Ammonia-induced apoptosis is accelerated at higher pH in gastric surface mucous cells

HIDEO SUZUKI,1 AKINORI YANAKA,1 TAKESHI SHIBAHARA,1 HIROFUMI MATSU1, AKIRA NAKAHARA,2 NAOMI TANAKA,3 HIROSHI MUTO,2 TAKASHI MOMOI,3 AND YASUO UCHIYAMA4
1Departments of Gastroenterology and Endoscopy, Institute of Clinical Medicine, University of Tsukuba, Ibaraki 305-8575; 2Toride Ishikai Hospital, Toride 300-0032; 3Department of Biochemistry, Juntendo University School of Medicine, Tokyo 113-8421; and 4Department of Cell Biology and Neuroscience, Osaka University Graduate School of Medicine, Osaka 565-0871, Japan

Received 12 November 2001; accepted in final form 14 June 2002

Suzuki, Hideo, Akinori Yanaka, Takeshi Shibahara, Hirofumi Matsui, Akira Nakahara, Naomi Tanaka, Hiroshi Muto, Takashi Momoi, and Yasuo Uchiyama. Ammonia-induced apoptosis is accelerated at higher pH in gastric surface mucous cells. Am J Physiol Gastrointest Liver Physiol 283: G986–G995, 2002. First published June 20, 2002; 10.1152/ajpgi.00482.2001.—Gastric luminal ammonia produced by Helicobacter pylori has been shown to cause gastric mucosal injury. This study was conducted to examine the mechanisms by which gastric luminal ammonia causes apoptosis of gastric epithelial cells. Monolayers of GSM06 cells, developed from murine gastric surface mucous cells, were cultured in the absence or presence of 10–30 mM NH4Cl at ambient pH of 5.0, 6.0, and 7.0. In the presence of luminal NH4Cl, GSM06 cells showed 1) cell shrinkage and nuclear chromatin condensation, 2) DNA fragmentation into oligonucleosomes, 3) leakage of cytochrome c into cytosolic fraction without affecting bax expression, and 4) increases in activity of caspases-3 and -9. These changes were accentuated when the cells were cultured at pH 7.0. In the absence of NH4Cl, none of these changes was detected at any pH examined. These results suggest that gastric luminal ammonia, at concentrations detected in H. pylori-infected subjects, induces apoptosis of gastric epithelial cells by release of cytochrome c from mitochondria, followed by activation of caspases-3 and -9, especially at higher ambient pH.

Helicobacter pylori; gastric epithelial cells

PROLONGED INFECTION with Helicobacter pylori causes chronic gastritis and gastric atrophy, which are closely associated with the development of gastric carcinoma (2, 9, 30). A number of studies have shown that H. pylori induces apoptosis of surface mucous cells and cells in gastric glands (7, 16, 17, 20, 22, 31, 36, 50, 52), a process that may be associated with the progression of gastric atrophy (4, 32, 45). Although several factors have been proposed with regard to H. pylori-induced apoptosis of gastric epithelial cells, the precise mechanisms by which H. pylori causes apoptosis have not been well clarified.

One of the major factors for H. pylori-induced apoptosis is ammonia. H. pylori possesses strong urease activity and produces high concentrations of ammonia within the mucous gel layer as a result of hydrolysis of urea (10, 30). H. pylori-derived ammonia causes injury in isolated human gastric epithelial cells in vitro (40). Ammonia, at concentrations detected in gastric juice in H. pylori-infected subjects, causes gastric mucosal injury (10, 33, 44, 48), retards gastric mucosal restitution (43), and induces apoptosis of gastric epithelial cells (13, 40). The mechanisms by which ammonia induces apoptosis of gastric epithelial cells remain unclear, however.

Ammonia exists as two different forms, NH3 and NH4+. NH3, a small lipophylic molecule (mol wt 17.0), is readily permeable across the phospholipid bilayer of cell membranes (18). In contrast, NH4+, a monovalent cation, does not passively diffuse cell membrane but passes through only cation channels located mainly on the basolateral cell membrane of gastric epithelial cells (3). Because ammonia has a pK of 9.0 (18), most of the ammonia exists as NH4+ within acidic gastric lumen, under physiological conditions. However, treatment of gastric mucosa with acid inhibitors increases luminal pH and thereby enhances generation of NH3 (18). Therefore, it seems reasonable to assume that gastric luminal ammonia diffuses across gastric mucosa readily at high luminal pH and thereby causes severe damage at high luminal pH (53). These considerations suggest that an increase in luminal pH by treatment with acid inhibitors may play an important role in the progression of ammonia-induced gastric injury.

