demonstrated the importance of the gastric and intestinal phenotypic expression in the human and MG model, using gastric mucous (MUC5AC, MUC6) and endocrine (gastrin), and intestinal mucous (MUC2, villin) and endocrine (gastric inhibitory polypeptide; GIP) markers (Mizoshita et al., 2003; Mizoshita et al., 2006; Cao et al., 2008; Hirata et al., 2009). However, the relations between PPI use, tumor development, and phenotypic expression have hitherto remained unclear in the Hp-infected stomach.

In the present study, we therefore analyzed the influence of PPI use on development of cancers and enterochromaffin-like lesion (ECL lesions) in the glandular stomachs of Hp-infected and uninfected MGs, evaluated histologically and phenotypically.

Materials and Methods

PPI

Lansoprazole (Takeda Pharmaceutical Co., Ltd.), mixed with CE-2 (CLEA Japan INC., Tokyo, Japan) was given as the PPI.

Animals

A total of 53 (26 Hp-infected and 27 uninfected), 13-week-old male MGs (MGS/Sea, Kyudo Co., Ltd) were housed in plastic cages on hardwood chip bedding in an air-conditioned biohazard room with 12:12 h light:dark cycle. The experimental design was approved by the Animal Care Committee of the Nagoya City University Animal Research Institute and the animals were cared for in accordance with institutional guidelines, in compliance with the instructions of the Health, Labour and Welfare Ministry concerning animal experiments.

Experimental design

The experimental design is illustrated in Figure 1. Fifty-three gerbils were divided into 6 groups. Hp+25PPI and 25PPI groups were given the PPI at 25mg/kg/day, and the Hp+5PPI and 5PPI groups at 5mg/kg/day. The animals were sacrificed humanely at 50 or 100 weeks (Figure 1).

After 24h fasting, all animals were deeply anesthetized, laparotomized, and exsanguinated from the inferior vena cava, followed by excision of their stomachs. Each glandular stomach was fixed in 10% formalin neutral buffer solution, and routinely processed for histological examination (Sasaki et al., 2007). The glandular stomach samples were serially cut into 5-mm slices in parallel with the lesser curvature and embedded in paraffin, and then sectioned and stained with hematoxylin and eosin (H&E).

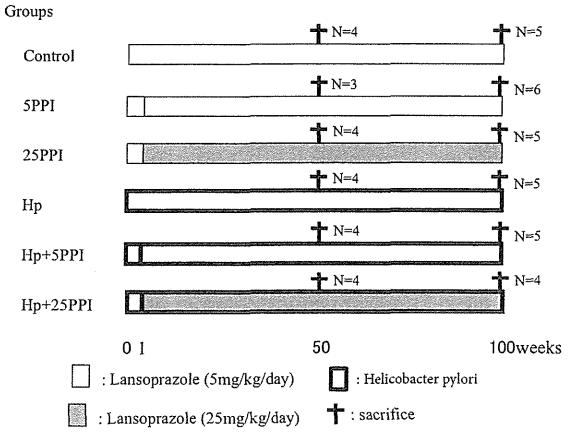

Figure 1. Experimental Design

Immunohistochemistry

Immunohistochemical staining was carried out with polyclonal antibodies against chromogranin A (CgA) (Yanaihara Institute Inc., Fujinomia, Japan), gastrin (Yanaihara Institute Inc., Fujinomia, Japan) and gastric inhibitory polypeptide (GIP) (Yanaihara Institute Inc., Fujinomia, Japan). The precise procedures were as described previously (Takenaka et al., 2006). Briefly, 4 μm-thick consecutive sections were deparaffinized and hydrated through a graded series of ethanols. After inhibition of endogenous peroxidase activity by immersion in 3% H_2O_2 methanol solution, sections were incubated with the primary antibody, washed thoroughly in phosphate-buffered saline (PBS), then incubated with biotinylated secondary antibody followed by the avidin-biotinylated horseradish peroxidase complex (Vectastain Elite ABC kit; Vector Laboratories, Burlingame, CA, USA). Finally, immune complexes were visualized by incubation with 0.01% H2O2 and 0.05% 3.3'-diaminobinzidine tetrachloride (DAB). Nuclear counterstaining was accomplished with Mayer's hematoxylin. Two independent investigators (HT and TM) judged the histology and immunohistochemical staining.

Diagnosis of ECL lesions

The gastric ECL lesions were evaluated histologically, basically according to TNM staging classification for the NET of the foregut published by a working group of the European Neuroendocrine Tumor Society (ENETS) in 2006 (Rindi et al., 2006), but with modifications (Cao et al., 2008). The lesions were divided into hyperplasia, NET-Tis, and NET-≥T1. The NET-≥T1 lesions were tumors invading lamina propria or submucosa, while NET-Tis lesions exhibited features of in situ tumors or dysplasia. Micronodular lesions, excepting NET-Tis and NET-≥T1, were defined as hyperplasia. Areas of hyperplasia, NET-Tis, and NET-≥T1 were assessed using a micrometer; lesions per length of glandular stomach epithelium examined (mm^2/cm) were then calculated.

Phenotypic classification of ECL lesions

Gastrin is the marker of the gastric endocrine cell phenotype, whereas GIP is typical of the intestinal one (Takenaka et al., 2007). ECL lesions were classified as endocrine-gastric (e-G) type or endocrine-intestinal (e-I) type, respectively with at least one gastric or intestinal cell phenotype, and as the endocrine-gastric-and-intestinal mixed (e-GI) type when both gastric and intestinal endocrine cell markers were present. Those showing neither gastric nor intestinal phenotypic expression were grouped as endocrine-null (e-N) type, as previously described (Takenaka et al., 2007; Hirata et al., 2009).

Serum gastrin level

Serum gastrin levels were examined using a radioimmunoassay kit Gastrin-RIA KIT II inhibition (Dainabot Co., Ltd., Tokyo) after sacrifice and expressed as pg/ml values.

Statistical analyses

The unpaired t test was applied to establish the significance of differences in titers of serum gastrin levels and the areas of ECL lesions. In the case of non-normal distribution, the Mann-Whitney's U test was applied. Incidences of ECL lesions and NET-≥T1 were assessed using the Fisher's exact test. Correlation analysis was performed using the Pearson's correlation coefficient test. Differences in serum gastrin levels in MGs with and without ECL lesions were assessed using the unpaired t test. P values < 0.05 were considered to be statistically significant.

Results

Expression of CgA, gastrin, and GIP

Immunohistochemical expression of CgA is shown in Figure 2. Limited numbers of CgA-positive cells were observed at the bottoms of the normal fundic and pyloric glands in non-infected MGs (red arrows). In the pyloric glands, expression of gastrin was clearly detected, but no GIP expression was observed. In the duodenum, GIP expression was detected, but no gastrin was observed. Neither gastrin nor GIP were observed in the fundic glands (data not shown).

Histopathological findings of ECL lesions

No adenocarcinomas were observed in the MG groups at 50 and 100 weeks. Some ECL lesions invaded the submucosa, but there was no involvement of the muscularis propria or subserosa. ECL lesions were always found in fundic mucosa near the forestomach in MGs, as previously described (Cao et al., 2008). Angio-invasion and metastasis were also not evident, and no gerbils died of NETs in the glandular stomach. All ECL lesions were positively immunostained for CgA (Figurex 2 and 3), but neither gastrin nor GIP expression was detected in the ECL lesions, which were classified phenotypically as e-N type (data not shown).

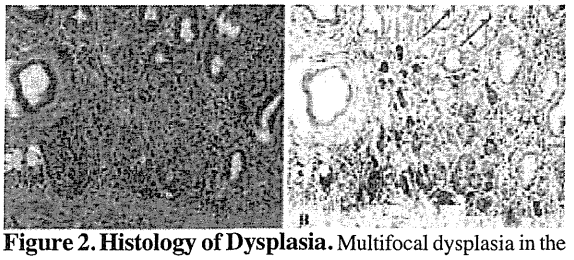

Figure 2. Histology of Dysplasia. Multifocal dysplasia in the fundic mucosa of the Hp-infected Mongolian gerbil treated with PPI (5mg/kg/day). Cytoplasmic CgA expression was detected in normal glands (red arrows) and dysplasia in the MG glandular stomach. (A: H&E staining, B: immunostaining for Cg A) (×200)

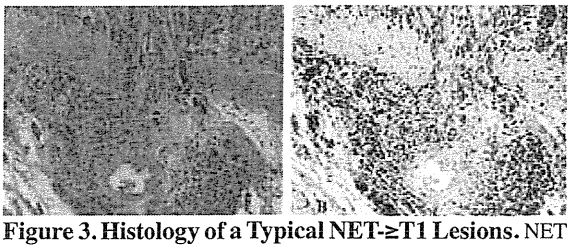

Figure 3. Histology of a Typical NET-≥T1 Lesions. NET invading the fundic submucosa through the muscularis mucosa in an Hp-infected Mongolian gerbil treated with PPI (5mg/kg/day). (A:H&E staining, B:immunostaining for CgA) (×200)

Table 1. Incidences of ECL Lesions in Mongolian Gerbils

Groups	Lansoprazole	Incidence 50 wks		Incidence 100 wks	
		NET-≥T1	NET-Tis*	NET-≥T1	NET-Tis*
Control	-	0/4[a]	0/4c	0/5[a]	0/5[c]
5PPI	5mg	0/3b	0/3d	0/6[b]	0/6[d]
25PPI	25mg	4/4[a,b]	4/4[c,d]	5/5[a,b]	5/5[c,d]
Hp	-	0/4	3/4	3/5	5/5
Hp+5PPI	5mg	0/4	1/4	2/5	2/5
Hp+25PPI	25mg	3/4	3/4	4/4	4/4

*NET-Tis + hyperplasia; [a,b,c,d]P<0.01 in Fisher's exact test

ECL lesions in the glandular stomachs of MGs

The incidences of the ECL lesions are summarized in Table 1. At 50 weeks, NET-≥T1 lesions occurred in the groups given PPI at high dose (25PPI and HP+25PPI), but not detected in the Control, 5PPI, Hp, and Hp+5PPI groups. Regarding hyperplasia and NET-Tis, they were detected in the Hp-infected (Hp, Hp+5PPI, and Hp+25PPI) and 25PPI groups. There were the statistical differences between Control and 25PPI, as well as 5PPI and 25PPI groups, while there were no statistical differences between Hp-infected groups.

At 100 weeks, NET-≥T1 lesions occurred in the Hp-infected (Hp, Hp+25PPI, and Hp+25PPI) and 25PPI groups. Hyperplasia and NET-Tis were also detected in the Hp-infected (Hp, Hp+5PPI, and Hp+25PPI) and 25PPI groups. There were the statistical differences between Control and 25PPI and also between the 5PPI and 25PPI groups, while there were no statistical differences between Hp-infected groups.

