— Regular Article —

Low-molecular Weight Amines Have Marked Potential to Promote Colitis in a Mouse Experimental Model: A Possible Proposal of *in vivo* Formation of Their Chloramines

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We have reported that methylamine dichloramine (CH_3NCl_2) causes colitis in mice and that in addition to its oxidative potentials, its cell membrane permeability is important for the onset of ulcerative colitis (UC). The aim of the present study was to determine if CH_3NH_2, a typical low-molecular weight biological amine, aggravates experimental UC in mice through *in vivo* formation of its chloramines. The biological oxidation potentials of low-molecular chloramines (50–200 μM) were evaluated by hemolysis and methemoglobin formation in sheep erythrocytes (1×10^8 cells/ml). ICR-strain mice were administered drinking water containing 1.5% dextran sulfate sodium (DSS), a potent UC inducer in mice, for 6 days. The mice were intraperitoneally administered CH_3NH_2 (5–40 mg/kg per day) for 5 days. The colonic lesions were characterized by visible parameters and microscopic analysis of histological alterations and the number of infiltrating and myeloperoxidase positive neutrophils, respectively. Methylamine chloramines showed considerably higher potentials for both hemolysis and methemoglobin formation than the other chloramines tested. The administration of CH_3NH_2 increased the excretion of CH_3NH_2 itself into feces in a dose-dependent manner and markedly aggravated experimental UC accompanying the increased neutrophil infiltration. These results strongly support the possibility that CH_3NH_2 causes serious aggravation in UC via the formation of its chloramines and suggest the participation of low-molecular weight biological amines in deteriorating colitis.

Key words —— methylamine dichloramine, chloramine, neutrophil, oxidative stress, reactive oxygen species, colitis

INTRODUCTION

Ulcerative colitis (UC) is an intractable disease characterized by chronic inflammation of mucosa in the large intestine.[1, 2] The majority of UC patients experience a course of repeated relapse and remission. Although many factors have been proposed to be involved in the induction and aggravation of UC, the main factor that leads to the disease has not yet been ascertained. In actuality, there may be many factors, such as genetic, immunological, and environmental factors, involved in the disease.[3] Among the autoimmune-related cells, activated neutrophils are thought to play a crucial role in the development of the injuries with their infiltration into the mucosal layer.[4] Several models of acute and chronic experimental UC have been developed in laboratory animals.[5] Dextran sulfate sodium (DSS)-induced experimental colitis models are the most widely used to study UC[6] because DSS models express many symptoms similar to those of human UC, such as mucosal injury accompanied by infiltration of neutrophils, diarrhea, rectal bleeding, and shortening of the colorectum.

Excessive generation of reactive oxygen species (ROS), hydrogen peroxide, hydroxyl radical, and peroxy radical derived from primed neutrophils

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may cause inflammatory injury.[7] In fact, several reports have indicated that treatment with ROS-eliminating antioxidants prevents the deterioration of UC in experimental animal models.[8,9] Moreover, hypochlorous acid (HOCl), which has a potent oxidation potential, is derived through the reaction of hydrogen peroxide with chloride ion by myeloperoxidase (MPO) from infiltrated neutrophils.[10] It is believed that HOCl which leaks into inflammatory sites may play an important role in the process of UC deterioration in experimental animal models.

HOCl is a very potent species, and it reacts with various amino compounds to yield chloramines, some of which retain sufficient oxidation potential to oxidize sulfur-containing amino acids in cellular proteins.[11,12] A recent study has indicated that intrarectal administration of ammonia chloramine (NH_2Cl), an active species produced by the reaction of HOCl with ammonia, induces inflammatory colorectal injury in rats.[13] Our previous study suggested that mucosal injury was induced by intrarectal administration of methylamine dichloramine (CH_3NCl_2) at lower dose (0.7 μmol/0.1 ml per mouse) than that of NH_2Cl, and also indicated that CH_3NCl_2 may promote UC and colon cancer in an experimental animal model and CH_3NCl_2 may have a greater cellular damaging action than methylamine monochloramine (CH_3NHCl), based on its higher cell permeability.[14]

Thus, we have focused our attention on the participation of chloramines, which have sufficient oxidation potential, in the aggravation of colorectal injuries with inflammation. Here we describe a possible relationship between low-molecular biological amines and the aggravation of mucosal injury associated with DSS-induced colitis in mice.