A number of clinical studies suggest that acid suppression therapy by proton pump inhibitors (PPIs) exaggerates H. pylori-induced corpus gastritis (5, 24, 28, 49) and that long-term treatment with PPIs accelerates progression of gastric atrophy in patients with H. pylori infection (23, 25). We have also shown that prolonged acid inhibition by long-term use of PPI ex-

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Address for reprint requests and other correspondence: A. Yanaka, Depts. of Gastroenterology and Endoscopy, Institute of Clinical Medicine, Univ. of Tsukuba, 1-1-1 Tennodai, Tsukuba, Ibaraki 305-8575, Japan (E-mail: ynk-aki@md.tsukuba.ac.jp).
aggregatepray corpus gastritis and enhances apoptosis of gastric corpus mucosa in *H. pylori*-infected mice in vivo (54). However, the issue of how or why PPIs exaggerate corpus gastritis in *H. pylori*-infected gastric mucosa has not been studied in detail.

On the basis of these background studies, we hypothesized that high luminal pH caused as a result of acid suppression therapy may enhance conversion of NH$_4^+$ to NH$_3$ at the apical surface of gastric epithelial cells, thereby accelerating diffusion of luminal ammonia into the mucosa and accelerating the apoptotic pathway of the gastric epithelial cells.

In the present study, we examined the effects of ammonia on the viability of gastric surface mucous cells at different luminal pH. In addition, we also analyzed mechanisms by which ammonia induces death of gastric epithelial cells using morphological and biochemical methods.

**MATERIALS AND METHODS**

**Cell Culture**

GSM06 cells, a gastric surface mucous cell line derived from transgenic mice harboring the simian virus 40 (SV40) large T-antigen gene (41), were cultured in DMEM (Dulbecco) supplemented with 10% fetal bovine serum, 1% insulin-transferrin-selenium-X (GIBCO), and 10 ng/ml epidermal growth factor (Wako Pure Chemical Industries, Osaka, Japan) until the cells established confluency. The incubation medium was replaced with a fresh solution at 3-day intervals. GSM06 cells were cultured in Earle’s buffered medium, containing (in mM) 1.8 CaCl$_2$, 5.4 KCl, 0.8 MgSO$_4$, 116 NaCl, 26.2 NaHCO$_3$, 1 NaH$_2$PO$_4$-H$_2$O, and 5.55 glucose as the standard medium. pH in the incubation medium was adjusted to 5.0, 6.0, or 7.0. The cells were incubated in the presence or absence of 10 or 30 mM NH$_4$Cl at different ambient pH.

**Electrophysiological Analysis**

Functional characteristics of GSM06 monolayers were evaluated by electrophysiological analysis. GSM06 cells were cultured on permeable supports (cell culture inserts 3092) (Falcon) for 7 days until confluence was established. The GSM06 monolayers were then incubated in lutein Ussing chambers in vitro. The serosal and the luminal sides were bathed with HEPES-Ringer-100% O$_2$ (pH 7.4) and with 150 mM NaCl (pH 7.4), respectively. Transmucosal electrical resistance was measured as an index of the gastric mucosal barrier function. Changes in electrical resistance during exposure to graded doses of HCl (pH 7.4–2.5) or NH$_4$Cl (1–100 mM) were examined. HCl or NH$_4$Cl was added to either the luminal or the serosal solution.

**Morphological Analysis by Electron Microscopy**

Some cells incubated under the experimental conditions were fixed for electron microscopy with the buffer containing 1.25% glutaraldehyde, 2.5% paraformaldehyde, 0.06% picric acid, and 0.06% CaCl$_2$ with 0.1 M cacodylate buffer (pH 7.2) for 5 min on ice. They were then postfixed with 1% OsO$_4$ buffered with 0.1 M cacodylate buffer (pH 7.2) for 5 min on ice. After dehydration with a graded series of ethanol, the samples were removed from the dishes by propylene oxide and were embedded in Epon 812 (TAAB). Thin sections, cut with an ultramicrotome (Ultracut-N, Reichert-Nissei) and stained with 1% uranyl acetate and lead citrate were observed with a Hitachi H-7100 electron microscope.