Areas of ECL lesions in the glandular stomachs of MGs

The results for areas of ECL lesions are summarized in Figure 4. Values for ECL lesions were 0±0 (×10-2mm2/cm, average±SE), 0±0, 4.63±0.582, 0.0316±0.0140, 0.0102±0.0102 and 1.79±0.738 in Control, 5PPI, 25PPI, Hp, Hp+5PPI and Hp+25PPI at 50 weeks, respectively. At 100 weeks they were 0±0, 0±0, 11.57±3.62, 1.613±0.846, 1.380±1.006 and 16.51±6.55. The areas of ECL lesions were increased by Hp infection (P<0.05, Control vs. Hp)

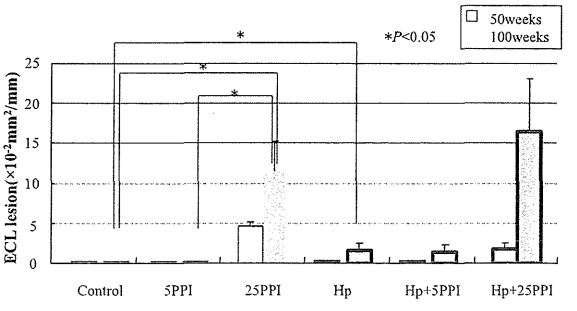

Figure 4. Areas of ECL Lesions in Each Group

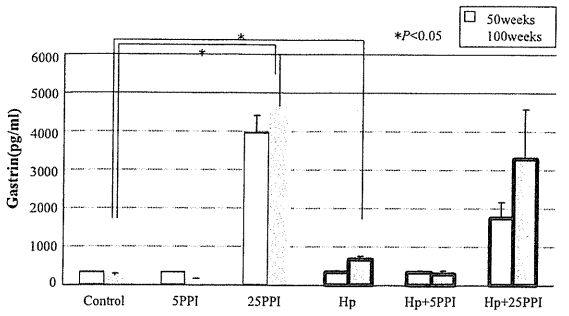

Figure 4. Serum Gastrin Levels in Each Group

and by 25PPI but not 5PPI. The areas of ECL lesions in Hp+25PPI group were greater than those in the Hp one, but without statistical significance.

Serum gastrin levels at 50 and 100 weeks

The serum gastrin levels are summarized in Figure 5. Values for the serum gastrin were 320±32 (pg/ml, average±SE), 320±26, 3950±483, 315±62, 315±56 and 1752±415 for Control, 5PPI, 25PPI, Hp, Hp+5PPI and Hp+25PPI groups at 50 weeks, respectively. At 100 weeks they were 246±65, 167±20, 4680±1324, 670±91, 277±93 and 3275±1328, respectively. The serum gastrin level was increased by Hp infection ($P<0.05$, Control vs. Hp) and by 25PPI (Fig.5). There were no significant differences between Hp-infected groups at 100 weeks.

Relationship between serum gastrin and ECL lesions

The areas of ECL lesions strongly correlated with serum gastrin levels ($P<0.01$, correlation coefficient=0.666). The serum gastrin level in the MGs with ECL lesions was 2951.9±505.3 (pg/ml, average±SE), significantly increased from the average in MGs without ECL lesions (282.8±24.7) ($P<0.01$).

Discussion

We here found that no stomach cancers developed in the Hp+PPI and PPI groups, although hypergastrinaemia is known to be induced by long-term PPI administration in the MG model. It has been reported that the long-term PPI use is associated with an increased incidence of atrophic gastritis (Kuipers et al., 1996), a precursor condition for stomach cancer (Uemura et al., 2001; YeNyren, 2003). However, long-term PPI treatment has not been documented to hasten the development of stomach cancer (Laine et al., 2000). Therefore long term PPI treatment is a safe therapy for acid peptic disorders (Kuipers, 2006), although, the development of atrophic gastritis in Hp-positive patients treated with PPIs, with the long-term concern of stomach cancer development, remains controversial (GillenMcColl, 2001). Gastric secretion may decrease during gastric cancer development in rat (Bralow et al., 1970), and long-lasting iatrogenic hypergastrinemia due to PPI might be expected to increase the occurrence of stomach cancer in the long-term (Waldum et al., 2005). Regarding the MG model, the gerbil can be easily infected with Hp, and the resultant chronic active gastritis, peptic ulcers, and intestinal metaplasia closely resemble lesions apparent in man (Hirayama et al., 1996; Tatematsu et al., 2005). The Hp-infected and chemical carcinogen-treated MG has proved very useful for the analysis of stomach carcinogenesis, providing clear evidence that Hp exerts a promoting effect on stomach carcinogenesis (Tatematsu et al., 2005). Indeed, Hp infection has been reported to induce the development of cancers in the glandular stomach of MGs (Honda et al., 1998; Watanabe et al., 1998; Hirayama et al., 1999). Thus, we consider that the finding of no cancerous lesions in the Hp-infected and uninfected stomach with PPI treatment, suggests that PPI is not a carcinogen. However, it was reported that the use of high dose (100mg/kg/day) omeprazole induced gastric adenocarcinoma (Hagiwara, 2011). This difference may be due to difference of the medicines or the doses. Further studies for the risk of carcinogenesis of PPI are needed.

In the present study, suppression of gastric acid by high-dose PPI (25PPI) induced hypergastrinaemia, and NET occurrence, although no NET-≥T1 tumor development was observed in the low-dose PPI (5PPI) groups. In rats, hypergastrinaemia is associated with an increased risk of stomach carcinoid (Havu, 1986). Gastric carcinoids also occur in patients with type-A chronic gastritis and the Zollinger-Ellison syndrome when it is associated with multiple endocrine neoplasia-1 (Borch et al., 1985; Borch et al., 1987; Solcia et al., 1990). Pronounced acid suppression has been shown to lead to elevated serum gastrin in many individuals (Lanzon-Miller et al., 1987). Whereas the relation between carcinoids and hypergastrinanemia induced by PPI has not been made clear in human, the differences between human and rodents need to be considered. Firstly, rats demonstrate a relatively greater increase in serum gastrin levels in response to inhibition of gastric acid secretion than do humans. In the present study, the serum gastrin level in the 25PPI group was approximately twentyfold the group control value. In contrast, long-term use of proton pump inhibitor therapy generally results in a two- to fourfold increase in gastrin serum levels in man (Freston, 1992). Patients with carcinoids associated with type-A chronic gastritis have marked long-standing hypergastrinanemia (>500pg/mL; normal limit <100pg/mL). Actually, in this study the MGs receiving 5PPI had a similar serum gastrin level as the control group, and no ECL lesions, including NETs, occurred. Secondly, several reports suggested that humans have a much lower density of enterochromaffin-like cells than do rats (Hakanson et al., 1976; Simonsson et al., 1988). Thus, we consider that the PPI use at clinical dose is safe, and no NET lesions would be expected to develop with standard dosing.

We also have shown that PPI at low dose (5PPI) is not associated with the occurrence of ECL lesions and serum gastrin level in the Hp-infected MG (Fig. 5). In the MG model, we and others have shown that long-term Hp colonization produces hyperplasia of gastrin-producing antral G-cells and carcinoid tumors (Hirayama et al., 1999; Kagawa et al., 2002; Cao et al., 2008). We also have previously reported that Hp infection induces NET development, and Hp eradication prevents its occurrence in the glandular MG stomach (Cao et al., 2008). In humans, Hp-infected individuals show hypergastrinemia, possibly due to alteration of G-cell function by specific Hp-products (McColl et al., 1997), or because of inflammation-

stimulating gastrin hypersecretion (McGowan et al., 1996). In Japan, Hp infection and hypergastrinemia are found in patients with NET without autoimmune gastritis, suggesting that Hp infection may induce corporal mucosal atrophy and hypergastrinemia that can produce a NET over time (Sato et al., 2002). It has been reported that Hp is an important factor in the progression of fundic gastritis and the development of ECL cell hyperplasia during long-term treatment with lansoprazole (Eissele et al., 1997). The Hp-induced hypergastrinemia and stomach NETs are thought to be closely linked (Bordi et al., 1991; Nilsson et al., 1993). Thus, we consider that Hp infection induces NET development, and PPI use at a clinical dose would have no influence on its occurrence in man.

After Hp infection, glands in the glandular stomach of MG start to proliferate into the submucosa, disrupting the laminal muscularis mucosa (Nozaki et al., 2002). Resultant lesions, termed heterotopic proliferative glands (HPGs) frequently develop with Hp infection in the glandular stomach of MG (Nozaki et al., 2002). HPGs often resemble differentiated adenocarcinomas, but do not appear to be malignant (Tatematsu et al., 2005). In our study, HPGs occurred in the Hp group, while no HPGs occurred in HP+5PPI and HP+25PPI groups (data not shown).

In 2006, a working group of the ENETS published a proposal for a TNM staging classification of NETs of the foregut (Rindi et al., 2006). Subsequent publication of a TNM staging classification of the midgut and hindgut NETs from the same group followed in 2007 (Rindi et al., 2007), both clearly distinguishing them from other tumors, including carcinomas. In the present study, we used the above-mentioned classification with some modifications. We have previously shown that most stomach NETs exhibit the e-G type in humans, in contrast to the e-N type predominating in MGs (Takenaka et al., 2007; Hirata et al., 2009). However, in the present study, ECL lesions had the e-N type, suggesting development from progenitor cells specializing towards the endocrine cell lineage in glandular ducts exhibiting neither gastric nor intestinal phenotypic expression.

In conclusion, PPI at low dose has no influence on development of carcinomas and NETs in the Hp-infected and uninfected glandular MG stomach, suggesting that PPI is clinically safe. However, at high dose it increases the NET development and serum gastrin level in the MG model, and clarification of whether these phenomenon are important for stomach tumorigenesis is needed.

Acknowledgments

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References

Bardram L, Thomsen P, Stadil F (1986). Gastric endocrine cells in omeprazole-treated and untreated patients with the Zollinger-Ellison syndrome. *Digestion*, **35**, 116-22.

Borch K, Renvall H, Kullman E, et al (1987). Gastric carcinoid associated with the syndrome of hypergastrinemic atrophic gastritis. A prospective analysis of 11 cases. *Am J Surg Pathol*, **11**, 435-44.

Borch K, Renvall H, Liedberg G (1985). Gastric endocrine cell hyperplasia and carcinoid tumors in pernicious anemia. *Gastroenterology*, **88**, 638-48.

Bordi C, Yu JY, Baggi MT, et al (1991). Gastric carcinoids and their precursor lesions. A histologic and immunohistochemical study of 23 cases. *Cancer*, **67**, 663-72.

Bralow SP, Gruenstein M, Meranze DR, et al (1970). Adenocarcinoma of glandular stomach and duodenum in Wistar rats ingesting N-methyl-N'-nitro-N-nitrosoguanidine, histopathology and associated secretory changes. *Cancer Res*, **30**, 1215-22.

Cao L, Mizoshita T, Tsukamoto T, et al (2008). Development of carcinoid tumors of the glandular stomach and effects of eradication in Helicobacter pylori-infected Mongolian gerbils. *Asian Pac J Cancer Prev*, **9**, 25-30.

Eissele R, Brunner G, Simon B, et al (1997). Gastric mucosa during treatment with lansoprazole: Helicobacter pylori is a risk factor for argyrophil cell hyperplasia. *Gastroenterology*, **112**, 707-17.