MATERIALS AND METHODS

Chemicals —— DSS (MW = 36000–50000) was obtained from MP Biomedicals, LLC (Illkirch, France). Sodium hypochlorite (NaOCl), ammonium chloride, and methylamine monochloride were obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). All other chemicals used were of reagent grade and were purchased from Wako Pure Chemical Industries, Ltd. They were used without further purification. Freshly prepared Milli-Q water from distilled water was used for the preparation of reagent solutions and chloramine solutions. The concentrations of HOCl were determined by iodometric titration. The amounts of free chlorine and chloramines were differentiated by the 2,5-dichlophenyldiazonium (DPD) method [American Public Health Association (APHA), American Water Works Association (AWWA), Water Environment Federation (WEF), 1992] after appropriate dilutions. In the present study, the concentrations of HOCl and chloramines are expressed as equivalent concentrations to chlorine (Cl_2), and HOCl is used to designate free chlorine, *i.e.* HOCl, hypochlorous ion (OCl^-), and their mixture.

Synthesis of Chloramines —— NaOCl solutions and amine solutions of Krebs-Ringer Phosphate (KRP) Buffer at pH 7.4 were prepared at the appropriate concentrations. Chloramine solutions for the experiments with erythrocytes were prepared by mixing equal volumes of NaOCl solution and the corresponding amine solution. The details of the preparation of the chloramines solutions have been described in an earlier report.[15] The chloramine solution was used within 30 min of preparation and diluted with saline solution to the desirable concentration.

Hemolysis and Hemoglobin Oxidation of Erythrocytes with Chloramines —— Bank blood obtained from sheep was purchased from Japan Biomaterial Co. Ltd. (Tokyo, Japan). Blood diluted with KRP was centrifuged at 3000 rpm at 4°C for 10 min to remove the plasma and buffy coat. This procedure was repeated two more times. The erythrocytes were suspended in KRP (1×10^8 cells/ml) and treated with chloramines prepared (50–200 μM) at 37°C for 5 min. The rates of hemolysis and hemoglobin oxidation in erythrocytes due to chloramines were determined and calculated according to the methods described in a previous report.[14]

Animals —— All animal experiments were performed in accordance with the Guidelines for Regulation of Animals of the Nihon University Animal Care and Use Committee. Five-week-old male ICR mice were purchased from Sankyo Labo Service (Tokyo, Japan). Mice were housed in plastic cages under specific pathogen free (SPF) conditions at 23°C and 55% humidity with a 12-hr light/dark cycle. The mice had free access to drinking water and a pelleted basal diet (FR-2, Funabashi Farm Co. Ltd., Chiba, Japan), and were acclimated for one-week prior to an experiment. The mice were treated with drinking water containing 1.5% DSS for 6 days and simultaneously injected intraperitoneally with 5–40 mg/kg CH_3NH_2 solution per day for 5 days

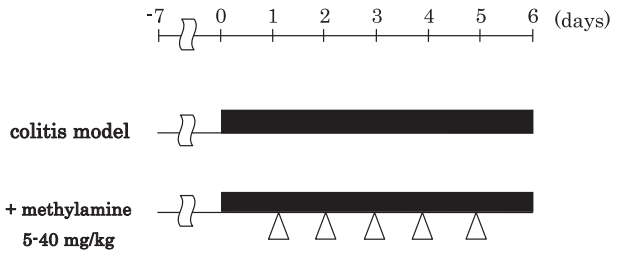

Fig. 1. Experimental Design for Determining the Aggravating Effect of Methylamine on Colitis in Mouse

■: Periods of treatment. Mice were treated with 1.5% DSS in drinking water. △: Injection points. Methylamine (5–40 mg/kg) was injected (i.p.) into ICR-strain mice.

as indicated in Fig. 1. CH_3NH_2 solution (pH 9.0) was prepared by adding 1N NaOH solution to saline containing CH_3NH_2 HCl. The control mice were treated with saline instead of CH_3NH_2.