**Cell Death Assay**

To examine viability of GSM06 cells, lactate dehydrogenase (LDH) release from the cells into the medium was determined. The LDH content in the culture medium was measured by using a LDH assay kit (Kyokuto Pharmaceutical, Tokyo, Japan). Total cellular LDH content was measured after lysis with 0.1% Tween 20. The cell death rate was estimated by calculating ratios of released LDH to total (cellular and released) LDH. In some experiments, LDH release was assessed in RGM-1 cells, a cell line derived from normal rat gastric mucosal cells (19).

**Morphological Analysis by Laser Scanning Microscopy**

The cells cultured in chambered slides (Nunc) were fixed for laser scanning microscopy, with 4% paraformaldehyde buffered with phosphate buffer, containing 4% sucrose, for 15 min at room temperature (15). In addition, cells were cultured in the presence of NH$_4$Cl with or without 100 μM acetyl-Asp-Glu-Val-Asp-CHO (Ac-DEVD-CHO), an inhibitor of caspase-3-like proteinases. These fixed cells were thoroughly washed with PBS and treated with 0.3% H$_2$O$_2$ in methanol for 30 min. To examine caspase-3 activity in dying cells, the cells were doubly stained for activated caspase-3 and terminal deoxynucleotidyl transferase (TdT)-mediated 2′-deoxyuridine 5′-triphosphate (dUTP)-biotin nick end labeling (TUNEL) as reported previously (15, 34). Briefly, the cells were stained with a site-specific antibody against caspase-3 for 24 h at 4°C, which recognizes the carboxy terminus of the cleaved site of p20/17 but not the proform (8, 15, 21), and then incubated with FITC-conjugated goat anti-rabbit IgG for 1 h at room temperature. To detect nuclear DNA fragmentation, the cells were incubated with 100 U/ml TdT and 10 nmol/ml biotinylated 16-2′-dUTP (Boehringer-Mannheim-Yamanouchi, Osaka, Japan) in TdT buffer (100 mM sodium cacodylate, pH 7.0, 1 mM cobalt chloride, 50 μg/ml gelatin) in a humid atmosphere at 37°C for 1 h. Further incubation with Texas red-conjugated avidin (Nichirei) was carried out for 30 min at room temperature. They were then viewed with a confocal laser scanning microscope (LSM-GB 200, Olympus, Tokyo, Japan).

**Biochemical Analysis**

To examine the molecular mechanism for ammonia-induced death of GSM06 cells, the cells cultured under various experimental conditions were biochemically analyzed. Fragmentation of DNA into oligonucleosomes in the treated cells was examined by electrophoresis of genomic DNA. Genomic DNA obtained from the cells was prepared for electrophoresis by a modification of the method described originally by Sambrook et al. (37). Each sample was subjected to electrophoresis on a 2% agarose gel and was visualized under ultraviolet light after staining with 250 ng/ml ethidium bromide.

To assess the potential involvement of the caspase family of proteinases, the activation of caspases-3 and -9 was determined by detecting their cleaved products by immunoblotting. The cells were lysed with a buffer containing 150 mM NaCl, 50 mM Tris, and 1% Triton X-100, including a proteinase inhibitor cocktail (Boehringer Mannheim). After being centrifuged twice at 15,000 g for 10 min at 4°C, the supernatants were measured for protein concentrations using the bicinchoninic acid protein assay system (Pierce, IL), and immunoblotting was performed. Each sample was separated...
by tricine SDS-PAGE (38) in 15% (wt/vol) acrylamide. Electrophoretic transfer of proteins from polyacrylamide gels to a polyvinylidene difluoride membrane (Immobilon-P; Millipore, Tokyo, Japan) was performed according to the method described by Towbin et al. (46). The sheets were soaked in PBS containing 5% bovine serum albumin (Sigma Chemical) to block nonspecific binding and then incubated with anticaspase-9 (MBL, Nagoya, Japan) or anti-caspase-3 (anti-p20/17). Immunodetection was carried out with a chemiluminescent enhanced chemiluminescence kit (Amersham, Pharmacia Biotech, Piscataway, NJ) according to the manufacturer's recommended protocol.