Freston JW (1992). Clinical significance of hypergastrinaemia: relevance to gastrin monitoring during omeprazole therapy. *Digestion*, **51**, 102-14.

Gillen DK, McColl E (2001). Problems associated with the clinical use of proton pump inhibitors. *Pharmacol Toxicol*, **89**, 281-6.

Hagiwara T, Mukaisho K, Nakayama T, et al (2011). Long-term proton pump inhibitor administration worsens atrophic corpus gastritis and promotes adenocarcinoma development in Mongolian gerbils infected with *Helicobacter pylori*. Gut, in press.

Hakanson R, Larsson LI, Liedberg G, et al (1976). Effects of antrectomy or porta-caval shunting on the histamine-storing endocrine-like cells in oxyntic mucosa of rat stomach. A fluorescence histochemical, electron microscopic and chemical study. *J Physiol*, **259**, 785-800.

Havu N (1986). Enterochromaffin-like cell carcinoids of gastric mucosa in rats after life-long inhibition of gastric secretion. *Digestion*, **35**, 42-55.

Hirata Y, Mizoshita T, Mizushima T, et al (2009). Gastric-and-intestinal mixed endocrine cell phenotypic expression of carcinoid tumors in the rectum. *Oncol Rep*, **21**, 107-12.

Hirayama F, Takagi S, Iwao E, et al (1999). Development of poorly differentiated adenocarcinoma and carcinoid due to long-term Helicobacter pylori colonization in Mongolian gerbils. *J Gastroenterol*, **34**, 450-4.

Hirayama F, Takagi S, Yokoyama Y, et al (1996). Establishment of gastric Helicobacter pylori infection in Mongolian gerbils. *J Gastroenterol*, **31**, 24-8.

Honda S, Fujioka T, Tokieda M, et al (1998). Development of Helicobacter pylori-induced gastric carcinoma in Mongolian gerbils. *Cancer Res*, **58**, 4255-9.

Kagawa J, Honda S, Kodama M, et al (2002). Enterocromaffin-like cell tumor induced by Helicobacter pylori infection in Mongolian gerbils. *Helicobacter*, **7**, 390-7.

Klinkenberg-Knol EC, Festen HP, Jansen JB, et al (1994). Long-term treatment with omeprazole for refractory reflux esophagitis: efficacy and safety. *Ann Intern Med*, **121**, 161-7.

Kuipers EJ (2006). Proton pump inhibitors and gastric neoplasia. *Gut*, **55**, 1217-21.

Kuipers EJ, Lundell L, Klinkenberg-Knol EC, et al (1996). Atrophic gastritis and *Helicobacter pylori* infection in patients with reflux esophagitis treated with omeprazole or fundoplication. *N Engl J Med*, **334**, 1018-22.

Laine L, Ahnen D, McClain C, et al (2000). Review article: potential gastrointestinal effects of long-term acid

suppression with proton pump inhibitors. *Aliment Pharmacol Ther*, **14**, 651-68.

Lamberts R, Creutzfeldt W, Stockmann F, et al (1988). Long-term omeprazole treatment in man: effects on gastric endocrine cell populations. *Digestion*, **39**, 126-35.

Lanzon-Miller S, Pounder RE, Hamilton MR, et al (1987). Twenty-four-hour intragastric acidity and plasma gastrin concentration before and during treatment with either ranitidine or omeprazole. *Aliment Pharmacol Ther*, **1**, 239-51.

McColl KE, E-Omar EM, Gillen D (1997). Alterations in gastric physiology in *Helicobacter pylori* infection: causes of different diseases or all epiphenomena? *Ital J Gastroenterol Hepatol*, **29**, 459-64.

McGowan CC, T. L. CoverM. J Blaser (1996). Helicobacter pylori and gastric acid: biological and therapeutic implications. *Gastroenterology*, **110**, 926-38.

Mizoshita T, Tsukamota T, Nakanishi H, et al (2003). Expression of Cdx2 and the phenotype of advanced gastric cancers: relationship with prognosis. *J Cancer Res Clin Oncol*, **129**, 727-34.

Mizoshita T, Tsukamoto T, Takenaka Y, et al (2006). Gastric and intestinal phenotypes and histogenesis of advanced glandular stomach cancers in carcinogen-treated, Helicobacter pylori-infected Mongolian gerbils. *Cancer Sci*, **97**, 38-44.

Nilsson O, Wangberg B, Johansson L, et al (1993). Rapid induction of enterochromaffinlike cell tumors by histamine2-receptor blockade. *Am J Pathol*, **142**, 1173-85.

Nozaki K, Shimizu N, Tsukamoto T, et al (2002). Reversibility of heterotopic proliferative glands in glandular stomach of Helicobacter pylori-infected Mongolian gerbils on eradication. *Jpn J Cancer Res*, **93**, 374-81.

Poulsen AH, Christensen S, McLaughlin JK, et al (2009). Proton pump inhibitors and risk of gastric cancer: a population-based cohort study. *Br J Cancer*, **100**, 1503-7.

Rindi G, Kloppel G, Alhman H, et al (2006). TNM staging of foregut (neuro)endocrine tumors: a consensus proposal including a grading system. *Virchows Arch*, **449**, 395-401.

Rindi G, Kloppel G, Couvelard A, et al (2007). TNM staging of midgut and hindgut (neuro) endocrine tumors: a consensus proposal including a grading system. *Virchows Arch*, **451**, 757-62.

Sasaki M, Mizoshita T, Mizushima T, et al (2007). Effect of plaunotol in combination with clarithromycin against clarithromycin-resistant Helicobacter pylori in vitro and in vivo. *J Antimicrob Chemother*, **60**, 1060-3.

Sato Y, Iwafuchi M, Ueki J, et al (2002). Gastric carcinoid tumors without autoimmune gastritis in Japan: a relationship with Helicobacter pylori infection. *Dig Dis Sci*, **47**, 579-85.

Simonsson M, Eriksson S, Hakanson R, et al (1988). Endocrine cells in the human oxyntic mucosa. A histochemical study. *Scand J Gastroenterol*, **23**, 1089-99.

Solcia E, Capella C, Fiocca R, et al (1990). Gastric argyrophil carcinoidosis in patients with Zollinger-Ellison syndrome due to type 1 multiple endocrine neoplasia. A newly recognized association. *Am J Surg Pathol*, **14**, 503-13.

Takenaka Y, Tsukamoto T, Mizoshita T, et al (2006). *Helicobacter pylori* infection stimulates intestinalization of endocrine cells in glandular stomach of Mongolian gerbils. *Cancer Sci*, **97**, 1015-22.

Takenaka Y, Tsukamoto T, Mizoshita T, et al (2007). Gastric and intestinal phenotypic correlation between exocrine and endocrine components in human stomach tumors. *Histol Histopathol*, **22**, 273-84.

Tatematsu M, Tsukamoto T, Mizoshita T (2005). Role of *Helicobacter pylori* in gastric carcinogenesis: the origin of gastric cancers and heterotopic proliferative glands in Mongolian gerbils. *Helicobacter*, **10**, 97-106.

Uemura N, Okamoto S, Yamamoto S, et al (2001). *Helicobacter pylori* infection and the development of gastric cancer. *N Engl J Med*, **345**, 784-9.

Waldum HL, Gustafsson B, Fossmark R, et al (2005). Antiulcer drugs and gastric cancer. *Dig Dis Sci*, **50**, S39-44.

Watanabe T, Tada M, Nagai H, et al (1998). *Helicobacter pylori* infection induces gastric cancer in Mongolian gerbils. *Gastroenterology*, **115**, 642-8.

Ye W, Nyren O (2003). Risk of cancers of the oesophagus and stomach by histology or subsite in patients hospitalised for pernicious anaemia. *Gut*, **52**, 938-41.

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Induction of glandular stomach cancers in *Helicobacter pylori*-infected Mongolian Gerbils by 1-nitrosoindole-3-acetonitrile

Satoshi Matsubara[1,2], Shinji Takasu[1], Tetsuya Tsukamoto[3], Michihiro Mutoh[1], Shuichi Masuda[4], Takashi Sugimura[1], Keiji Wakabayashi[1,4] and Yukari Totsuka[1]

[1] Cancer Prevention Basic Research Project, National Cancer Center Research Institute, Chuo-ku, Tokyo, Japan
[2] Food Research Department, Yakult Central Institute for Microbiological Research, Kunitachi-shi, Tokyo, Japan
[3] Department of Pathology and Matrix Biology, Mie University Graduate School of Medicine, Tsu-shi, Mie, Japan
[4] Department of Food and Nutritional Sciences, Graduate School of Nutritional and Environmental Sciences, University of Shizuoka, Yada, Shizuoka, Japan

Helicobacter pylori (_H. pylori_) infection and high intake of various traditional salt-preserved foods are regarded as risk factors for human gastric cancer. We previously reported that Chinese cabbage contains indole compounds, such as indole-3-acetonitrile, a mutagen precursor. 1-Nitrosoindole-3-acetonitrile (NIAN), formed by the treatment of indole-3-acetonitrile with nitrite under acidic conditions, shows direct-acting mutagenicity. In the present study, NIAN administration by gavage to Mongolian gerbils (MGs) at the dose of 100 mg/kg two times a week resulted in three adduct spots (1.6 adducts/10^8 nucleotides in total), detected in DNA samples from the glandular stomach by ^{32}P-postlabeling methods. Treatment with six consecutive doses of 100 mg/kg of NIAN, two times a week for 3 weeks, induced well—and moderately—differentiated glandular stomach adenocarcinomas in the MGs at the incidence of 31% under _H. pylori_ infection at 54–104 weeks. Such lesions were not induced in MGs given broth alone, broth + NIAN or infection with _H. pylori_ alone. Thus, endogenous carcinogens formed from nitrosation of indole compounds could be critical risk factors for human gastric cancer development under the influence of _H. pylori_ infection.

