4-methoxyindole-3-acetonitrile and 4-methoxyindole-3-aldehyde as mutagen precursors. 1-Nitrosoindole-3-acetonitrile (NIAN), an *N*-nitroso-substituted compound formed by treatment of indole-3-acetonitrile with nitrite under acidic conditions, is a direct-acting mutagen in *S. typhimurium* and Chinese hamster lung cells,[20-22] and it is confirmed to form DNA adducts and to induce DNA single-strand scission in the rat glandular stomach.[23, 24] Therefore, NIAN could play some role in gastric cancer development, as in the case of the well-known direct-acting mutagens, MNNG and MNU, in animal experiments.[16, 17, 25]

The Mongolian gerbil (MG) is reported to be susceptible to colonization by *H. pylori*, and *H. pylori* infection greatly enhances MNNG or MNU-induced gastric carcinogenesis in MGs.[26, 27] Therefore, the MG is considered to be a useful animal model for evaluating the gastric cancer risk of direct-acting *N*-nitroso compounds, with or without *H. pylori* infection.

Chinese cabbage, containing nitrate and indole compounds, is commonly consumed in East Asian countries, including Japan,

Korea and China, in which gastric cancer mortality is very high. In the present study, DNA adducts were detected with NIAN treatment in the glandular stomach of MGs, and the carcinogenicity of NIAN for gastric cancer *in vivo* was examined. The results clearly demonstrated that gastric cancer developed with a combination of NIAN administration and *H. pylori* infection in MGs. Possible involvement of indole compounds and nitrate derived from various foodstuffs, including Chinese cabbage, in gastric cancer development in humans is discussed.

Materials and Methods

Materials

Indole-3-acetonitrile was purchased from Tokyo Food Techno Co., Ltd. (Tokyo, Japan), sodium nitrite from Wako Pure Chemical Industries, Ltd. (Osaka, Japan), and ammonium sulfamate from Kanto Chemical Co., Inc. (Tokyo, Japan). Brucella broth was obtained from Becton Dickinson Co. (Cockeysville, MD, USA), and horse serum from Nippon Bio-Supply (Tokyo, Japan).

Preparation of NIAN

The chemical structure of NIAN is shown in Figure 1A. Indole-3-acetonitrile in 27 mM citrate-phosphate buffer (pH 3.0) was treated with 50 mM sodium nitrite for 1 hour at room temperature in the dark, as previously reported.[21] Nitrosation was stopped by addition of ammonium sulfamate at a final concentration of 50 mM. The reaction solution was filtered and the residue was washed with deionized water, then with n-hexane. The residual paste was dried and stored at -80°C until use. The preparation was >93% pure as judged by its UV absorbance on HPLC.

Bacterial culture

H. pylori (ATCC 43504; American Type Culture Collection, Manassas, VA) was cultured in brucella broth supplemented with 10% heat-inactivated horse serum for 24 hours at 37 °C under microaerobic conditions (5% O_2, 10% CO_2 and 85% N_2), as previously described.[28]

Animal treatment

Specific pathogen-free male, 6-week-old MGs (MGS/Sea, Kyudo,

Fukuoka, Japan) were housed in a biohazard room, air-conditioned at 24 °C ± 2 °C and 55% humidity, on a 12 hours light-dark cycle and were allowed free access to commercial diet (CE-2; CLEA Japan Inc., Tokyo, Japan) and water.

In order to analyze the formation of DNA adducts in the glandular stomach of MGs by NIAN treatment, NIAN was dissolved in 50% dimethyl sulfoxide (DMSO), and administered to three MGs by gavage of 0.5 mL solution, two times a week at a level of 100 mg/kg body weight. Two further MGs served as a control group receiving the solvent alone (0.5 mL). At 8 hours after administration of NIAN, both groups of animals were sacrificed under ether anesthesia, and their stomachs were resected and stored at -80 °C until use. DNA was extracted by a standard procedure with enzymatic digestion of protein and RNA followed by extraction with phenol and chloroform/isoamyl alcohol (24:1, v/v).