Proteolytic activity of caspase-3-like proteinases was also examined in cells cultured under various experimental conditions, using the method described elsewhere (6). Briefly, cytosolic extracts of the cells were prepared by repeated freezing and thawing of cells in 100 μl of extraction buffer (50 mM PIPES-NaOH, pH 7.0, 50 mM KCl, 5 mM EGTA, 2 mM MgCl₂, 1 mM DTT, 20 μM cytochalasin B, 1 mM PMSF, 1 μg/ml leupeptin, 1 μg/ml pepstatin A, 50 μg/ml antipain, and 10 μg/ml chymopain) as described. Cell lysates were then diluted with 0.5 ml IL-1β-converting enzyme standard buffer (100 mM HEPES-KOH buffer, pH 7.5, 10% sucrose, 0.1% 3-(3-cholamidopropyl)dimethylammonio)-1-propanesulfonate, 10 mM DTT, and 0.1 mg/ml ovalbumin) and were incubated at 30°C for 30 min with 1 μM of the fluorescent substrate (7-methoxycoumarin-4-yl)acetyl-Asp-Glu-Val-Asp-Ala-Pro-Lys(Dnp)-NH₂ (MOCAc-DEVDAPK) (The Peptide Institute, Osaka, Japan). Specific caspase-3-like activity was determined by subtracting the values obtained in the presence of 1 μM Ac-DEVD-CHO and expressed as optical density (OD) (× 10⁶) per milligram protein.

The involvement of cytochrome c and bax in the cell death cascade was also examined in GSM06 cells under various experimental conditions. The cells were rinsed with PBS after removal of medium, scraped from dishes, and centrifuged at 1,200 g for 5 min at 4°C. Pelleted cells were homogenized in 0.25 M sucrose by passing them six times through a 27-gauge needle at 4°C and then centrifuged at 1,200 g for 5 min at 4°C. The supernatant was referred to as postnuclear supernatant. After the postnuclear supernatants were centrifuged at 20,000 g for 30 min, the supernatants were further centrifuged at 100,000 g for 1 h, and their supernatants were used as the cytosolic fractions. This fraction was then incubated with the lysis buffer and subjected to SDS-PAGE and immunoblotting. Immunostaining was performed using anti-bax and anti-cytochrome c antibody (PharMingen, San Diego, CA) and detected using the method described above.

RESULTS

Electrophysiological Features of GSM06 Cell Monolayers

Effects of luminal or serosal acidification on electrical resistance. In this series of experiments, serosal or luminal acidification was induced by a stepwise decrease in the serosal or the luminal pH, respectively. During the serosal or the luminal acidification, the luminal or the serosal pH was kept constant at 7.4 throughout the experiment, respectively. Electrical resistance at each pH value was measured after the cells were exposed to the pH for 10 min.

During serosal acidification, electrical resistance significantly decreased in parallel with the decrease in the serosal pH from 7.4 to 4.0. (Fig. 1A). In contrast, during luminal acidification, electrical resistance did not change during the decrease in the luminal pH from 7.4 to 4.0. Further reduction in the luminal pH to <3.5 caused a significant decrease in electrical resistance. These results suggest the GSM06 cell monolayers have excellent polarity as gastric epithelial cells, in terms of
the relatively impermeable nature of the apical membrane to H⁺.

**Effects of luminal or serosal NH₄Cl on electrical resistance.** In this series of experiments, serosal or luminal NH₄Cl was added by a stepwise increase in the serosal or the luminal NH₄Cl concentration ([NH₄Cl]) from 0 to 100 mM, respectively. During exposure to the serosal or the luminal NH₄Cl, the luminal or the serosal side was bathed with NH₄Cl-free HEPES-buffered Ringer solution throughout the experiment, respectively. Electrical resistance at each [NH₄Cl] value was measured after the cells were exposed to the [NH₄Cl] for 10 min.

Both the luminal and the serosal NH₄Cl, at concentrations between 3 and 100 mM, induced dose-dependent decreases in electrical resistance. The magnitude of the decrease in electrical resistance induced by the serosal NH₄Cl was significantly greater than that induced by the equivalent concentrations of the luminal NH₄Cl, indicating that serosal membrane of the GSM06 cells is relatively more permeable to ammonia than the apical membrane. These results are in concert with our previous results in intact sheets of guinea pig gastric mucosa in vitro (43).