Gastric cancer is the second most frequent cause of cancer death worldwide.[1] Although gastric cancer has become a relatively rare cancer in North America and most Northern and Western European countries, it remains common in East Asia, Eastern Europe, Russia, and selected areas of Central and South America.[2] *Helicobacter pylori* (*H. pylori*) is a well-established major risk factor for gastric cancer,[3–5] and the prevalence of *H. pylori* infection in East Asia countries, including Japan and Korea is reported to be relatively high.[6,7] In addition, the risk of gastric cancer is increased with a high intake of various traditional salt-preserved foods.[3] In fact, pickled vegetable consumption is reported to increase gastric cancer risk in Japan and Korea.[8–10] In Korea, kimchi, commonly prepared with Chinese cabbage or radish, is a traditional and popular food, which contains high levels of nitrate (median 1550 mg/kg).[11] Furthermore, Chinese cabbage is well known as a pickled vegetable commonly consumed in Japan. Moreover, ingestion of nitrate, mainly from food, is suggested to correlate with mortality from gastric cancer.[12–14] Ingested nitrate is mainly converted to nitrite by bacteria in the oral cavity after secretion into saliva.[15] Carcinogenic *N*-nitroso compounds can be formed from nitrite and secondary amines under acidic conditions. Furthermore, direct-acting *N*-nitroso compounds, such as *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (MNNG)[16] and *N*-methyl-*N*-nitrosourea (MNU),[17] are known to induce cancer in the glandular stomach of experimental animals. Thus, it is suggested that *N*-nitroso compounds that are formed in the stomach under acidic conditions could be positively associated with the risk of gastric cancer. Nitric oxide, formed by nitric oxide synthase, is also reported to contribute to production of *N*-nitroso compounds.[18]

We have previously reported that treatments of various foodstuffs with nitrite under acidic conditions produce direct-acting mutagens towards *Salmonella* tester strains.[19,20] Among those foodstuffs, Chinese cabbage is shown to contain three indole compounds, indole-3-acetonitrile, 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-

Key words: gastric cancer, *Helicobacter pylori*, Mongolian gerbil 1-nitrosoindole-3-acetonitrile, indole-3-acetonitrile
Abbreviations: DMSO: dimethyl sulfoxide; H&E: hematoxylin and eosin; *H. pylori*: *Helicobacter pylori*; MG: Mongolian gerbil; MNNG: *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine; MNU: *N*-methyl-*N*-nitrosourea; NIAN: 1-nitrosoindole-3-acetonitrile.
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Correspondence to: Yukari Totsuka, Cancer Prevention Basic Research Project, National Cancer Center Research Institute, 1-1, Tsukiji 5-chome, Chuo-ku, Tokyo 104-0045, Japan, Tel.: +81-3-3542-2511, Fax: +81-3-3543-9305, E-mail: ytotsuka@ncc.go.jp

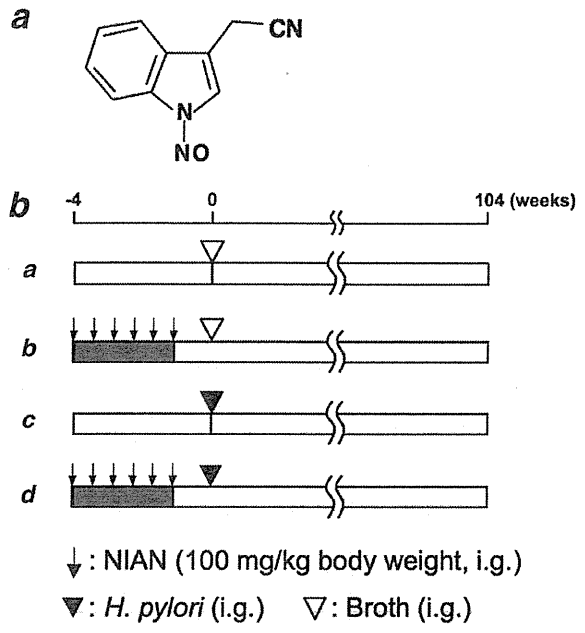

Figure 1. Chemical structure of NIAN and experimental protocol for the carcinogenicity study. (*a*) Chemical structure of NIAN. (*b*) Male 6-week-old MGs were orally administered NIAN (100 mg/kg) in 50% DMSO (groups B and D) or 50% DMSO alone (groups A and C) two times a week for 3 weeks. One week after the final administration, the animals were inoculated with *H. pylori* (ATCC 43504) (groups C and D) or sterilized broth (groups A and B).

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.

Material and Methods

Materials

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

Preparation of NIAN

The chemical structure of NIAN is shown in Figure 1*a*. Indole-3-acetonitrile in 27 mM citrate-phosphate buffer (pH 3.0) was treated with 50 mM sodium nitrite for 1 hr 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 hr 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 hr light–dark cycle and were allowed free access to commercial diet (CE-2; CLEA Japan, Tokyo, Japan) and water.

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 hr 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 1*b*. The animals were randomly divided into four groups (groups A–D). Groups A and C were given 50% DMSO without NIAN (0.5 ml) whereas 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 3 weeks. At one week after the last administration, the

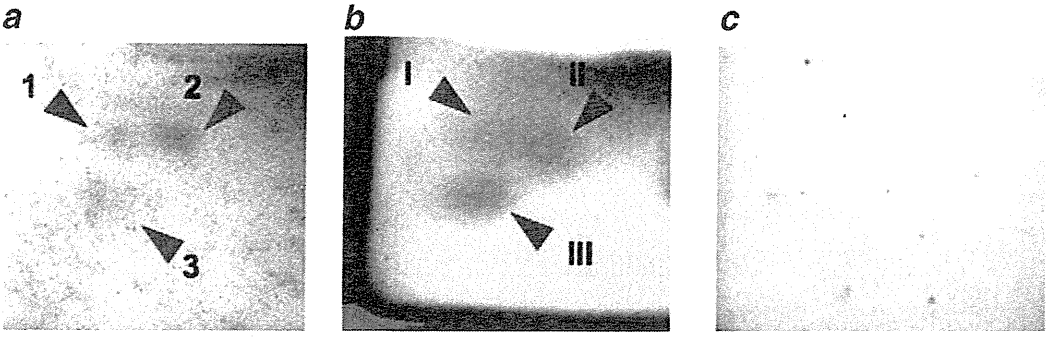

Figure 2. Autoradiograms of NIAN-DNA adducts in glandular stomach of MGs or calf thymus DNA treated with NIAN. Adducts were analyzed by ^{32}P-postlabeling method, as described in the Material and Methods. DNA samples were isolated from glandular stomach of MGs (*a*) or calf thymus DNA (*b*) after treatment with NIAN. DNA samples were also prepared from glandular stomach of MGs without NIAN treatment (*c*). Arrowheads indicate adducts. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

animals of groups C and D were given an intragastric inoculation of *H. pylori* broth culture (0.5 ml, 0.9 × 10^8 CFU/animal) whereas 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) treated with NIAN (3 mg) for 12 hr 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, 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 Tukey'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 (Fig. 2*a*). 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

Table 1. *H. pylori* infection induced-gastritis in MGs

Group	Treatment	Effective No.	Stomach wet weight (g)	Inflammation score
A	Broth	15	0.647 ± 0.097	0
B	NIAN + Broth	22	0.631 ± 0.094	0
C	*H. pylori*	18	1.432 ± 0.445*	2.22 ± 0.43*
D	NIAN + *H. pylori*	26	1.483 ± 0.445*	2.38 ± 0.64*

*$p < 0.01$ versus group A and B.
Values for results are expressed as averages ± SD.

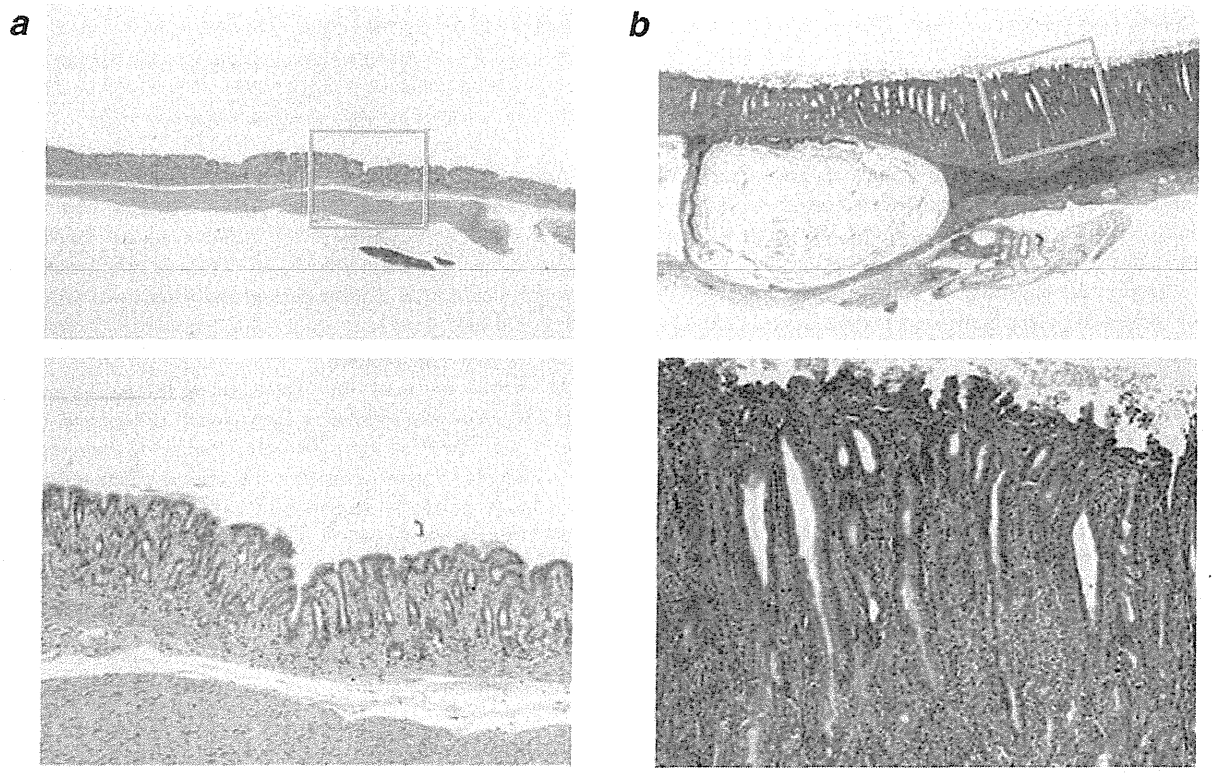

Figure 3. Macroscopic and microscopic views of gastritis in MGs infected or uninfected with *H. pylori*. (*a*) Normal gastric mucosa in group A. (*b*) Severe infiltration of many inflammatory cells with development of heterophilic proliferative glands in group C; H&E staining, ×40. Yellow boxes are shown at greater magnification below, ×200.

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, Fig. 2*b*). In the case of DNA samples derived from control animals, no adduct spots were seen on the TLC sheets (Fig. 2*c*).

Macroscopical 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

Table 2. Incidence of glandular stomach adenocarcinoma in MGs

Group	Treatment	Effective No.	No. of animals with glandular stomach adenocarcinoma (%)		
			Total	Well dif.	Moderately dif.
A	Broth	15	0 (0)	0 (0)	0 (0)
B	NIAN + Broth	22	0 (0)	0 (0)	0 (0)
C	*H. pylori*	18	0 (0)	0 (0)	0 (0)
D	NIAN + *H. pylori*	26	8 (31)*	7 (27)	1 (4)

Well dif., well differentiated adenocarcinoma; Moderately dif., moderately differentiated adenocarcinoma.
*$p < 0.05$ versus group A and C and $p < 0.01$ versus group B.

