Monochloramine induced DNA fragmentation in gastric cell line MKN45

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1Department of Internal Medicine, School of Medicine, Keio University, Shinjuku-ku, Tokyo 160-8582; 2Department of Pharmacology, Central Research Laboratory, Zeria Pharmaceutical Co., Ltd., Saitama 369-0100; 3Department of Gastroenterology, National Tokyo Medical Center, Meguro-ku, Tokyo 152-0021; and 4Second Department of Internal Medicine, National Defense Medical College, Saitama 359-8513, Japan

Suzuki, Hidekazu, Koichi Seto, Mikiji Mori, Masayuki Suzuki, Soichiro Miura, and Hiromasa Ishii. Monochloramine induced DNA fragmentation in gastric cell line MKN45. Am. J. Physiol. 275 (Gastrointest. Liver Physiol. 38): G712–G716, 1998.—Monochloramine (NH2Cl) is known to be one of the virulence factors in the course of gastric cancer (13), the mechanism of malignant transformation remains obscure. Although colonization of the gastric mucosa by H. pylori is also reported to play an important role in the pathogenesis of gastric cancer (13), the mechanism of malignant transformation remains obscure. Oxygen free radicals derived from H. pylori-activated neutrophils are also known to be one of the virulence factors in the course of gastric mucosal injury.

Myeloperoxidase in neutrophils catalyzes the oxidation of chloride by H2O2 to yield hypochlorous acid (HClO). The interaction between H. pylori-derived NH3 and HClO produces monochloramine (NH2Cl), which is reported to be exceptionally reactive and toxic because of its high lipophilic property and low molecular weight. We previously found (18) that H. pylori directly elicited human neutrophils and then led to the gastric mucosal cell injury mediated by NH2Cl. The value of luminal-dependent chemiluminescence, which depicts the contents of HClO, an NH2Cl precursor, is increased in the H. pylori-colonized gastric mucosa (16). We have previously reported (17) that NH2Cl induced gastric cellular DNA double-strand break and chromatin condensation.

Apoptosis is a physiological suicide mechanism that preserves homeostasis, in which cell death naturally occurs during normal tissue turnover (21). In general, cells undergoing apoptosis display profound structural changes, including a rapid blebbing of the plasma membrane and nuclear disintegration. The nuclear collapse is associated with extensive damage to chromatin and DNA cleavage into oligonucleosomal length DNA fragments after activation of calcium-dependent endogenous endonuclease (3). In patients chronically infected with H. pylori, increased numbers of apoptotic epithelial cells were found along with increased numbers of proliferating epithelial cells (6, 10). Accelerated rates of proliferation may be the stimulus to increased apoptosis, or vice versa, and an imbalance between apoptosis and proliferation may explain the diverse clinical outcomes of infection, including neoplasia (19).

The present study demonstrates quantitatively the effect of NH2Cl on the level of DNA cleavage in the gastric cell line MKN45.

MATeRIAls AND METHODS

Reagents. RPMI 1640, fetal bovine serum (FBS), penicillin, streptomycin sulfate, and Hanks’ balanced salt solution (HBSS) were obtained from Gibco BRL (Rockville, MD). Sodium hypochlorite (NaClO) and NH2Cl solution were obtained from Kanto Chemical (Tokyo, Japan). Taurine was obtained from Sigma Chemical (St. Louis, MO).

Cells. In the present set of experiments, we used the gastric epithelial cell line MKN45 (Japanese Cancer Research Bank no. 0254), because this cell line is adherent enough to assess the cytoplasmic release of mono- and oligonucleosomes in the present protocol and does not constitutively express the proinflammatory chemokine interleukin-8, which may itself cause apoptosis (1), as observed in KATO III cells (2). The MKN45 cell line used for this study was obtained from Jap an Health Sciences Foundation (Osaka, Japan). Cells were maintained in RPMI 1640 containing 10% FBS, 2 mM L-glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin sulfate. The cells were rinsed with PBS and then transferred in HBSS at a...
concentration of 10^6 cells/well. Separate sets of cell cultures were pretreated with the different concentrations of taurine.