**Morphological Features of GSM06 Cells by Electron Microscopy**

The cells cultured at pH 7.0 in the absence of NH₄Cl showed cuboidal shape with a few cytoplasmic processes. The cells possessed large irregularly shaped nuclei with one or two distinct nucleoli (Fig. 2A). The cells had some vacuolar structures and microvilli on the apical membrane. Tight junctions were observed between the cells, indicating that the GSM06 cell monolayers were polarized, as do normal gastric epithelial cells (Fig. 2A). The same cellular features were observed in the cells incubated at pH 5.0 and pH 6.0 in the absence of NH₄Cl (data not shown).

Because 3–100 mM NH₄Cl caused significant decrease in electrical resistance at pH 7.4 (Fig. 1B), we examined the effects of 10 and 30 mM NH₄Cl on morphology of GSM06 cells. Incubation of the cells with 10 mM NH₄Cl for up to 6 h did not cause dramatic changes in morphology at any pH between 5.0 and 7.0 (data not shown), suggesting that electrical resistance may be a more sensitive index than morphological alteration in response to exposure to NH₄Cl. In contrast, the cells incubated with 30 mM NH₄Cl for 3 h showed numerous vacuolar structures containing part of the cytoplasm. Tight junctions were not observed between the cells (Fig. 2B). Further incubation of the cells with NH₄Cl for 6 h induced not only cell shrinkage but also electron-dense materials and myelin-like figures in the cytoplasm. More than 30% of the cells demonstrated typical morphological configuration of apoptotic cells, characterized by pyknosis with margination and condensation of nuclear chromatin and shrinkage of cytoplasm especially when the cells were incubated at pH 7.0 (Fig. 2C), suggesting that GSM06 cells undergo apoptosis in the presence of NH₄Cl at neutral ambient pH. These features were rarely observed in the cells incubated for 6 h at lower pH even in the presence of NH₄Cl (data not shown).

**Effects of NH₄Cl on Cell Viability**

Incubation of the GSM06 cells in the presence of 30 mM NH₄Cl for 6 h increased LDH release significantly in a dose-dependent (10–30 mM) and pH-dependent (pH 5.0–7.0) manner (Fig. 3A). Incubation of the cells for 6 h did not increase LDH release at any pH (Fig. 3A).

Incubation of the RGM-1 cells with 30 mM NH₄Cl for 6 h also caused a significant increase in LDH release, in a dose-dependent (10–30 mM) and pH-dependent (pH 5.0–7.0) manner (Fig. 3B).

**Morphological Features of GSM06 Cells by Laser Scanning Microscopy**

To examine the ultrastructural features of the dying cells, the GSM06 cells cultured in the presence of 30 mM NH₄Cl for 6 h were doubly stained with TUNEL and an anti-activated form of caspase-3, which recognizes the carboxy terminus of cleaved caspase-3 (p20/17) (15). The GSM06 cells cultured in the absence of NH₄Cl did not show any cells stained positively with TUNEL and activated caspase-3 at all pHs examined (Fig. 4, A–C). In contrast, positive staining for both
TUNEL (Texas red labeling) and activated caspase-3 (FITC labeling) were detected in the cells cultured in the presence of NH₄Cl at pH of 5.0, 6.0, and 7.0 (Fig. 4, D–F). The number of the doubly stained cells increased significantly in parallel with the elevation of ambient pH (Fig. 4, D–F). The double staining of the GSM06 cells with TUNEL and activated caspase-3 induced by NH₄Cl was completely abolished in the presence of a specific inhibitor of caspase-3, Ac-DEVD-CHO (Fig. 4, G–I), indicating that activation of caspase-3 is involved in this dying process.

Biochemical Analysis of GSM06 Cells Cultured in the Presence of NH₄Cl

Effects of NH₄Cl on electrophoresis of genomic DNA. To further confirm nuclear alterations in GSM06 cells during incubation with NH₄Cl, DNA fragmentation into oligonucleosomes was analyzed by electrophoresis. Genomic DNAs obtained from the cells at 6 h after addition of NH₄Cl clearly showed clear formation of a ladder at pH 7.0, less so at pH 6.0, and very faintly at pH 5.0 (Fig. 5). No fragmentation was observed in genomic DNAs obtained from the cells cultured in the absence of NH₄Cl (Fig. 5). These results suggest that death of GSM06 cells induced by NH₄Cl was apoptotic and that ammonia-induced apoptosis occurs more favorably at higher ambient pH.