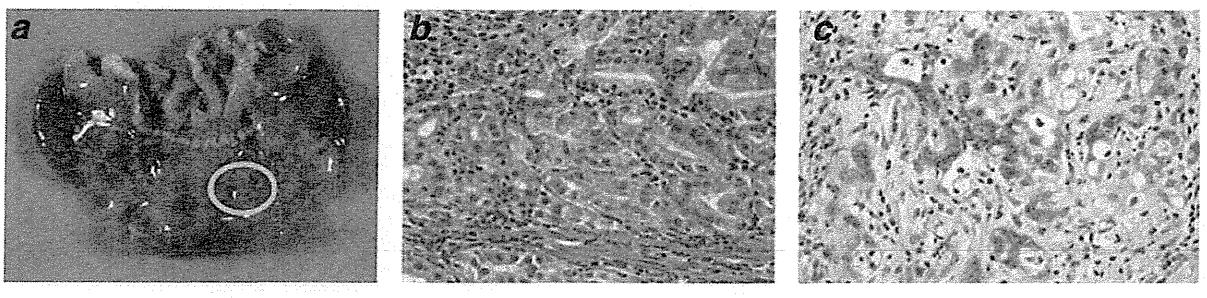

Figure 4. Histological findings of gastric adenocarcinoma in the animals treated with both NIAN and *H. pylori*. (*a*) Typical macrograph of a stomach. The yellow circle shows the suspected lesion of gastric cancer. (*b*) Well differentiated adenocarcinoma. (*c*) Moderately differentiated adenocarcinoma. (*b* and *c*) H&E staining, ×400.

the animals infected with *H. pylori* (groups C and D) (Fig. 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 eight 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 (Fig. 4*a*). The observed adenocarcinomas in seven animals were of well differentiated (Fig. 4*b*), and a moderately differentiated lesion was observed in one animal (Fig. 4*c*). 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 $p < 0.05$, 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 one animal (1/15 = 7%) in group A, three animals (3/22 = 14%) in group B, two animals (2/18 = 11%) in group C and five animals (5/26 = 19%) in group D. A hemangioma was also observed in a kidney of one 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 analog, 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] On the basis of 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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References

1. Boyle P, Ferlay J. Cancer incidence and mortality in Europe, 2004. *Ann Oncol* 2005;16:481–8.
2. Bertuccio P, Chatenoud L, Levi F, Praud D, Ferlay J, Negri E, Malvezzi M, La Vecchia C. Recent patterns in gastric cancer: a global overview. *Int J Cancer* 2009;125:666–73.
3. Tsugane S, Sasazuki S. Diet and the risk of gastric cancer: review of epidemiological evidence. *Gastric Cancer* 2007;10:75–83.
4. Parsonnet J, Friedman GD, Vandersteen DP, Chang Y, Vogelman JH, Orentreich N, Sibley RK. Helicobacter pylori infection and the risk of gastric carcinoma. *N Engl J Med* 1991;325:1127–31.

5. Uemura N, Okamoto S, Yamamoto S, Matsumura N, Yamaguchi S, Yamakido M, Taniyama K, Sasaki N, Schlemper RJ. Helicobacter pylori infection and the development of gastric cancer. *N Engl J Med* 2001;345:784–9.
6. Parkin DM. The global health burden of infection-associated cancers in the year 2002. *Int J Cancer* 2006;118:3030–44.
7. Yim JY, Kim N, Choi SH, Kim YS, Cho KR, Kim SS, Seo GS, Kim HU, Baik GH, Sin CS, Cho SH, Oh BH. Seroprevalence of Helicobacter pylori in South Korea. *Helicobacter* 2007;12:333–40.
8. Tajima K, Tominaga S. Dietary habits and gastro-intestinal cancers: a comparative case-control study of stomach and large intestinal cancers in Nagoya, Japan. *Jpn J Cancer Res* 1985;76:705–16.
9. Kim HJ, Chang WK, Kim MK, Lee SS, Choi BY. Dietary factors and gastric cancer in Korea: a case-control study. *Int J Cancer* 2002;97:531–5.
10. Nan HM, Park JW, Song YJ, Yun HY, Park JS, Hyun T, Youn SJ, Kim YD, Kang JW, Kim H. Kimchi and soybean pastes are risk factors of gastric cancer. *World J Gastroenterol* 2005;11:3175–81.
11. Seel DJ, Kawabata T, Nakamura M, Ishibashi T, Hamano M, Mashimo M, Shin SH, Sakamoto K, Jhee EC, Watanabe S. N-Nitroso compounds in two nitrosated food products in southwest Korea. *Food Chem Toxicol* 1994;32:1117–23.
12. Jakszyn P, Bingham S, Pera G, Agudo A, Luben R, Welch A, Boeing H, Del Giudice G, Palli D, Saieva C, Krogh V, Sacerdote C, *et al.* Endogenous *versus* exogenous exposure to N-nitroso compounds and gastric cancer risk in the European Prospective Investigation into Cancer and Nutrition (EPIC-EURGAST) study. *Carcinogenesis* 2006;27:1497–501.
13. Joossens JV, Hill MJ, Elliott P, Stamler R, Lesaffre E, Dyer A, Nichols R, Kesteloot H. Dietary salt, nitrate and stomach cancer mortality in 24 countries. European Cancer Prevention (ECP) and the INTERSALT Cooperative Research Group. *Int J Epidemiol* 1996;25:494–504.
14. van Velzen AG, Sips AJ, Schothorst RC, Lambers AC, Meulenbelt J. The oral bioavailability of nitrate from nitrate-rich vegetables in humans. *Toxicol Lett* 2008; 181:177–81.
15. Spiegelhalder B, Eisenbrand G, Preussmann R. Influence of dietary nitrate on nitrite content of human saliva: possible relevance to *in vivo* formation of N-nitroso compounds. *Food Cosmet Toxicol* 1976;14: 545–8.
16. Sugimura T, Fujimura S. Tumour production in glandular stomach of rat by N-methyl-N′-nitro-N-nitrosoguanidine. *Nature* 1967;216:943–4.
17. Hirota N, Aonuma T, Yamada S, Kawai T, Saito K, Yokoyama T. Selective induction of glandular stomach carcinoma in F344 rats by N-methyl-N-nitrosourea. *Jpn J Cancer Res* 1987;78:634–8.
18. Ohshima H, Bartsch H. Chronic infections and inflammatory processes as cancer risk factors: possible role of nitric oxide in carcinogenesis. *Mutat Res* 1994;305:253–64.
19. Wakabayashi K, Nagao M, Ochiai M, Tahira T, Yamaizumi Z, Sugimura T. A mutagen precursor in Chinese cabbage, indole-3-acetonitrile, which becomes mutagenic on nitrite treatment. *Mutat Res* 1985;143:17–21.
20. Wakabayashi K, Nagao M, Ochiai M, Fujita Y, Tahira T, Nakayasu M, Ohgaki H, Takayama S, Sugimura T. Recently identified nitrite-reactive compounds in food: occurrence and biological properties of the nitrosated products. *IARC Sci Publ* 1987:287–91.
21. Wakabayashi K, Nagao M, Tahira T, Saito H, Katayama M, Marumo S, Sugimura T. 1-Nitrosoindole-3-acetonitrile, a mutagen produced by nitrite treatment of indole-3-acetonitrile. *Proc Jpn Acad Ser* 1985;B61: 190–92.
22. Tiedink HG, Davies JA, Visser NA, Jongen WM, van Broekhoven LW. The stability of the nitrosated products of indole, indole-3-acetonitrile, indole-3-carbinol and 4-chloroindole. *Food Chem Toxicol* 1989; 27:723–30.
23. Yamashita K, Wakabayashi K, Kitagawa Y, Nagao M, Sugimura T. ^{32}P-postlabeling analysis of DNA adducts in rat stomach with 1-nitrosoindole-3-acetonitrile, a direct-acting mutagenic indole compound formed by nitrosation. *Carcinogenesis* 1988; 9:1905–7.
24. Furihata C, Ikui E, Matsushima T. DNA single-strand scission in the pyloric mucosa of rat stomach induced by four glandular stomach carcinogens and three other chemicals. *Mutat Res* 1996; 368:1–6.
25. Tatematsu M, Yamamoto M, Iwata H, Fukami H, Yuasa H, Tezuka N, Masui T, Nakanishi H. Induction of glandular stomach cancers in C3H mice treated with N-methyl-N-nitrosourea in the drinking water. *Jpn J Cancer Res* 1993;84: 1258–64.
26. Sugiyama A, Maruta F, Ikeno T, Ishida K, Kawasaki S, Katsuyama T, Shimizu N, Tatematsu M. Helicobacter pylori infection enhances N-methyl-N-nitrosourea-induced stomach carcinogenesis in the Mongolian gerbil. *Cancer Res* 1998;58:2067–9.
27. Shimizu N, Inada K, Nakanishi H, Tsukamoto T, Ikehara Y, Kaminishi M, Kuramoto S, Sugiyama A, Katsuyama T, Tatematsu M. Helicobacter pylori infection enhances glandular stomach carcinogenesis in Mongolian gerbils treated with chemical carcinogens. *Carcinogenesis* 1999;20: 669–76.
28. Matsubara S, Shibata H, Ishikawa F, Yokokura T, Takahashi M, Sugimura T, Wakabayashi K. Suppression of Helicobacter pylori-induced gastritis by green tea extract in Mongolian gerbils. *Biochem Biophys Res Commun* 2003;310: 715–9.
29. Reddy MV, Randerath K. Nuclease P1-mediated enhancement of sensitivity of ^{32}P-postlabeling test for structurally diverse DNA adducts. *Carcinogenesis* 1986;7: 1543–51.
30. Dixon MF, Genta RM, Yardley JH, Correa P. Classification and grading of gastritis. The updated Sydney System. International Workshop on the Histopathology of Gastritis, Houston 1994. *Am J Surg Pathol* 1996;20:1161–81.
31. Lucas LT, Gatehouse D, Shuker DE. Efficient nitroso group transfer from N-nitrosoindoles to nucleotides and 2′-deoxyguanosine at physiological pH. A new pathway for N-nitrosocompounds to exert genotoxicity. *J Biol Chem* 1999;274: 18319–26.
32. Burney S, Caulfield JL, Niles JC, Wishnok JS, Tannenbaum SR. The chemistry of DNA damage from nitric oxide and peroxynitrite. *Mutat Res* 1999;424:37–49.
33. Lucas LT, Gatehouse D, Jones GD, Shuker DE. Characterization of DNA damage at purine residues in oligonucleotides and calf thymus DNA induced by the mutagen 1-nitrosoindole-3-acetonitrile. *Chem Res Toxicol* 2001;14:158–64
34. Vincent AL, Ash LR. Further observations on spontaneous neoplasms in the Mongolian gerbil, Meriones unguiculatus. *Lab Anim Sci* 1978;28:297–300.
35. Okamoto T, Isogai Y, Koizumi T, Fujishiro H, Sato Y. Studies on plant growth regulators, III. Isolation of indole-3-acetonitrile and methyl indole-3-acetate from the neutral fraction of the Moyashi extract. *Chem Pharm Bull* 1967;15: 163–68.
36. Piacek-Llanes BG, Tannenbaum SR. Formation of an activated N-nitroso compound in nitrite-treated fava beans (Vicia faba). *Carcinogenesis* 1982;3: 1379–84.
37. Yang D, Tannenbaum SR, Buchi G, Lee GC. 4-Chloro-6-methoxyindole is the precursor of a potent mutagen (4-chloro-6-methoxy-2-hydroxy-1-nitroso-indolin-3-one oxime) that forms during nitrosation of the fava bean (Vicia faba). *Carcinogenesis* 1984;5:1219–24.
38. Ochiai M, Wakabayashi K, Sugimura T, Nagao M. Mutagenicities of indole and 30 derivatives after nitrite treatment. *Mutat Res* 1986;172:189–97.