Preparation of oxidant solutions. NaClO and NH3 solution were prepared, and NH2Cl solution was produced by mixing 100 mM NaClO with 1.3-fold volume of 100 mM NH3 solution. The concentration of NH2Cl in the mixture was measured photometrically by the optical density of 242 nm. The NH2Cl concentration of these original mixtures ranged between 40 and 50 mM. With the appropriate dilution, stock solutions at 0.1, 1, and 10 mM NH2Cl were prepared as were suspensions with the comparable concentrations of NH3 or NaClO.

Cell incubation. Cells grown to confluent condition for several days were incubated with HBSS containing NH2Cl, NH3, or NaClO for 5 h. Then the supernatant of the cell cultures were sampled for the measurement of lactate dehydrogenase (LDH) activity. The cells were lysed and used for the analysis of cytoplasmic mono- and oligonucleosomes.

In separate sets of experiments, human neutrophils were isolated from the peripheral blood of one healthy volunteer with the use of standard dextran sedimentation and gradient separation on Histopaque-1077 (Sigma Chemical). This procedure yields a 95% viable (trypan blue exclusion) and 98% separation on Histoplaque-1077 (Sigma Chemical). This protocol was performed by mixing 100 mM NaClO with 1.3-fold volume of 100 mM NH3 solution.

Some fractions of isolated neutrophils were preincubated with sodium azide (NaN3; 0.3 mM), a myeloperoxidase inhibitor, to inactivate the hypochlorous anion generation by neutrophils. MKN45 cells were incubated with neutrophils (1.0 x 10^6 cells/ml) in the presence or absence of NH3 (0.0001 mM) for 5 h. To test the NH2Cl scavenger, we applied taurine (0.0001 mM) to some wells of cell mixtures. Then the cells were lysed and used for the analysis of cytoplasmic mono- and oligonucleosomes.

Assay for cytoplasmic mono- and oligonucleosomes. Apoptosis was evaluated by photometric enzyme immunoassay of cytoplasmic mono- and oligonucleosomes (histone-associated DNA fragments) determination (Boehringer Mannheim). Briefly, cells treated with several inducers were harvested, and an aliquot of the cell suspension was transferred into a streptavidin-precoated 96-well microplate. Then biotinylated anti-histone antibody, which binds to histone I, histone IIA, histone IIB, histone III, or histone IV, and peroxidase-labeled anti-DNA antibody were added. Because mitochondrial DNA is not covered by histones (14), the present value of cytoplasmic mono- and oligonucleosomes specifically addresses the damage of nuclear DNA, which is covered by histones. Two hours later, the supernatant was discarded and then incubated with substrate solutions. The absorbance (405 nm) was analyzed by a microplate reader (Bio-Rad Laboratories, Hercules, CA).

Assay for LDH activity. Plasma membrane damage, which is linked to necrosis, was evaluated by measuring the LDH activity in the cell supernatant formazan colorimetric methods (LDH-CII test; Wako Pure Chemical, Osaka, Japan) (4).

DNA electrophoresis. DNA fragmentation was assessed by DNA agarose gel electrophoresis, which was performed as described previously (15). Briefly, cells were incubated with 0.001 mM NH2Cl or HBSS alone for 5 h and harvested by using lysis buffer [50 mM Tris·HCl, pH 7.8, 10 mM EDTA-Na, and 0.5% (wt/vol) sodium N-lauroyl sarcosinate], and then cell numbers were counted. The cell suspension (2 x 10^6 cells) was pelleted by centrifugation at 500 g for 5 min and resuspended with lysis buffer. RNase A (1 mg/ml) was added in cell suspension, incubated at 50°C for 30 min, and then incubated with proteinase K (1 mg/ml) at 50°C for 30 min. Each sample was resuspended in the gel loading buffer. Then 18 µl of each sample solution were loaded to a 1.5% agarose gel and electrophoresed at 100 V for 1 h.