Effects of NH₄Cl on immunoblotting of caspase-3 and caspase-9. Because the active form of caspase-3 was immunopositive in GSM06 cells incubated in the presence of NH₄Cl, the caspase cascade was further examined by immunoblotting. Activated forms of caspases-9 and -3 were detected in the extracts of GSM06 cells, which had been incubated for 6 h in the presence of NH₄Cl at any pHs (Fig. 6). The amounts of proteins of caspase-9 and caspase-3 increased significantly as the elevation of ambient pH (Fig. 6). In contrast, the activated forms of caspases-9 and -3 were not detected at any pHs, when the cells were cultured in the absence of NH₄Cl for 6 h.

Effects of NH₄Cl on DEVDase activity. DEVDase (caspase-3-like proteinase) activity was measured in the cells cultured with or without 30 mM NH₄Cl. The DEVDase activity was low in the cells cultured in the absence of NH₄Cl, whereas it showed a pH-dependent increase when 30 mM NH₄Cl was present in the culture media (Fig. 7).

Effects of NH₄Cl on cytochrome c release and expression of bax protein. Because the activation of caspase-9 was involved in the ammonia-dependent apoptosis of GSM06 cells, the next series of experiments was conducted to examine if ammonia induces cytochrome c release from mitochondria into the cytosol and to determine if bax protein is involved in the ammonia-induced release of mitochondrial cytochrome c.

Incubation of the GSM06 cells with 30 mM NH₄Cl at pH 7.0 induced release of cytochrome c from mitochondria to cytosol (Fig. 8A). In addition, the NH₄Cl-induced release of cytochrome c increased over time for 6 h after addition of NH₄Cl, without altering expression of bax protein (Fig. 8B). These results support the contention that ammonia-dependent apoptosis of GSM06 cells is mediated by the release of cytochrome c from mitochondria, followed by the activation of caspases-9 and -3, and further suggest that bax is not involved in the upstream initiation of the ammonia-induced cytochrome c release.

DISCUSSION

The present study demonstrates that NH₄Cl decreases viability of GSM06 cells, especially at high ambient pH. Morphological examination shows that
many of the cells incubated with NH₄Cl underwent shrinkage and condensation of nuclear chromatin. Biochemical analysis revealed that NH₄Cl induces release of cytochrome c from mitochondria into the cytosol, activation of caspase cascade, caspases-9 and -3, and fragmentation of genomic DNA into oligonucleosomes. Dual staining of TUNEL and activated caspase-3 using a cleavage site-directed antibody against caspase-3 (8, 15, 21) revealed that DNA fragmentation in the cells was associated with the activation of caspase-3. In addition to TUNEL staining, DNA fragmentation into oligonucleosomes was also confirmed by electrophoresis. These data strongly suggest that NH₄Cl-induced apoptosis of GSM06 cells is mediated by cytochrome c and caspases-9 and -3.

It has been well known that release of cytochrome c triggers activation of caspase 9, leading to activation of caspase-3, and eventually causes apoptosis in a variety of conditions (12, 26, 39). Cytochrome c released from mitochondria, together with dATP, has been shown to bind Apaf-1, which resembles CED-4 in Caenorhabditis elegans and recruits procaspase-9, which is autoactivated (26). The activation of caspase-3 activates caspase-activated DNase (CAD) by cleaving an inhibitor of CAD, ICAD, resulting in DNA fragmentation into oligonucleosomes in nuclei (27).

Recent studies in human gastric mucosa have shown that bax protein is involved in H. pylori-induced apoptosis of gastric epithelial cells (20). Because bax protein has been shown to increase mitochondrial membrane permeability and to enhance release of cytochrome c...
It is well known that \textit{H. pylori} has strong urease activity and produces high concentrations of ammonia, which diffuses into the gastric lumen (47). Concentrations of ammonia in the gastric juice of \textit{H. pylori}-infected subjects have been shown to reach 30 mM (47). Because \textit{H. pylori} resides largely beneath the surface mucous layer adjacent to the gastric epithelial cells, the luminal surface of gastric epithelial cells is exposed to high concentrations of ammonia. Because the present study suggests that the GSM06 cell monolayers possess excellent functional polarity, as do normal gastric epithelial cells (Fig. 1, A and B), it seems reasonable to assume that in the present study we have added \text{NH}_4\text{Cl} on the luminal surface of the GSM06 cell monolayers. Thus we believe that the concentrations of ammonia (10–30 mM) used in the present study are clinically relevant with those in the gastric lumen in \textit{H. pylori}-infected subjects.