The protocol for long-term gastric carcinogenicity in MGs treated with NIAN + *H. pylori* infection is illustrated in Figure 1B. The animals were randomly divided into four groups (groups A-D). Groups A and C were given 50% DMSO without NIAN (0.5 mL),

while groups B and D were orally administrated NIAN (0.5 mL, 100 mg/kg body weight) dissolved in 50% DMSO by gavage, two times a week for three weeks. At 1 week after the last administration, the animals of groups C and D were given an intragastric inoculation of *H. pylori* broth culture (0.5 mL, 0.9 x 10^8 CFU/animal), while animals of groups A and B were given sterilized broth alone (0.5 mL).[28]

During the experiments, animals which became moribund or emaciated (<80 g body weight) were sacrificed. At 104 weeks after *H. pylori* infection, all surviving animals were sacrificed under ether anesthesia. At performance of necropsy, all tissues were carefully checked macroscopically and the stomachs and major organs were removed and assessed for macroscopic lesion development. Effective numbers of animals were defined as those surviving until week 54 of the study, when gastric tumors were observed for the first time. In addition, in the *H. pylori*-infected groups, the animals developing gastritis observed on histological examination were regarded as effective. The percentages of gastritis-bearing animals by the single inoculation of *H. pylori* were 62% for group C and

76% for group D, being similar to those previously reported.[27] All animal experiments were performed according to the "Guidelines for Animal Experiments in the National Cancer Center" and were approved by the Institutional Ethics Review Committee for Animal Experimentation in the National Cancer Center.

Detection of DNA adducts by ^{32}P-postlabeling method

Calf thymus DNA (0.5 mg, Sigma, St. Louis, MO, USA) treated with NIAN (3 mg) for 12 hours under neutral conditions was used for authentic NIAN-DNA adducts.[23] DNA samples from the glandular stomach of MGs and calf thymus DNA samples were digested with micrococcal nuclease and phosphodiesterase II, and subjected to ^{32}P-postlabeling analysis using the same procedure as described previously[23] except with solvent systems for two-dimensional development. The solvent system consisted of buffer A (4.0 M lithium formate, 7.7 M urea, pH 3.5) from bottom to top, and buffer B (0.90 M lithium chloride, 0.45 M Tris-HCl, 7.7 M urea, pH 8.0) from left to right, followed by 1.7 M sodium phosphate buffer, pH 6.0, from left to right, with 3.5 cm filter

paper. Adducts were detected with a Bio-Image Analyzer (BAS 3000; Fuji Photo Film Co., Tokyo, Japan) after exposing the TLC sheets to Fuji imaging plates. Relative adduct labeling was determined by the methods of Reddy *et al.*[29], and values were calculated as averages using data from three assays.

Histological examination

All excised stomachs were opened along the greater curvature and washed twice with saline, then fixed in 10% neutral-buffered formalin. The fixed stomachs were sliced along the longitudinal axis into 9-12 strips of equal width, and routinely processed to sections stained with hematoxylin and eosin (H&E). The degree of chronic active gastritis was graded according to criteria modified from the Updated Sydney System,[30] by scoring the infiltration of neutrophils and mononuclear cells. Other organs, in which macroscopic lesions were observed, were also fixed in 10% neutral-buffered formalin and routinely processed to sections stained with H&E for histological examination.

Statistical analysis

The significance of differences in quantitative data for gastric inflammation, gastric adenocarcinoma and tumors of other organs was analyzed by Fisher's exact test. Data for stomach wet weight and inflammation score were examined using Turkey's multiple comparison test. Significance was concluded at $p<0.05$.

Results

DNA adduct formation by NIAN administration in the glandular stomach of MGs

To confirm the formation of NIAN-DNA adducts in the glandular stomach of MGs, NIAN was injected two times a week at a dose of 100 mg/kg by gavage, and then analyzed by ^{32}P-postlabeling method. Three adduct spots were observed in DNA samples derived from NIAN-treated animals (Figure 2A). The adduct levels were 0.3 for adduct 1, 1.1 for adduct 2, 0.2 for adduct 3, and 1.6 adducts/10^8 nucleotides in total. This TLC pattern was similar to that in the *in vitro* reaction of calf thymus DNA with NIAN (total adduct level of 4.8 adducts/10^7 nucleotides, Figure 2B). In the case of DNA samples derived from control animals, no

adduct spots were seen on the TLC sheets (Figure 2C).