39. Wakabayashi K, Ochiai M, Saito H, Tsuda M, Suwa Y, Nagao M, Sugimura T. Presence of 1-methyl-1,2,3,4-tetrahydro-beta-carboline-3-carboxylic acid, a precursor of a mutagenic nitroso compound, in soy sauce. *Proc Natl Acad Sci USA* 1983;80:2912–6.
40. Suzuki T, Mower HF, Friesen MD, Gilibert I, Sawa T, Ohshima H. Nitration and nitrosation of N-acetyl-L-tryptophan and tryptophan residues in proteins by various reactive nitrogen species. *Free Radic Biol Med* 2004;37:671–81.
41. Inoue M, Tsugane S. Epidemiology of gastric cancer in Japan. *Postgrad Med J* 2005;81:419–24.
42. Kobayashi T, Kikuchi S, Lin Y, Yagyu K, Obata Y, Ogihara A, Hasegawa A, Miki K, Kaneko E, Mizukoshi H, Sakiyama T, Tenjin H. Trends in the incidence of gastric cancer in Japan and their associations with Helicobacter pylori infection and gastric mucosal atrophy. *Gastric Cancer* 2004;7:233–9.
43. Plummer M, Franceschi S, Munoz N. Epidemiology of gastric cancer. *IARC Sci Publ* 2004;157:311–26.

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Clinical Cancer Research

Cancer Therapy: Clinical

See commentary by Thompson and Gerner, p. 3509

Randomized Double-Blind Trial of Sulindac and Etodolac to Eradicate Aberrant Crypt Foci and to Prevent Sporadic Colorectal Polyps

Tetsuji Takayama[1], Hiroyuki Nagashima[5], Masahiro Maeda[2], Shuichi Nojiri[2], Michiaki Hirayama[3], Yoichiro Nakano[6], Yasuo Takahashi[7], Yasushi Sato[5], Hitoshi Sekikawa[4], Mitsuru Mori[8], Tomoko Sonoda[8], Tetsuo Kimura[1], Junji Kato[5], and Yoshiro Niitsu[9]

Abstract

Purpose: On the basis of the results of our preliminary trial suggesting that aberrant crypt foci (ACF) could be eradicated by short-term administration of sulindac, in the present study, we explored the feasibility of using ACF as surrogate markers for chemoprevention of colorectal cancer.

Experimental design: Randomly assigned to sulindac (300 mg daily), etodolac (400 mg daily), and placebo groups were 189 subjects without polyps or who had undergone polypectomy. Drugs were administered for 2 months. ACF in the rectal region were counted by magnifying endoscopy. Occurrence of polyps was evaluated at 12 months. A planned interim analysis was conducted.

Results: ACF number at 2 months was significantly suppressed in the sulindac group ($P = 0.0075$), but not in the etodolac group ($P = 0.73$). In the sulindac group, the numbers of adenomas plus hyperplastic polyps (total polyps) and adenomas at 12 months were significantly ($P = 0.02$) and marginally ($P = 0.064$) lower, respectively, in comparison with the placebo group; no such difference was observed in the etodolac group. In analysis of only polypectomized subjects, the numbers of total polyps and adenomas in the sulindac group were even more markedly lower, with P values of 0.014 and 0.034, respectively. A similar tendency was confirmed by analyses of the incidence of polyps at 12 months. Suppression rates of total polyps and adenomas in ACF responders to sulindac were significantly greater than in nonresponders. In all groups, compliance was more than 90% and no intolerable adverse effects were observed.

Conclusions: ACF may be useful as surrogate lesions for chemoprevention of colorectal cancer. *Clin Cancer Res; 17(11); 3803–11. ©2011 AACR.*

Introduction

Despite the recent introduction of various therapeutic modalities, colorectal cancer remains one of the most common causes of cancer deaths worldwide (1–3). Several chemopreventive modalities have been introduced in the past decade or so, and agents such as calcium (4), cyclooxygenase-2 (COX-2) inhibitors (5,6), aspirin (7–9), and sulindac, a nonsteroidal anti-inflammatory drug (NSAID; ref. 10), have been shown to inhibit the recurrence of colorectal polyps after polypectomy or the development of colorectal polyps. A major obstacle in the development of chemopreventive drugs is that they are administered for relatively long periods to cancer-free subjects; therefore, poor compliance and adverse effects frequently hamper trials.

We previously showed that aberrant crypt foci (ACF), tiny lesions that expressed the K-ras mutation and are identifiable only by magnifying endoscopy, correlated in number and size with the number of adenomas in patients with adenoma, and proposed these lesions to be precursors of colorectal adenoma and cancer (11–13). Subsequently, several investigators have confirmed our proposal of the ACF-adenoma-carcinoma sequence through demonstrating a close relationship between ACF and adenomas or cancers in terms of number, size, and pathologic features (14–19).

We then, though preliminarily, showed that ACF could be eradicated by short-term administration of sulindac (20,21) and proposed the possibility that discontinuous use of the drug may be just as effective as continuous use and may make the daunting task of chemoprevention more realistic. However, results of multicenter trials of

Authors' Affiliations: [1]Department of Gastroenterology and Oncology, Institutes of Health Biosciences, University of Tokushima Graduate School, Tokushima; [2]Department of Gastroenterology, Muroran Shinnittetsu Hospital, Muroran; [3]Department of Internal Medicine, Otaru Ekisaikai Hospital, Otaru; [4]Department of Pharmacology, Hokkaido Medical University, Tobetsu; [5]Fourth Department of Internal Medicine, Sapporo Medical University; Departments of [6]Gastroenterology, Gorinbashi Hospital; [7]Department of Gastroenterology, Sapporo Cancer Center; [8]Public Health; and [9]Molecular Target Exploration, Sapporo Medical University, Sapporo, Japan

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Online registry: http://upload.umin.ac.jp; clinical trial no. C000000100.

Corresponding Author: Yoshiro Niitsu, Department of Molecular Target Exploration, Sapporo Medical University, South-1, West-16, Chuo-ku, Sapporo, Japan, 060-8556. Phone: 81-11-611-2111; Fax: 81-11-611-9196. E-mail: niitsu@sapmed.ac.jp

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Translational Relevance

Trials for chemoprevention of cancer are generally daunting because drugs are administered for years to cancer-free subjects, resulting in low compliance and are sometimes associated with adverse effects. In the present randomized controlled study of chemoprevention of colorectal cancer, we successfully showed that a short-term (2 months) administration of a nonsteroidal anti-inflammatory drug (sulindac) to eradicate aberrant crypt foci, minute precursor lesions of polyps, was as effective as long-term administration of similar drugs in previously reported trials by using polyps as a surrogate marker for colorectal cancer. The results therefore suggest that a short-term and discontinuous administration of drugs is pertinent for chemoprevention because every cancer should be derived from precursor lesions (seeds), which could be readily eradicated by drugs.

chemoprevention by others have raised controversy over the ACF-adenoma-carcinoma sequence theory or the use of ACF as biomarkers for recurrent colorectal adenomas (22, 23). Results showed dissociation of ACF prevalence and adenoma history (22) or no significant modulation of ACF by celecoxib (23). However, accuracy as to the technical aspects of ACF detection in these studies might be questioned because the number of ACF detected was very low compared with that in other reports and inter-rater agreement rates were also low (11,14,15). Further, utilizing a COX-2 inhibitor might not have been appropriate in a trial to examine the effect on ACF because COX-2 was mostly negative in ACF and became positive in adenoma, although COX-1 was expressed in both ACF and adenoma (24).

In the present randomized controlled trial, to directly validate the usefulness of ACF as a surrogate marker for chemoprevention and to address the controversial issues described above, we explored the effect of short-term (2 months) administration of sulindac, an inhibitor of both COX-1 and COX-2, or etodolac, a selective COX-2 inhibitor, on ACF by employing magnifying endoscopy, which is a suitable method for detection of ACF as we reported previously (11,12). We also elucidated the relevancy of the effects of the drugs on ACF to that on polyp development 1 year after the start of the study.

Patients and Methods

Study design and subjects

This randomized, double-blinded, and placebo-controlled study was conducted between February 2002 and October 2007 at the 4th Department of Internal Medicine, Sapporo Medical University Hospital and its 4 affiliate hospitals. Actual recruitment was carried out from 2002 to 2006. The rather long-term recruitment period was because of the delay in approval by the institutional review board in 3 hospitals and the delay in dispensing the drug to 1 hospital. According to reduction rates in ACF number by sulindac administration in our preliminary trials (20, 21), we estimated that 360 subjects would generate 90% power for a difference in the ACF number among the groups. Because it was possible that the estimate of an adequate sample size to show significant efficacy of the investigational drug was too conservative, a planned interim analysis was carried out when half of the subjects had been enrolled.

Subjects were recruited from patients who had undergone colonoscopy for abdominal symptoms including discomfort, distension, and a feeling of tightness on defecation. Eligible criteria were (i) positive for ACF in the lower rectal region from the middle Houston valve to the dentate line, (ii) age from 20 to 75 years, (iii) no colorectal polyps or polyps had been resected by polypectomy, (iv) not pregnant, (v) no malignant disease, (vi) no active infection, (vii) no history of gastroduodenal ulcer, (viii) no use of NSAIDs or aspirin in the previous 2 months, (ix) no abnormal findings by laboratory tests [blood cell count, aspartate amino transferase, alanine aminotransferase (ALT), total protein, albumin, blood urea nitrogen, creatinine, total bilirubin, lactate dehydrogenase, creatine kinase, and electrolytes (Na, K, Cl)], and (x) no familial adenomatous polyposis.

Subjects were randomly assigned to 1 of the 3 treatment groups at a 1:1:1 ratio (sulindac, etodolac, or placebo) by an independent statistician in the study center. We administered each drug for only 2 months on the basis of our unpublished observation in the preliminary open trial, which showed that administration of sulindac brought about a significant reduction in ACF number in 6 of 7 patients with 2 months treatment, in 4 of 4 patients with 3 months of treatment, and in 5 of 5 with 5 months treatment, whereas in 6 patients with 1 month of treatment, the reduction rate was not significant. Therefore, in this study, the number of ACF was assessed after 2 months of drug administration (primary endpoint). The participants did not receive any further treatment, and assessment was made for the occurrence of polyps 1 year after initiation of the study (secondary endpoint).

There are reportedly 2 types of ACF, dysplastic, and non-dysplastic ACF, with the former type being suggested to be more likely a precursor of polyps than the latter (11, 17). However, in the present investigation, it was impossible to analyze these 2 types separately because the proportion of the dysplastic type among all ACF was too small for statistical analysis.

At the start of the study, we carried out a baseline colonoscopy on all patients to determine the presence of polyps in the entire colorectum and to count ACF in the rectal area. After 2 months of drug administration, we conducted only rectosigmoidoscopy on these patients to determine the number of ACF in the rectal area because the number of ACF in the rectal region correlates well with that in the total colorectum (11).