Measurement of CH_3NH_2 in Feces —— After the administration of DSS and CH_3NH_2 as described above, the mice were euthanized and the colon was cut open longitudinally. The feces (*ca.* 0.1 g) were collected from the colon into 1 ml of 1M HCl and the mixture was centrifuged at 15000 rpm for 10 min. The supernatants were pooled. This procedure was repeated 5 times. CH_3NH_2 in the combined supernatants was converted to its benzene sulfonyl derivative, and measured by gas chromatography with a flame ionization detector-gas chromatogrophy (FID-GC, Shimadzu GC17, Shimadzu, Kyoto, Japan) using a DB-1 capillary column.[16]

Aggravating Effects of Methylamine on DSS-induced Mouse Colitis —— Colonic damage was evaluated by scoring using 2 visible parameters, diarrhea and rectal bleeding, on a scale from 0 (normal) to 3 (severe rectal bleeding) according to the method described by Tamaki *et al.*[17] The appearance of rectal bleeding was defined as trace hematochezia in the anal region and a trace amount of blood in the cage. After the collection of feces, the colon was rinsed with ice-cold 0.9% saline. The specimens were fixed in 10% buffered formalin. The colon specimens were embedded in paraffin and then 5-μm sections were prepared. The sections stained with hematoxylin and eosin were examined histopathologically under a microscope equipped with a digital camera system (Olympus BX41-FX380, Olympus Corporation, Tokyo, Japan). The colonic lesions were characterized by the histological appearance of atrophy of crypt architecture, loss of goblet cells, and hypertrophy of mucosa by microscopic analysis. The scores for colonic lesions were evaluated according to mucosal damage area estimated from the method of Cooper *et al.*[18] The number of neutrophils that had infiltrated into each area (0.6–0.8 mm^2, n = 3–5) at 5 mm from the anal opening was counted under a microscope at a high resolution.

Immunohistochemical Analysis of MPO Positive Cells —— Paraffin embedded colorectal sections were deparaffinized. The slide glasses were heated in 0.01 M citric acid buffer using a microwave for 5 min. This was repeated 3 times. Non-specific binding was blocked by incubating the slides in phosphate buffered saline (PBS) containing 10% normal goat serum (Chemicon, Billerica, MA, U.S.A.) and 20% Avidin D (Vector Laboratories, Burlingame, CA, U.S.A.) overnight at 4°C. The sections were then incubated overnight with a primary antibody, rabbit polyclonal antibody for myeloperoxidase (Thermo Fisher Scientific, Cheshire, U.K.), and diluted 1 : 200 with PBS containing 1% BSA and 20% biotin (Vector Laboratories). The sections were immersed in a second antibody, biotinylated anti-rabbit IgG (KPL, Gaithersburg, MD, U.S.A.), diluted 1 : 100 with PBS containing 1% BSA for 2 hr, and then rinsed in PBS containing 0.1% Tween 20 and incubated in avidin-biotin complex (Vectastain Elite ABC kit, Vector Laboratories). Reaction products were developed by immersing the section in 3,3′-diaminobenzidine 4HCl (Sigma Chemical Co. St. Louis, MO, U.S.A.) containing 0.01% H_2O_2.

Statistical Analysis —— The level of significance between different groups was calculated using Williams' or Dunnett's test as a *post hoc* test. The software used in the statistical analysis was Excel-Toukei 2006, an add-in-software of Microsoft Excel (Social Survey Research Information Co., Ltd., Tokyo, Japan).

RESULTS

Erythrocyte Oxidation by Chloramines

To assess oxidative cellular damage caused by chloramines, hemolysis and methemoglobin formation caused by various biological chloramines were examined and compared. Figure 2 (a) shows the percentages of hemolysis following exposure to chloramines at 100 or 200 μM. Hemolysis was observed in over 25% of erythrocytes exposed to methylamine chloramines at 200 μM. On the other hand, among the chloramines tested here,