Macro- and microscopical observation of *H. pylori*-induced gastritis in MGs

MGs were sacrificed until 104 weeks after *H. pylori* infection, and gastric disorders were analyzed. Stomach wet weights and gastric inflammation scores are shown in Table 1. Macroscopically, edematous thickening with hemorrhagic spots was apparent in the gastric mucosa in *H. pylori*-infected MGs (groups C and D), but not in animals uninfected with *H. pylori* (groups A and B). The stomach wet weight, reflecting edematous thickening, in animals infected with *H. pylori* (groups C and D) was significantly increased compared with that of animals not infected with *H. pylori* (groups A and B) ($p<0.01$). No significant differences of stomach wet weight were detected between groups A and B and also between groups C and D.

Microscopically, gastritis, featuring infiltration of many inflammatory cells, and hyperplastic change of glandular epithelium, and erosion were observed in the pyloric regions of the animals infected with *H. pylori* (groups C and D) (Figure

3). Heterotopic proliferative glands, whose development is related to severe gastritis in *H. pylori*-infected MGs, were sometimes observed in *H. pylori*-infected groups (groups C and D). No gastritis was found in animals not infected with *H. pylori* (groups A and B). The gastric inflammation score in *H. pylori*-infected animals was significantly increased compared with that of animals uninfected with *H. pylori* ($p<0.01$). There were no significant differences of gastric inflammation score between groups C and D.

Development of glandular stomach adenocarcinomas in MGs treated with both NIAN and *H. pylori*

The observed incidences of glandular stomach adenocarcinomas are shown in Table 2. Glandular stomach adenocarcinomas, histologically featuring tubular structures with cellular atypia infiltrating into the muscle layer, were found in 8 animals treated with both NIAN and *H. pylori* (8/26=31%) at 54 - 104 weeks. All adenocarcinomas were observed in the pyloric mucosa and located in the lesser curvature of the stomach, where macroscopically severe edematous thickening was also seen

(Figure 4A). The observed adenocarcinomas in 7 animals were of well differentiated (Figure 4B), and a moderately differentiated lesion was observed in 1 animal (Figure 4C). In the animals treated with broth alone, broth + NIAN and *H. pylori* alone (groups A, B and C), no glandular stomach adenocarcinomas were observed. The incidence of glandular stomach adenocarcinomas in group D was significantly higher than that in groups A, B and C ($p<0.05$, $p<0.01$ and $p<0.01$, respectively).

Irrespective of NIAN treatment and *H. pylori* infection, skin tumors, which histologically were well to poor differentiated squamous cell carcinomas, sebaceous carcinomas and melanomas, were found in 1 animal (1/15=7%) in group A, 3 animals (3/22=14%) in group B, 2 animals (2/18=11%) in group C and 5 animals (5/26=19%) in group D. A hemangioma was also observed in a kidney of 1 animal in group D (1/26=4%). No significant differences were apparent in these tumor incidences among groups A-D.

Discussion

In the present study, NIAN was found to induce glandular stomach

adenocarcinomas in MGs in combination with *H. pylori* infection. NIAN-DNA adducts were also detected in the glandular stomach of MGs after treatment with NIAN, although clarification of their chemical structure(s) has yet to be performed. DNA adducts observed in the glandular stomachs of NIAN-treated MGs probably contain an indole-3-acetonitrile moiety. However, it is further likely that NIAN would act as an NO donor under aqueous conditions, thereby causing DNA modifications.[31-33] In fact, Lucas *et al.* demonstrated that NIAN can efficiently transfer nitroso groups to nucleophilic targets in purine nucleotides, causing N-nitrosation, deamination, and the formation of a novel guanine analogue, oxanine.[33]