All patients provided written informed consent. The protocol and informed consent forms were approved

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by the institutional review board at each participating institution.

Endoscopy

Magnifying endoscopy (model EZW450, Fujinon-Toshiba ES System Co.) was used throughout the examination as previously reported (11, 12). The day before endoscopy, the patients consumed a low-residue diet, and were given orally 4 g magnesium sulfate and 5 mg of sodium picosulfate. On the day of endoscopy, 2,000 mL of polyethylene glycol (PEG) was given orally. When the feces were not sufficiently clear, they were given another 1,000–2,000 mL of PEG to ensure sufficient bowel cleansing.

A total of 5 endoscopists from Muroran Shinnittesu Hospital, Otaru Ekisaikai Hospital, Gorinbashi Hospital, and Sapporo Cancer Center Hospital, were engaged in the endoscopic examinations. They were all trained for at least 1 month at the 4th Department of Sapporo Medical University Hospital for detection of ACF. They were all blinded to the treatment arms. Each endoscopist carried out almost the same number of examinations in each arm.

At the baseline colonoscopy, the endoscope was inserted into the cecum, and the entire colorectum was carefully observed as the endoscope was pulled back. Insertion into the cecum was verified by videotape as described below. When the endoscope was pulled back to the rectum, the lower rectal region from the middle Houston valve to the dentate line was washed thoroughly with water, sprayed with 0.25% methylene blue, which was left to stand for 2 minutes, then washed again thoroughly with water. The number of ACF in the rectal region, which has been shown to correlate with that in entire colon (11), was counted by magnifying endoscope.

For the 2-month survey for ACF by rectosigmoidoscopy, we simply used a 110 mL glycerin enema to cleanse the region, and if the rectum was not sufficiently cleansed, the subject was orally administered 2,000–4,000 mL PEG.

One year after the initiation of the study, all patients underwent total colonoscopy to detect polyps in the entire colorectum. The same cleansing preparation was used as for the baseline colonoscopy. The same endoscopist carried out the baseline, 2-month, and 1-year endoscopic examinations for each subject. All procedures were recorded on videotape, and all ACF and polyps were photographed. The numbers of ACF and polyps were first counted by the operators during performance of the colonoscopy or rectosigmoidoscopy. To further ensure validity, another count was made through observation of the recorded videotapes by 3 expert endoscopists (M.M., Y.N., and Y.T.) from the Assessment Panel of Endoscopy.

Drug administration and monitoring for adverse effects

For blinding of subjects and trial staff, identical looking capsules were filled with either 150 mg of sulindac (Banyu, Tokyo, and Japan), 200 mg of etodolac (Wyeth Pharmaceutical Co. Ltd.), or 200 mg of lactose as the placebo. All subjects were also administered 15 mg of lansoplazole twice daily. The drug set for each subject was labeled by an identification code unrelated to the allocation to conceal the allocation from subjects and trial staff until the blind was opened.

Subjects were instructed to take 1 capsule after food in the morning and 1 capsule after food in the evening. Study patients visited the hospital every 2 to 4 weeks to be evaluated for subjective symptoms of any adverse events, including abdominal and cardiovascular symptoms and to receive a new supply of medication. Two weeks after initiation of the treatment, liver and renal function was evaluated. Adverse events were graded according to the National Cancer Institute Common Toxicity Criteria (NCI-CTC) Version 2.0. Compliance was monitored by counting the capsules returned by patients every month at outpatient clinic. A Safety Monitoring Board reviewed the study semi-annually.

Statistical analysis

To maintain the overall type I error rate at 5%, Pocock's method (25) was applied for the interim analysis with a significance level of 0.0294. The number of ACF, the primary endpoint, was compared by the Mann–Whitney U test. For adjustment of multiplicity of 2 times pairwise comparison (placebo versus sulindac and placebo versus etodolac), the level of significance (0.0294) was modified by Bonferroni's method, that is, $0.0294/2 = 0.0147$. The number of polyps after 1 year was compared by Mann–Whitney U test. A comparison of the incidence of polyps after 1 year was made according to logistic regression analysis, and the level of significance was also set at $P = 0.0147$.

Results

Patients

A total of 304 subjects were screened for eligibility, and 115 subjects were excluded from the study for the reasons shown in Figure 1. The remaining 189 patients underwent randomization: 63 were assigned to the sulindac group, 64 to the etodolac group, and 62 to the placebo group (Fig. 1). Of these, 10 subjects withdrew their informed consent within 2 weeks: 5 after consulting with their family at home, 4 after deep reconsideration, and 1 after consultation with a supervisor at work. These subjects did not allow use of their data. Of the remaining 179, 4 were dropped from the study for taking NSAIDs for common cold, 1 for taking aspirin for headache, and 1 because of having orthopedic surgery during the study. The 177 patients (59, sulindac group; 60, etodolac group; 58, placebo group) underwent the 2-month endoscopy. The analysis was based on the intent-to-treat principle. Table 1 shows baseline characteristics of the subjects. No particular difference in each characteristic among the 3 groups was observed. In all groups, the number of polypectomized subjects was almost 5 times that of polyp-free subjects. No patient with hereditary nonpolyposis colorectal cancer (HNPCC) was included when we reviewed the subjects with regard to diagnostic criteria for HNPCC.

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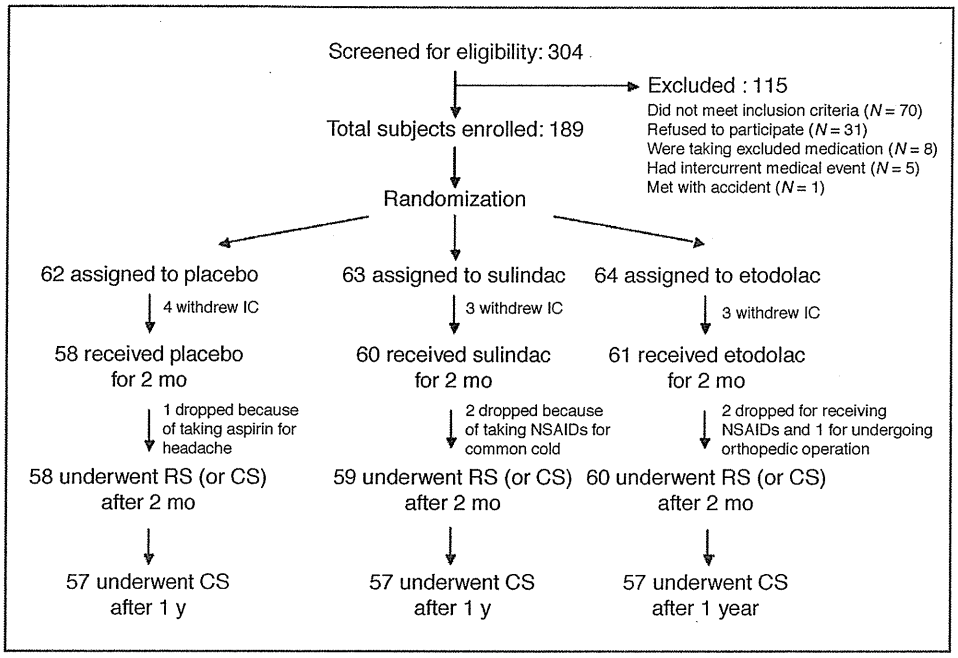

Figure 1. Trial profile. A total of 304 patients were screened for eligibility, and 115 were excluded. Enrolled were 189 patients who were randomly assigned to either the sulindac, etodolac, or placebo group. Drugs were administered for only 2 months, and the number of rectal ACF was assessed by rectosigmoidoscopy (RS). One year after the initiation of treatment, subjects underwent total colonoscopy (CS) to detect all polyps in the entire colorectum. The number of subjects is based on the intent-to-treat (ITT) population.

Validity of endoscopic assessment of ACF

The number of ACF in 26 patients randomly selected was evaluated through review of videotapes independently by 3 endoscopists from the Assessment Panel of Endoscopy (M. M., Y.N., and Y.T.) to assess the degree of concordance with the endoscopic findings. The mean counts of ACF were 8.5 ± 3.7 for M.M., 8.8 ± 4.6 for Y.N., and 8.6 ± 3.9 for Y.T. The inter-rater agreement rates within +/− 1 ACF between M.M. and Y.N., M.M. and Y.T., and Y.N. and Y.T. were 88.5%, 84.6%, and 92.3%, respectively. The Cronbach's alpha was 0.89, proving the validity of the endoscopic count of ACF.

Number of ACF before and after the 2-month treatment

The number of ACF before and after the 2-month treatment period in the 3 groups is shown in Table 2. In the polypectomized subjects, the ACF number after 2 months in the sulindac group was significantly lower than that in the placebo group ($P < 0.001$), whereas the number in the

Table 1. Baseline characteristics of subjects

	Placebo ($n = 58$)	Sulindac ($n = 60$)	Etodolac ($n = 61$)
Age, (mean ± SD), y	64.0 ± 9.9	65.8 ± 10.2	63.1 ± 9.7
Sex (M/F)	36/22	36/24	37/26
Colorectal cancer in parent no. (%)	8 (13.8)	8 (13.3)	10 (16.4)
Current smoker, no. (%)	11 (19.0)	12 (20.0)	11 (18.0)
History of diabetes, no. (%)[a]	5 (8.6)	6 (10.0)	5 (8.2)
History of hyperlipidemia, no. (%)[b]	14 (24.1)	15 (25.0)	16 (26.2)
History of hypertension, no. (%)[c]	18 (31.0)	20 (33.3)	18 (29.5)
Findings at baseline CS			
No. of polyps, median (interquartile range)	1.0 (0.5–2.0)	1.0 (0.0–2.0)	1.0 (0.0–2.0)
No. of adenomas, median (interquartile range)	1.0 (0.0–2.0)	1.0 (0.0–2.0)	1.0 (0.0–2.0)
Polypectomy/polyp-free subjects	48/10	50/10	50/11

[a]History of diabetes was defined as use of antidiabetic medication or participant report of clinically diagnosed diabetes.
[b]History of hyperlipidemia was defined as use of cholesterol-lowering medication or participant report of clinically diagnosed hyperlipidemina.
[c]History of hypertension was defined as use of antihypertensive medication or participant report of clinically diagnosed high blood pressure.