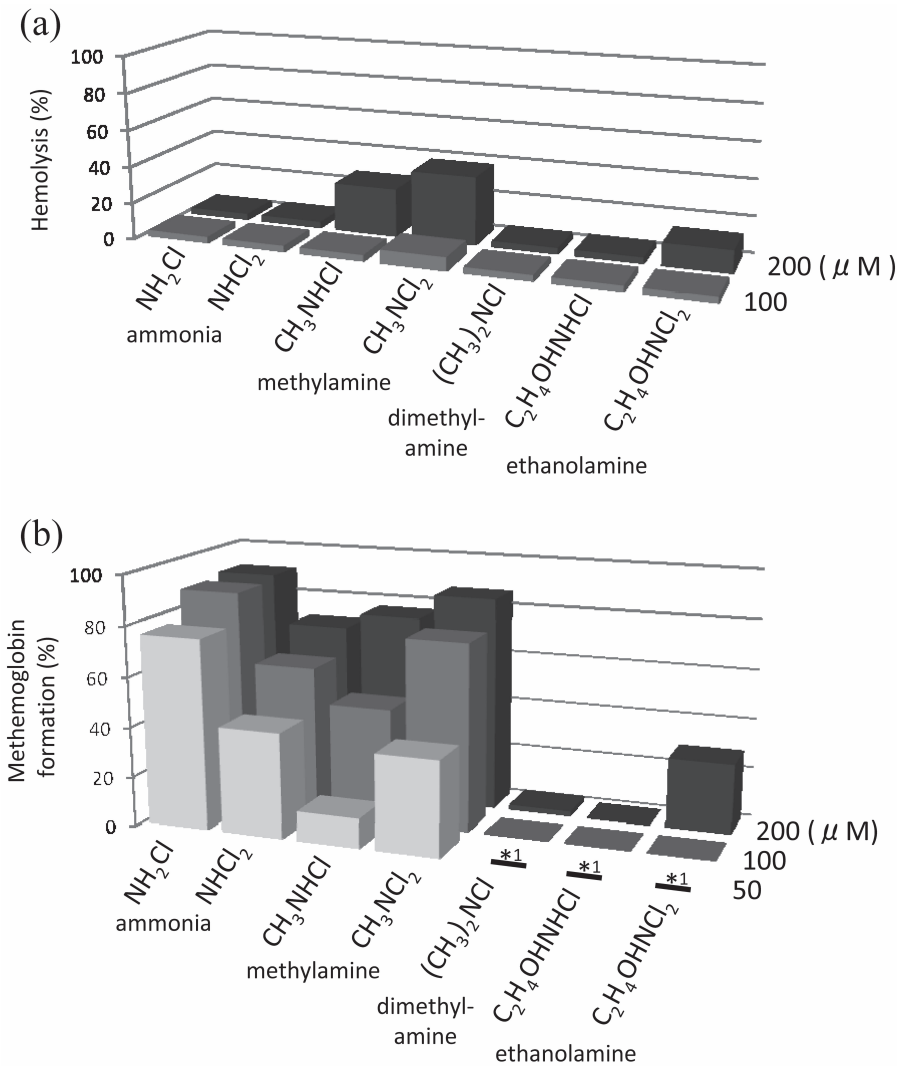

Fig. 2. Percentages of Hemolysis (a) and Methemoglobin Formation (b) in Sheep Erythrocytes Treated with Ammonia and Low Molecular Chloramines

Sheep erythrocytes, 1×10^8 cells/ml suspended in KRP at pH 7.4, were treated with chloramines, 50–200 μM (as available chlorine) for 5 min at 37°C. The amounts of hydrolysis and methemoglobin were determined by the methods described in a previous paper.[14] *1 Not tested.

methemoglobin formation by ammonia chloramines and methylamine chloramines at 50–200 μM was more substantial than that by dimethylamine and ethanolamine chloramines [Fig. 2 (b)]. Hemolysis and methemoglobin formation by chloramines derived from other biological nitrogen-containing compounds such as creatinine and taurine was not observed (data not shown). These results suggest that typical low-molecular chloramines, particularly ammonia chloramines (NH_2Cl and $NHCl_2$) and methylamine chloramines (CH_3NHCl and CH_3NCl_2), are more substantial than others concerning the effective formation of methemoglobin without hemolysis and that the phenomena may occur due to their greater ability to permeate cell membranes, as mentioned in our previous report.[14] Among the chloramines tested, methylamine chloramines exhibited higher efficacy in both hemolysis and methemoglobin formation.