It has been reported that numerous apoptotic cells are detected in the gastric surface mucous epithelium and gastric glands of \textit{H. pylori}-infected subjects (31, 33). However, the mechanisms by which \textit{H. pylori} induces apoptosis of gastric epithelial cells have remained unclear. A number of studies have proposed several different mechanisms by which \textit{H. pylori} causes apoptosis of gastric epithelial cells. First, \textit{H. pylori} produces a variety of toxic substances, which may cause apoptosis of gastric epithelial cells. For example, ammonia, known to dissipate mitochondrial membrane potential and to inhibit mitochondrial energy consumption (48), causes apoptosis of gastric mucosal cells (13, 40). Monochloramine (\text{NH}_2\text{Cl}), a highly toxic substance generated as a result of the reaction of ammonia with neutrophil-derived free radicals, induces DNA fragmentation in gastric epithelial cells

(12), one would assume that bax may be involved in the ammonia-induced apoptosis of GSM06 cells. Our results, however, strongly suggest that bax is not involved in the upstream initiation of ammonia-induced cytochrome c release in the GSM06 cells (Fig. 8B), because ammonia enhanced release of cytochrome c cells over time for 6 h but did not affect bax expression throughout the experiment (Fig. 8B). Because ammonia has been shown to open mitochondrial permeability transition pore (1), which plays an important role in cytochrome c release from mitochondria (56), we assume that ammonia induces cytochrome c release presumably through enhancement of mitochondrial membrane permeability without altering bax expression. Such a case has also been reported in apoptosis induced by selenium (56) or by free radicals (11).

It has been suggested that \textit{H. pylori} causes mutation of p53 gene, which affects apoptosis of gastric epithelial cells (52, 55). Because p53 gene in GSM06 cells is inactivated by induction of SV40 large T gene, our results obtained from GSM06 cells may not be directly comparable to those of other native gastric epithelial cells, in which p53 gene is normally operative. We believe, however, that our postulated pathway for the ammonia-induced apoptosis of gastric epithelial cells is not influenced by inactivation of p53 gene in GSM06 cells, because a recent study in primary cultures of guinea pig chief cells, which are considered to have no mutation in p53 gene, has also shown that ammonia induces apoptosis of chief cells by enhancing cytochrome c release, followed by activation of caspase-9 and caspase-3 (51), which is exactly the same pathway as we proposed in the present study.

\textbf{Fig. 7.} Effects of \text{NH}_4\text{Cl} on caspase-3-like activity. The caspase-3-like activity was measured using a fluorescent substrate, (7-methoxyxoumarin-4-yl)acetyl-Asp-Glu-Val-Asp-Ala-Pro-Lys(Dnp)-\text{NH}_2 (MOCAc-DEVDAKP). \text{NH}_4\text{Cl} increased DEVDase (caspase-3-like) activity in GSM06 cells in a concentration-dependent and pH-dependent manner. In particular, the activity was highest in cells cultured in the presence of either 10 or 30 mM \text{NH}_4\text{Cl} at ambient pH 7.0. In contrast, no activity was shown in the cells cultured without \text{NH}_4\text{Cl}. Data are expressed as means ± SD. *\textit{P} < 0.05 and \textit{bP} < 0.05, significant difference from the corresponding values at pH 5.0 and at pH 6.0, respectively. *\textit{P} < 0.001, significant difference from the corresponding values in the presence of 10 mM \text{NH}_4\text{Cl}.