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Table 2. Comparison of ACF number among the sulindac, etodolac, and placebo groups before and after treatment

	Before[a]	After 2 mo	
Subjects with polypectomy			
Placebo			
Mean ± SD	7.77 ± 4.66	6.87 ± 6.03	
Median (interquartile range)	7.0 (5.0–10.0)	6.0 (3.0–8.5)	$P < 0.001$[b]
Sulindac			
Mean ± SD	7.70 ± 4.04	4.00 ± 2.95	
Median (interquartile range)	7.0 (4.5–10.0)	4.0 (1.0–6.0)	
Etodolac			
Mean ± SD	7.52 ± 4.01	6.28 ± 5.21	$P = 0.67$
Median (interquartile range)	7.0 (4.0–10.0)	5.0 (2.5–8.5)	
Polyp-free subjects			
Placebo			
Mean ± SD	4.00 ± 1.82	3.90 ± 2.72	
Median (interquartile range)	4.0 (2.0–6.0)	3.0 (1.0–7.0)	$P = 0.38$
Sulindac			
Mean ± SD	4.40 ±2.21	2.70 ±2.16	
Median (interquartile range)	4.0 (2.0–6.0)	3.0 (0–4.5)	
Etodolac			
Mean ± SD	4.73 ± 2.32	4.10 ± 2.60	$P = 0.54$
Median (interquartile range)	4.0 (0–5.5)	4.0 (2.0–9.0)	
Ali subjects			
Placebo			
Mean ± SD	7.12 ± 4.53	6.35 ± 5.69	
Median (interquartile range)	6.5 (4.0–9.0)	5.0 (2.3–8.0)	$P = 0.0075$[b]
Sulindac			
Mean ± SD	7.15 ± 3.98	3.77 ± 2.86	
Median (interquartile range)	6.5 (4.0–9.0)	4.0 (1.0–5.3)	
Etodolac			
Mean ± SD	7.01 ± 3.89	5.91 ± 4.93	$P = 0.73$
Median (interquartile range)	6.0 (4.0–9.0)	5.0 (2.0–7.5)	

[a] There were no significant differences before treatment among the 3 groups by the Kruskal-Wallis test.
[b] Significant *P* value by Mann–Whitney *U* test.

etodolac group was not significantly different from the placebo group ($P = 0.67$). Among polyp-free subjects, neither the sulindac nor etodolac group had a significant reduction in ACF compared with the placebo group. When polypectomized and polyp-free subjects were combined in the analysis, results were similar to those in the polypectomized subjects with a significant suppression of ACF ($P = 0.0075$) only in the sulindac group, reflecting the fact that polypectomized subjects were dominant in the subject population. Intraindividual comparison in ACF numbers before and after treatment (Supplementary Table S1) also showed a significant decrement in ACF in the sulindac group of polypectomized subjects ($P < 0.001$) as well as in all subjects in the sulindac group ($P < 0.001$). In the analysis of subjects receiving etodolac, ACF number tended to decline slightly after 2 months but without significance ($P = 0.09$). Thus, because the superiority of the test drug (sulindac) over the placebo was confirmed, termination of the study was recommended by the Data Monitoring Board. Incidentally, before the treatment, ACF number in subjects who had adenoma (subjects with polypectomy) was significantly higher than that in the subjects without adenoma (polyp-free subjects) in each group ($P < 0.001$), which was in good agreement with our previous finding (10).

Number and incidence of total polyps and adenomas 1 year after treatment

A total of 107 polyps were found after 1 year. Of these, 96 were adenomas and 11 were hyperplastic polyps (Supplementary Table S2). There was no significant difference in location and histology among the 3 groups. The average size in the sulindac group was slightly smaller than in the placebo group ($P = 0.16$ by Mann–Whitney *U* test).

In polypectomized subjects, the mean numbers of total polyps (adenoma plus hyperplastic polyp) and adenomas

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Table 3. Numbers of total polyps and adenomas 1 year after initiation of treatment

	Placebo	Sulindac	Etodolac
Subjects with polypectomy	$n = 48$	$n = 48$	$n = 47$
No. of total polyps			
Mean ± SD	0.92 ± 1.05	0.42 ± 0.68	0.73 ± 0.94
Median (range)	1 (0–4)	0 (0–2)	0 (0–3)
P value		$P = 0.014$[a]	$P = 0.64$
No. of adenomas			
Mean ± SD	0.81 ±1.0	0.42 ± 0.71	0.68 ± 0.86
Median (range)	1 (0–4)	0 (0–2)	0 (0–3)
P value		$P = 0.034$	$P = 0.61$
Polyp-free subjects	$n = 9$	$n = 9$	$n = 10$
No. of total polyps			
Mean ± SD	0.22 ± 0.44	0.22 ± 0.44	0.20 ± 0.42
Median (range)	0 (0–1)	0 (0–1)	0 (0–1)
P value		$P = 1.00$	$P = 0.94$
No. of adenomas			
Mean ± SD	0.11 ±0.33	0.22 ± 0.44	0.20 ± 0.67
Median (range)	0 (0–1)	0 (0–1)	0 (0–2)
P value		$P = 0.54$	$P = 0.93$
All subjects	$n = 57$	$n = 57$	$n = 57$
No. of total polyps			
Mean ± SD	0.81 ± 1.01	0.40 ± 0.70	0.68 ± 0.89
Median (range)	0 (0–4)	0 (0–2)	0 (0–3)
P value		$P = 0.020$	$P = 0.61$
No. of adenomas			
Mean ± SD	0.70 ± 0.96	0.39 ± 0.68	0.60 ± 0.84
Median (range)	0 (0–4)	0 (0–2)	0 (0–3)
P value		$P = 0.064$	$P = 0.63$

[a]Significant *P* value by Mann–Whitney *U* test.

in the sulindac group were significantly ($P = 0.014$) and marginally ($P = 0.034$) lower, respectively, whereas those in the etodolac group were not lower with statistical significance ($P = 0.64$ for total polyps and $P = 0.61$ for adenomas) in comparison with the placebo group (Table 3). In polyp-free subjects, neither total polyp number nor adenoma number was lower in either the sulindac or etodolac group compared with the placebo group. In analyses of all subjects (polypectomized plus polyp-free subjects), the numbers of total polyps and adenomas were markedly ($P = 0.020$) and marginally ($P = 0.064$) lower in the sulindac group but not in the etodolac group. The incidences of total polyps and adenomas in polypectomized subjects were markedly ($P = 0.025$) and marginally ($P = 0.039$), respectively, lower in the sulindac group but not in the etodolac group in comparison with the placebo group (Table 4). In polyp-free subjects, there were no differences in incidence among the groups. When incidence was analyzed in all subjects, the incidence of total polyps was marginally lower ($P = 0.037$) and that of adenomas tended to be lower ($P = 0.08$) in the sulindac group but not in the etodolac group. Though statistically not significant because of the small number for analysis, there was a tendency ($P = 0.25$) for the incidence of multiple adenomas to decrease by treatment with sulindac; however, there were no apparent differences between the etodolac and placebo groups ($P = 0.81$).

Comparison of number and incidence of total polyps and adenomas between ACF responders and nonresponders to drugs

We selected out as responders those subjects whose ACF number became zero at 2 months or whose ACF reduction rate by 2 months was above the 90th percentile of the placebo group. We then compared polyp number and incidence at 12 months of the responders with those in the remaining subjects, which were designated as "nonresponders" (Supplementary Table S3). In the sulindac group, the numbers of total polyps and adenomas in responders were significantly lower than in nonresponders ($P = 0.017$ and $P = 0.023$, respectively). Moreover, the incidences of total polyps and adenomas in responders

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Table 4. Incidence of total polyps and adenomas 1 year after treatment

	Placebo	Sulindac	Etodolac
Subjects with polypectomy	$n = 48$	$n = 48$	$n = 47$
Incidence of total polyps	26/48 (54.2%)	15/48 (31.3%)	24/47 (46.8%)
Risk ratio (95% CI)		0.39 (0.17–0.89)	0.96 (0.43–2.15)
P value[a]		0.025	0.92
Incidence of adenomas	24/48 (50.0%)	14/48 (29.2%)	21/47 (44.7%)
Risk ratio (95% CI)		0.41 (0.18–0.96)	0.81 (0.36–1.81)
P value[a]		0.039	0.60
Polyp-free subjects	$n = 9$	$n = 9$	$n = 10$
Incidence of total polyps	2/9 (22.2%)	2/9 (22.2%)	2/10 (20.0%)
Risk ratio (95% CI)		1.00 (0.11–9.23)	0.88 (0.10–7.95)
P value[a]		1.00	0.91
Incidence of adenomas	1/9 (11.1%)	2/9 (22.2%)	2/10 (20.0%)
Risk ratio (95% CI)		2.29 (0.17–31.0)	2.00 (0.15–26.7)
P value[a]		0.53	0.60
All subjects	$n = 57$	$n = 57$	$n = 57$
Incidence of total polyps	28/57 (49.1%)	17/57 (29.3%)	26/57 (45.6%)
Risk ratio (95% CI)		0.44 (0.20–0.95)	0.87 (0.42–1.81)
P value[a]		0.037	0.71
Incidence of adenomas	25/57 (43.9%)	16/57 (28.1%)	23/57 (40.4%)
Risk ratio (95% CI)		0.50 (0.23–1.09)	0.87 (0.41–1.82)
P value[a]		0.08	0.70

[a]Logistic regression analysis was used to calculate *P* values.

were significantly lower than in nonresponders ($P = 0.011$ and $P = 0.022$, respectively). A similar tendency was observed but not with significance in the etodolac group.

Adverse effects and compliance

The incidence of adverse events, including symptoms such as abdominal pain, heartburn, diarrhea, and exanthema, and abnormal laboratory test results such as a transient elevation of ALT or creatinine was less than 4% (Table 5); all were grade 1. Differences were not significant among the 3 groups. No cardiovascular events, including myocardial infarction, angina, stroke, and transient ischemic attacks, were observed during the 2 months of treatment. Average compliance with medication was 92.7%: 93.9% in the placebo group, 91.7% in the sulindac group, and 92.5% in the etodolac group.

Discussion

For the present study, we selected 2 types of drugs, a NSAID (sulindac) and a COX-2 inhibitor (etodolac) because both were proved to be effective as chemopreventive agents for colon adenoma (10, 26–28). Drug dosages were selected according to information in previous reports (11, 29). Although one of the COX-2 inhibitors, high-dose celecoxib, was reported to increase the risk of cardiovascular events (30), we considered that our protocol, using etodolac in 1 arm, was safe because the cardiac adverse event related to etodolac was reportedly negligible at the dosage we used (31). We administered lansoprazole to all subjects, including those in the placebo group, to prevent any possible gastrointestinal damage caused by sulindac or etodolac.

In most previous chemopreventive trials for colon cancer, only the polypectomized subjects were enrolled (4–9). In the present trial, we recruited both polypectomized and polyp-free subjects in view of the possibility of differences between the 2 subject groups in sensitivity of ACF and polyps to drugs. However, to our disappointment, the number of polyp-free subjects enrolled was so small that we were, practically, not able to draw any meaningful conclusion from comparisons of these 2 groups. Incidentally, a possible reason for the high rate of polyps detected by the baseline colonoscopy was because patients who underwent the colonoscopic examination were those at high risk for colorectal polyps, such as those with fecal occult blood. Further, the relatively high proportion of polypectomized subjects compared with polyp-free subjects was probably because of their higher motivation to participate in the current trial. Nevertheless, the results of the 2-month treatment on ACF both in comparison analysis among groups (Table 2) and in the intragroup analysis (Supplementary Table S1) clearly indicated the effectiveness of sulindac in eradicating the lesions, particularly in polypectomized subjects. Thus, the primary endpoint of the present study was achieved. The failure of etodolac to eradicate ACF is probably explained by the fact that most ACF do not express COX-2 (20). Moreover, it is surmised