Statistical analysis. Statistical analysis was performed using one-way ANOVA and compared by Fisher’s multiple comparison test. *P < 0.05 was considered significant.

RESULTS

Figure 1 shows the levels of cytoplasmic mono- and oligonucleosomes under incubation with various concentrations of NH2Cl for 5 h. The levels of cytoplasmic mono- and oligonucleosomes incubated with NH2Cl were significantly increased compared with the control (0 mM). Cytoplasmic mono- and oligonucleosome levels after incubation with 0.001 and 0.01 mM NH2Cl were significantly higher than those after incubation with 0.1 mM NH2Cl.

Such an increase in the levels of cytoplasmic mono- and oligonucleosomes at 0.001 mM NH2Cl was significantly attenuated by taurine at a concentration of 0.0001 mM (Fig. 2). This suggests that the effect of NH2Cl on the cytoplasmic mono- and oligonucleosome levels was inhibited by at least a 0.3-fold greater concentration of taurine. Incubation for 5 h with NH3 as well as NaClO at a concentration of 0.001 mM did not evoke levels of cytoplasmic mono- and oligonucleosomes similar to those evoked by NH2Cl (Fig. 3), suggesting this phenomenon was mainly induced by NH2Cl and not by its substrates, NH3 and NaClO.

Figure 4A shows the DNA ladder formation after a 5-h treatment with (Fig. 4A, lane 2) and without (Fig. 4A, lane 1) 0.001 mM NH2Cl. NH2Cl enhanced the levels of DNA ladder formation (Fig. 4A, lane 2). Figure 4B depicts the DNA ladder formation of MKN45 cells under the same conditions as MKN45 cells. MKN45 cells did not evoke clear DNA ladder formation with 0.001 mM NH2Cl.

The level of LDH activity in the supernatant of MKN45 culture is shown in Fig. Although LDH activity level did not significantly increase at an NH2Cl concentration of 0.001 or 0.01 mM after 5 h of incubation, it
was significantly increased at 0.1 mM NH₃Cl. This phenomenon, together with the above-mentioned fact that mono- and oligonucleosome release was less at 0.1 mM NH₃Cl than at 0.001 or 0.01 mM (Fig. 1), suggests that NH₃Cl at a concentration of 0.1 mM had already evoked direct damage to the cell membrane and that mono- and oligonucleosomes were further released from the cytosol to the extracellular space. The incubation for 5 h with NH₃ as well as NaClO at a concentration of 0.1 mM did not evoke levels of LDH activity similar to those evoked by NH₃Cl (Fig. 6), suggesting this phenomenon was also induced by NH₃Cl, not by its substrates, NH₃ or NaClO.

Figure 7 shows the levels of cytoplasmic mono- and oligonucleosomes under incubation with neutrophils for 5 h. Although the levels of cytoplasmic mono- and oligonucleosomes were slightly but significantly increased with the addition of neutrophils even in the absence of NH₃, the values were further increased in the presence of NH₃ (0.0001 mM). Such an increase in the levels of cytoplasmic mono- and oligonucleosomes under incubation with neutrophils in the presence of NH₃ was significantly attenuated by taurine (0.0001 mM). The pretreatment of neutrophils with NaN₃ attenuated significantly such an increase in the levels of cytoplasmic mono- and oligonucleosomes under incubation with neutrophils in the presence of NH₃.

**DISCUSSION**

The present investigation demonstrated that NH₃Cl leads to DNA fragmentation of MKN45 cells (Figs.
and 4). Treatment with taurine significantly attenuated the DNA fragmentation evoked by NH$_2$Cl (Fig. 2). Neither NH$_3$ nor NaClO evoked levels of DNA fragmentation that were higher than those evoked by NH$_2$Cl (Fig. 3). The injurious effect was mainly due to the specific effect of NH$_2$Cl. The DNA fragmentation of MKN45 cells was also demonstrated by the mixture of isolated human neutrophils with NH$_3$ and attenuated by neutrophil inactivation by NaN$_3$ or treatment with taurine (Fig. 7).