\textbf{Fig. 8.} Effects of \text{NH}_4\text{Cl} on cytochrome c release and bax expression. \textit{A}: effects of \text{NH}_4\text{Cl} on cytochrome c release from mitochondria (Mt) to cytosol. \textit{B}: changes in bax expression and cytochrome c release after addition of \text{NH}_4\text{Cl}. Postnuclear supernatant (PNS) prepared from the GSM06 cells was separated into cytosolic fractions by centrifugation at 100,000 g for 1 h. The immunoblot demonstrated that cytochrome c was released from mitochondria to cytosol at 6 h after addition of \text{NH}_4\text{Cl} at pH 7.0 (\textit{A}). Incubation of the cells with 30 mM \text{NH}_4\text{Cl} at pH 7.0 increased release of cytochrome c over time for 6 h but did not affect bax expression throughout the experiment (\textit{B}).
H. pylori urease itself has been shown to induce apoptosis of gastric epithelial cells, which depend on the expression of class II major histocompatibility complex molecules by the target cells (7). H. pylori also produces other substances such as vacuolating cytotoxin (vac A) and lipopolysaccharide, which cause apoptosis of gastric epithelial cells (17, 22). In addition to these H. pylori-derived substances, H. pylori induces inflammatory responses in host gastric mucosa, which also modulate apoptosis in gastric epithelial cells. For instance, H. pylori infection upregulates expression of inducible nitric oxide synthase (29), IL-1β/H11001, and TNF-α (35), which enhances Fas-mediated apoptosis in host gastric mucosa (14, 36), all of which have been thought to play important roles in H. pylori-induced apoptosis of gastric epithelial cells in vivo.

The present study demonstrates that high ambient pH accelerates ammonia-induced death of both GSM06 cells and RGM-1 cells. Because the elevation of luminal pH accelerates ammonia-induced death of both GSM06 and RGM-1 cells and because the elevation of luminal pH enhances conversion of NH4⁺ to NH3 (18), it seems reasonable to assume that at higher ambient pH relatively high concentrations of NH3 diffuse the cell membrane more readily and enhance cytochrome c release from mitochondria, thereby accelerating the apoptotic pathway.

In addition to the present findings, our preliminary results in human gastric mucosal biopsies suggest that ammonia-induced apoptosis of human gastric mucosal cells is also enhanced at high ambient pH (data not shown). Furthermore, our previous studies in intact sheets of bullfrog gastric mucosa in vitro have shown that gastric luminal ammonia decreases gastric mucosal potential difference and resistance only at high luminal pH (53). The present results are in agreement with the findings by others. For example, ammonia accelerates cell death in rabbit isolated gastric glands in vitro at high ambient pH (13). Oral administration of ammonia causes severe damage in rat gastric mucosa only when the luminal pH was maintained at high values (48). Taken together, we believe that the pH dependency of the effects of ammonia on cell death is not a specific finding of GSM06 cells but rather is a common feature of different cell types.

We believe that the present results are relevant with the clinical observations that long-term use of PPI exaggerates corpus gastritis and accelerates gastric mucosal atrophy in patients with H. pylori infection (5, 23–25, 28, 49). Although no studies have directly shown that acid suppression treatment enhances apoptosis of H. pylori-infected human gastric mucosa, we have previously shown that prolonged acid inhibition by long-term use of PPI enhances apoptosis of gastric corpus mucosa and accelerates progression of corpus atrophy in H. pylori-infected mice in vivo (54), suggesting that acid suppression therapy causes gastric epithelial cell apoptosis within H. pylori-infected mucosa. Because exaggeration of gastritis is accompanied by the increase in epithelial cell apoptosis in H. pylori-infected gastric mucosa (52), we believe that these clinical findings could be attributable at least in part to the enhancement of ammonia-induced apoptosis of gastric epithelial cells at high luminal pH.

In summary, the present results suggest that 1) ammonia at concentrations that are detectable in the H. pylori-infected gastric lumen causes apoptosis of GSM06 cells through release of cytochrome c from mitochondria, thereby activating the caspase cascade followed by DNA fragmentation, and 2) high ambient pH enhances ammonia-induced apoptosis of the cells, an effect that may be associated with the clinical findings that prolonged acid suppression therapy exaggerates corpus gastritis and accelerates gastric atrophy in patients with H. pylori infection.

We gratefully thank Drs. W. Silen and S. Ito (Harvard Medical School) for critical review of the manuscript. We also gratefully thank Drs. N. Sugiyama and Y. Tabuchi (Molecular Biology Research Laboratory and Exploratory Research Laboratories, Daiichi Pharmaceutical) for the kind gift of GSM06 cells and for technical advice.

This work was supported by Grant-in-Aid for Scientific Research 10670450 and by priority areas from the Ministry of Education, Science, Sports, and Culture of Japan.

REFERENCES