Levels of CH_3NH_2 Excreted into Feces after Administration of CH_3NH_2

We have previously proposed that CH_3NCl_2, which formed endogenously by the reaction of CH_3NH_2 and HOCl around nearby infiltrated neutrophils, may play a crucial role in the process of inflammatory colorectal disease.[14] It can be, therefore, presumed that administration of CH_3NH_2 leads to the aggravation of UC. To confirm this, we determined the levels of CH_3NH_2 excreted into feces after the injection [intraperitoneal (i.p.)] of CH_3NH_2 into mice according to the experimental

Table 1. Methylamine Levels in Feces of Methylamine-injected Mice

Administration of methylamine (mg/kg per day)	Methylamine in feces (μg/g)
0	17.8 ± 2.5
5	22.0 ± 7.1
10	24.4 ± 9.5
20	27.2 ± 5.3
40	34.0 ± 9.4*

DSS-treated mice were injected 5, 10, 20 and 40 mg/kg methylamine (i.p.), respectively, as indicated in Fig. 1. Methylamine in the feces was determined at the 6th days of the treatment. Each value indicates mean ± S.D. (n = 3–5). *Significant difference ($p < 0.025$) from methylamine none-administered group (Williams' test).

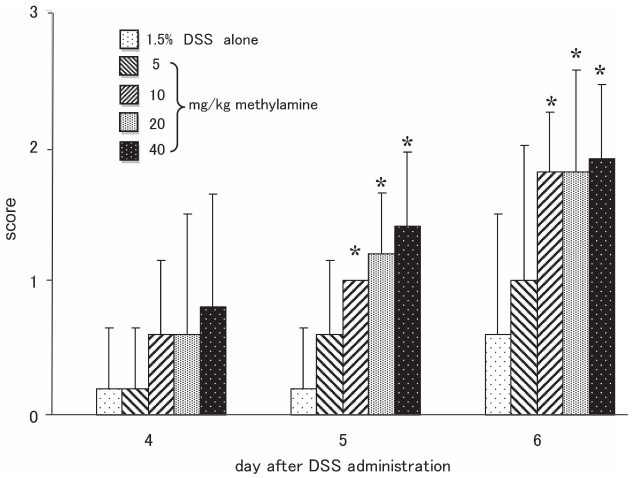

Fig. 3. Aggravation Effect of Methylamine on Colonic Damage Induced by 1.5% DSS Administration in Mice

DSS-treated mice were injected with 5, 10, 20, and 40 mg/kg methylamine, respectively, as indicated in Fig. 1. Colonic damage was scored by the induction of diarrhea (score of 1), rectal bleeding (score of 2), and severe rectal bleeding (score of 3). Bars represent mean ± S.D. (n = 5–6). *Significant difference ($p < 0.025$) from 1.5% DSS-alone-treated group (Williams' test).

design shown in Fig. 1. As shown in Table 1, the excretion levels of CH_3NH_2 into feces following a single i.p. injection at 5–40 mg/kg per day for 5 days increased in a dose-dependent manner. The results suggest that CH_3NH_2 which passes into the bloodstream is partly excreted into the intestinal tract.

Aggravating Effect of CH_3NH_2 Treatment on DSS-induced Colitis in Mice

The colonic damage scores in the treated mice are shown in Fig. 3. During the experiment, the body weight gain of all mouse treated with 1.5% DSS did not change compared to the untreated mice (data not shown). Only diarrhea was observed in the mice treated with DSS only. No colitis-like findings were observed in the treatment with CH_3NH_2 (40 mg/kg) alone (data not shown). DSS-mice treated with CH_3NH_2 exhibited more obvious and serious symptoms of experimental colitis, such as diarrhea and rectal bleeding, in a dose-dependent manner (5–40 mg/kg), indicating significant aggravation of colitis. The colitic aggravation by CH_3NH_2 was further characterized by histopathological analysis (Fig. 4). These typical colitis-like symptoms were followed by morphological changes. As shown in Fig. 4 (1), mucosal lesions such as crypt atrophy with architectural alterations, namely, the loss of goblet cells that made up the crypts, were induced in the mice treated with DSS alone for 6 days compared to the control. Both DSS and CH_3NH_2-treated mice (10–40 mg/kg) revealed more severe mucosal lesions than those treated with DSS alone. The degree of colonic lesion elevated gradually with the increases of methylamine dose [Fig. 4 (2)]. As shown in Fig. 5 (1), neutrophils (a) and MPO-positive cells (b) infiltrated into mucosal layer, indicating a high degree of colorectal injury with inflammation.[19] The numbers of these cells were significantly increased in the group treated both with DSS and CH_3NH_2, compared to that treated with DSS alone [Fig. 5 (2)].