We previously reported that the high level of chromatin condensation was evoked by the treatment of rabbit gastric mucosal cells or KATO III cells with 0.1 mM NH$_2$Cl (17). In that study (17), the level of chromatin condensation was significantly higher in NH$_2$Cl-treated cells than in NH$_3$- or HClO-treated cells. Although it has been estimated that activated neutrophils can generate OCl$^-$ and NH$_2$Cl in the range of 0.1–0.6 mM (11, 18), the mucosal epithelial concentration at the site distant from the surface of activated neutrophils should be <0.1 mM. In the present study, we examined the effect of lower concentrations of NH$_2$Cl (0.001 and 0.01 mM) from the viewpoint of DNA fragmentation (cytoplasmic mono- and oligonucleosomes) as well as the direct cell membrane damage (LDH activity) and obtained the results that although a lower concentration of NH$_2$Cl (0.001 and 0.01 mM) evoked DNA fragmentation without cell membrane damage, a higher concentration (0.1 mM) evoked the direct cell membrane damage compatible with necrotic cell death rather than DNA fragmentation. As shown in Fig. 4B, although DNA fragmentation was not clearly induced in KATO III cells with NH$_2$Cl, it was evoked significantly in MKN45 cells (Fig. 4A). These observations support the present selection of MKN45 for evaluating the DNA fragmentation.

Although Chen et al. (7) recently reported that H. pylori directly induced gastric epithelial apoptosis by a Bak (a Bcl-2 family protein)-dependent pathway, other independent regulators of apoptosis may also be important in gastric mucosa. Among them, recruited leukocytes as well as their products, excessive amounts of oxygen free radicals exhibited in the damaged gastric mucosa (20), can become candidates for the inducer of apoptosis. In a previous study (9), a positive correlation was found between oxygen free radical production and the H. pylori status of patients. There was even a positive association between mucosal oxygen free radical production and quantitative histological and microbiological H. pylori assessments (8). We have previously reported (17, 18) that the level of gastric cell membrane damage assessed by the cytotoxicity assay using the extracellular release of 2',7'-bis(2-carboxyethyl)-5(6)-carboxyfluorescein and its level of DNA double-strand break and chromatin condensation of KATO III cells as well as of isolated rabbit gastric
mucosal cells were higher with NH2Cl treatment than with HClO or NH3. The present study further examined the incidence of DNA cleavage by the levels of cytoplasmic mono- and oligonucleosomes and DNA ladder formation, which could be strongly related to DNA fragmentation, one of the aspects of apoptosis.

With regard to oxidative DNA damage in H. pylori-associated gastric mucosal injury, Baik et al. (5) recently reported that the 8-hydroxy-2′-deoxyguanosine content, a marker of oxidative DNA damage, of the gastric mucosal DNA of the H. pylori-positive group was 2.3-fold higher than that of the H. pylori-negative group. This finding (5) of higher levels of oxidative DNA damage in the gastric mucosa during the early phase of H. pylori infection supports the hypothesis that the oxygen radicals persistently produced in the gastric mucosa due to H. pylori infection are the driving force that transforms the chronic gastritis ultimately into gastric cancer. If cell death is the major effect of oxygen radicals on the rapidly proliferating gastric epithelial stem cells, the extensive gastric atrophy would be encountered. Mutation on the DNA in the stem cells induced by the challenge of oxygen free radicals might lead to intestinal metaplasia, dysplasia, and neoplasia in the long term.

In conclusion, NH2Cl originating from H. pylori-infected gastric mucosa remarkably induces DNA fragmentation, which is comparable to apoptosis, at its pathophysiological concentration (0.001–0.01 mM) in gastric epithelial cells, suggesting the possible involvement of NH2Cl in gastric epithelial apoptosis.

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