Glandular stomach adenocarcinomas induced by NIAN treatment plus *H. pylori* infection were located in the pyloric region, similar to MNNG or MNU treatment plus *H. pylori* infection-induced glandular stomach adenocarcinomas in MGs.[26, 27] Meanwhile, no glandular stomach cancers were observed in the groups of *H. pylori*-infected MGs without NIAN treatment, which is consistent with previous studies,[26, 27] nor in the group treated with only NIAN. These findings indicated that *H. pylori* is a

strong promoter of gastric carcinogenesis. Histological examination revealed that the tumors developed by NIAN + *H. pylori* were of well or moderately differentiated adenocarcinomas. Well or poorly differentiated adenocarcinomas and signet ring cell carcinomas were observed in *H. pylori*-infected MGs treated with MNNG or MNU.[26, 27] Further studies are required to clarify the histological variety of stomach adenocarcinomas induced by NIAN, MNNG or MNU, since the type of cancer might depend on the genotoxic action of chemical carcinogens, rather than the effects of *H. pylori* infection.[27] In addition tumors were observed in skin and kidney, which were suspected to spontaneously develop. The MGs have been reported to develop spontaneous skin tumors such as sebaceous and squamous cell carcinoma.[34]

Epidemiological studies have indicated that nitrate intake increases gastric cancer risk, and major sources are vegetables including Chinese cabbage, spinach and parsley.[14] Indole-3-acetonitrile, a precursor of NIAN, is distributed widely in cruciferous vegetables including Chinese cabbage and sprouts.[35] Furthermore, fava beans (*Vicia faba*), which are

commonly consumed in Colombia, give rise to a potent mutagen in the presence of nitrite under acidic conditions.[36] The nitrosatable precursor of the mutagen in fava beans and the major product of nitrosation are reported to be an indole compound, 4-chloro-6-methoxyindole, and an *N*-nitroso compound, 4-chloro-2-hydroxy-N^1-nitroso-indolin-3-one oxime, respectively.[37] Other indole compounds are also reported to produce direct-acting mutagens after nitrite treatment under acidic conditions.[38, 39] In general, conversion of indole derivatives to nitrosated forms in vitro is known to be rapid and efficient at physiologically feasible nitrite concentrations with the low pH of the human stomach.[37] Thus, it is conceivable that nitrosation of indole compounds such as indole-3-acetonitrile probably occurs in human stomach. On the other hand, nitric oxide is suggested to be produced by activated macrophages in inflamed organs with *H. pylori* infection.[18] Therefore, nitrosation of indole compounds could be mediated by both acid catalysis and inflammatory responses in the human stomach.[18, 20, 37-40] Based on the conversion rate of NIAN from indole-3-acetonitrile under physiological

conditions, the dose of NIAN used in the present study appears about 500-1000 fold the expected human exposure to NIAN via fresh or pickled Chinese cabbage. However, humans continually consume various kinds of foods containing indole compounds and nitrate during ordinary life. Thus it is probable that the total amount of nitroso-indole compounds would be much closer to the dose of NIAN used in the present study. Moreover, it has been reported that low doses of chemical carcinogens, such as MNNG and MNU, could induce glandular stomach cancers in rodents under inflammation conditions including NaCl treatment and *H. pylori* infection, but hardly induce glandular stomach cancer without NaCl treatment and *H. pylori* infection. Therefore, the continuous intake of indole compounds and nitrate may play an important role for gastric carcinogenesis in East Asian countries still with a high salt consumption and *H. pylori* infection rate.

Gastric cancer is tending to decline in most countries.[41-43] One of the explanations for this tendency is the reduced prevalence of *H. pylori* infection.[42] Changes in dietary habits, mainly being lower salt consumption, could be also related to

reduced gastric cancer incidence. However, the gastric cancer prevalence in East Asian countries, such as Japan and Korea, is still high.[2] At present, we have not succeeded in detecting NIAN in human bodies nor the exposure levels of the precursor, indole compounds for humans. Thus, it is necessary to estimate the human exposure levels to nitroso-indole compounds including NIAN, and to study further animal experiments and epidemiological analyses for clarification of contribution of nitroso-indole compounds under *H. pylori* infection in humans gastric carcinogenesis.

In conclusion, the present study demonstrated that NIAN can induce gastric cancer in *H. pylori*-infected MGs. It is noteworthy that nitrosatable precursors widely exist in foods. Thus, it is suggested that *N*-nitroso indole compounds including NIAN might contribute to the frequent development of gastric cancer in East Asian countries such as Japan and Korea in which the prevalence of *H. pylori* infection is relatively high. Further studies of interaction with other dietary elements appear warranted to promote the prevention of human gastric cancer.

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