DISCUSSION

HOCl is a neutrophil-derived ROS that reacts with a variety of biological amines to yield chloramines. It is believed that chloramines cause damage to the gastrointestinal mucosa. NH_2Cl is formed by the reaction of HOCl generated by activated neutrophils and ammonia produced through the action of urease of *Helicobacter pylori*,[20] and is considered to be responsible for this mucosal injury (gastric ulcer). The mucosal injury is often muted and is associated with chronic inflammatory infiltrates, chronic peptic ulcer disease, and with their malignancy.[21] The production of NH_3 and some species of low-molecular alkyl amines by intestinal bacteria metabolically have been detected in the lumen of intestine.[22] Several studies have reported that the level of NH_3 is approximately 10 mM in rat feces[23] and that an excessive intake of dietary protein by rats also leads to a significant increase in fecal NH_3.[23,24] Intrarectal administration of NH_2Cl, which is prepared by the reaction of NH_3 and HOCl, into rats induced

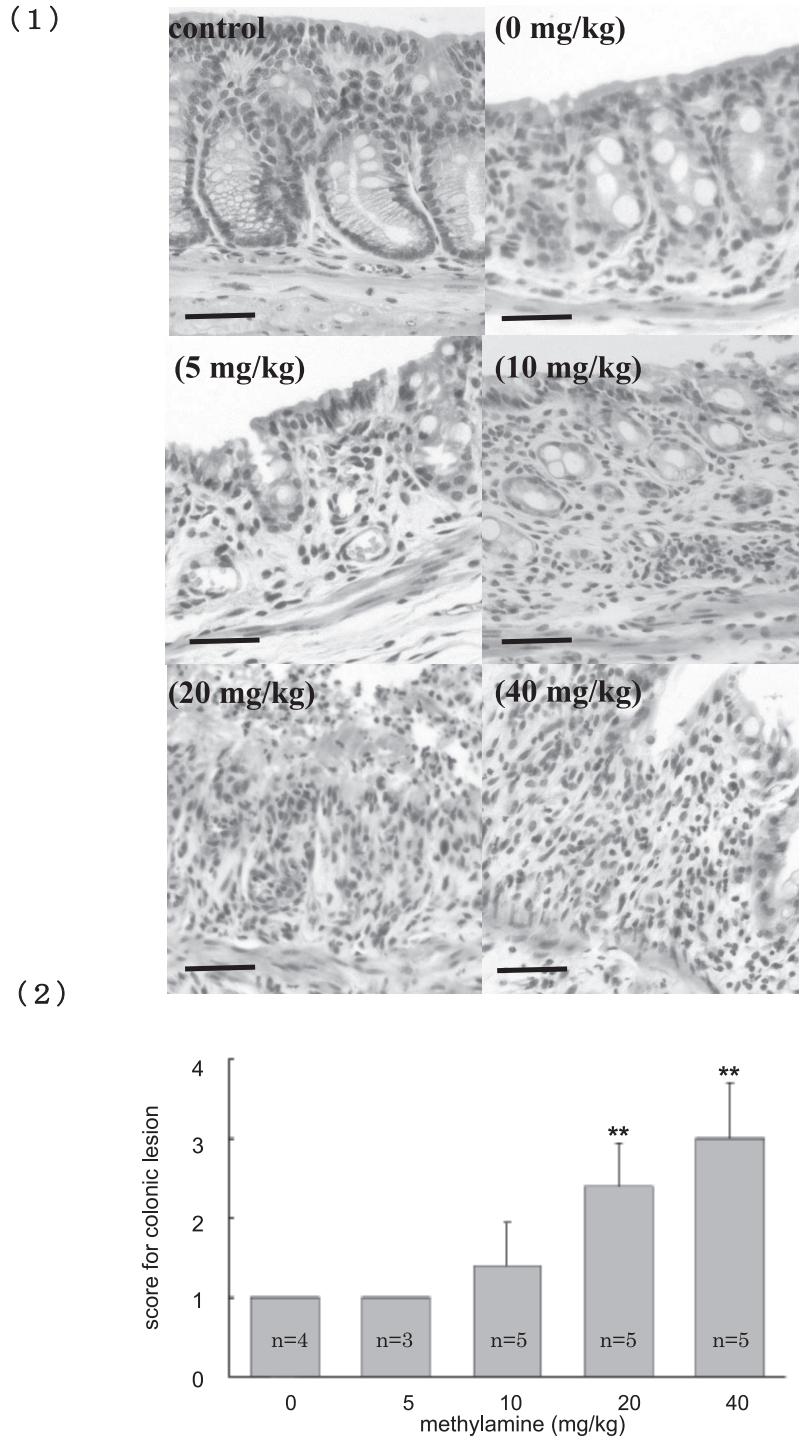

Fig. 4. Histological Evaluation of Colitis Site Aggravated by Injection (i.p.) of Methylamine

(1) Histological observations of colitis site: methylamine (0–40 mg/kg) was injected into 1.5% DSS-treated mice. Bars indicate 100 μm. (2) Scores for colonic lesions: bars represents mean ± S.D. The numbers of the mouse treated were indicated in each bar. **Significant difference ($p < 0.01$) from 1.5% DSS-alone-treated group (Dunnett's test).

intense inflammatory responses in the large intestine,[13] although the concentration of NH_2Cl used (3.2 mg, 250 mM/0.25 ml, 62.5 μmol/rat) was much higher than that of chloramines estimated in human phagocytes.[25] Since it has been reported that the concentration of fecal NH_3 in humans is approximately 0.2 mM (mmol/kg),[26] which is similar to that of CH_3NH_2 in feces,[27] experiments performed using biological levels are needed to further elucidate the possible mechanism(s) of inflammatory re-

