter pylori 臨床分離株の持つ空胞化毒素の産生と細胞障害性についての検討

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要　旨

Helicobacter pylori の病原因子のひとつに，*in vitro* で上皮細胞に空胞化変性を引き起こす，*vacA* 遺伝子にコードされた細胞外分泌蛋白（空胞化毒素）がある．この毒素の活性は全ての菌株には存在せず，この毒素を有する菌株と消化性潰瘍との関連が指摘されてきた．空胞化毒素と消化性胃潰瘍の発生との関連について，胃粘膜上皮細胞への障害性を検討することによって明らかにすることを本研究の目的とした．

H. pylori 感染を細菌学的，組織学的に証明した慢性胃炎患者（＝15），胃潰瘍患者（n＝21），十二指腸潰瘍患者（n＝21）から得られた *H. pylori* 臨床分離株57株の培養上清を用いて，ウサギ胃粘膜上皮細胞の空胞化の有無を検討し，MTT 法による細胞障害度の定量化を試みた．

57株のうち36株（63%）の培養上清が空胞化毒素活性を有していたが，3疾患群での空胞化毒素陽性率に有意差は認められなかった（慢性胃炎73%，胃潰瘍62%，十二指腸潰瘍57%）．一方，MTT 法では胃潰瘍患者由来株の培養上清の細胞障害活性は慢性胃炎患者由来のそれよりも有意に高かった（$p<0.01$）．空胞化毒素陽性株間でも同様の傾向が認められたが（$p<0.01$），空胞化毒素陰性株間では認められなかった．

以上の結果より，空胞化毒素は疾患特異性を規定する因子ではないが，胃潰瘍患者においてはその胃粘膜障害を担う一つの病原因子として重要な役割を果たしている可能性が示唆された．