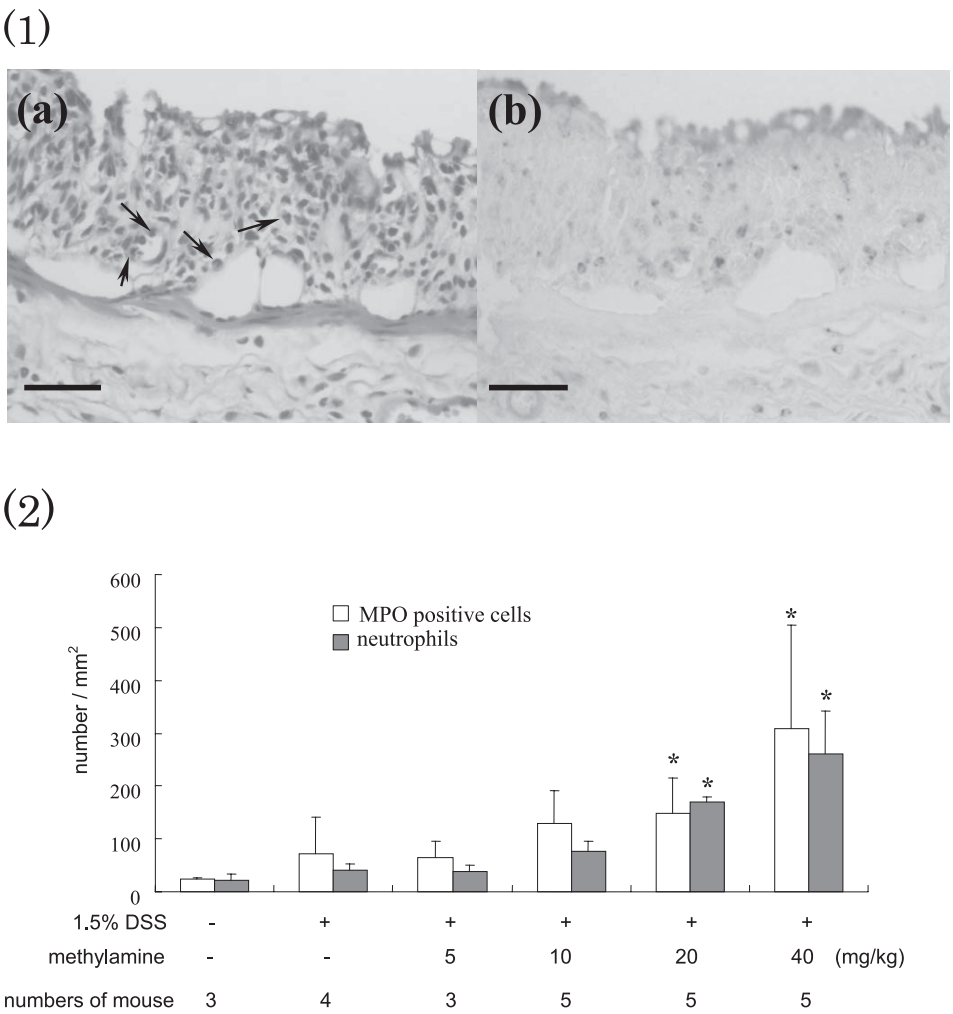

Fig. 5. Increase of MPO Positive Cells and Neutrophils by Injection (i.p) of Methylamine

(1) Histological observations of infiltrated neutrophils and MPO positive cells in colitis site aggravated by administration of methylamine (40 mg/kg): neutrophils were stained with hematoxylin and eosin (a). Arrows indicate neutrophils. MPO positive cells in the mucosal layer were immunostained with anti-MPO antibody (b). Bars indicate 100 μm. (2) The numbers of MPO positive cells and neutrophils in the mucosal layer: methylamine (0–40 mg/kg) was injected into DSS-treated mice. Bars represent mean ± S.D. *Significant difference ($p < 0.025$) from 1.5% DSS-alone-treated group (Williams' test).

sponses due to the formation of low-molecular chloramines. Methylamine chloramines, *i.e.*, CH_3NHCl and CH_3NCl_2, are believed to be generated endogenously by the reaction between CH_3NH_2 and HOCl from activated neutrophils. *In vitro* experiments suggest that CH_3NH_2 is produced metabolically from amino acids such as glycine[28] and creatinine[29] by human intestinal bacteria. In a previous study, we proposed that the induction of colorectal mucosa injury by CH_3NCl_2 is dependent not only on its oxidation potential but also on its high degree of cell permeability. This is because CH_3NCl_2 is a relatively stable pro-oxidant in biological environments, and may have a reactivity different from the two other neutrophil-derived oxidants, HOCl and CH_3NHCl.[14] CH_3NCl_2 is also likely to be more effective against colitis than common ROS generated from activated neutrophils, hydrogen peroxide, hydroxyl radical, and peroxy radical, a point that deserves special consideration in research concerning the aggravating mechanisms of inflammatory bowel disease.[14]

The present study followed-up the effect of low-molecular chloramines as aggravating factors in DSS-induced experimental colitis. A screening test using *in situ* erythrocyte-hemoglobin as an index of oxidation potential through the cell-permeability of chloramines showed that low-molecular chloramines, particularly ammonia chloramines and methylamine chloramines, have more potent oxidation potential in cell permeation and hemoglobin oxidation than other low-molecular chloramines

(Fig. 2). Furthermore, intraperitoneal administration of CH_3NH_2, which is believed to produce methylamine chloramines with HOCl generated from neutrophils in inflammatory sites, aggravated the experimental colitis (Figs. 3–5), suggesting that the aggravating action of CH_3NH_2 is likely attributable to *in vivo* production of methylamine chloramines.

The findings of the present study suggest that CH_3NH_2 and most likely NH_3 as well may aggravate colorectal diseases with inflammation. Taking these findings together, we therefore, propose here that the increase in the endogenous production of low-molecular chloramines, such as methylamine and/or ammonia chloramines, may play an important role in the etiology of colorectal disease, such as colitis and colon cancer, which has been increasing recently.

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