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Thioproline inhibits development of esophageal adenocarcinoma induced by gastroduodenal reflux in rats

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Several epidemiological cohort studies have suggested that duodeno-gastroesophageal reflux *per se* induces Barrett's esophagus leading to increased risk of the development of esophageal adenocarcinoma (EAC). However, the exact causative factors behind EAC remain unclear. Recently, we designed a new duodenal contents reflux model which retained normal stomach function. In this model, duodenal contents flowed back into the esophagus and stomach resulting in repeated re-entry into the esophagus through the site of esophagojejunostomy. To elucidate the factors underlying the development of EAC, thiazolidine-4-carboxylic acid (thioproline, TPRO) was applied to the new reflux models as a nitrite scavenger and as a probe to detect reactive nitrogen species (RNS). Post-operatively, 31 animals were divided into two groups according to diet. Animals belonging to the control group were given normal diet ($n = 18$), while the TPRO group was given food containing 0.5% TPRO ($n = 13$). All esophageal sections in both groups were examined using hematoxylin and eosin staining and immunohistochemical analysis of inducible nitric oxide synthase (iNOS). EACs developed in 7 of 18 rats (38.9%) of the control group, whereas no EACs were detected in the TPRO group (Fisher's exact test, $P < 0.05$). Conversely, esophageal squamous cell carcinoma (ESCC) was detected in 1 of 18 rats (5.6%) of the control group and in 1 of 13 rats (7.7%) of the TPRO group. The incidence of ESCC was not significantly different between the two groups ($P = 0.671$). iNOS protein was overexpressed in Barrett's esophagus of both groups. The present results suggest that RNS such as nitric oxide and peroxynitrite and nitroso compounds derived from reflux of duodenal contents play an important role in the development of EAC, and that the primary causes of ESCC and EAC may differ.

Abbreviations: BE, Barrett's esophagus; EAC, esophageal adenocarcinoma; ESCC, esophageal squamous cell carcinoma; GCA, *N*-nitroso-glycocholic acid; GERD, gastroesophageal reflux disease; HE, hematoxylin and eosin; iNOS, inducible nitric oxide synthase; RNS, reactive nitrogen species; TCA, *N*-nitroso-taurocholic acid; TPRO, thiazolidine-4-carboxylic acid.

Introduction

Gastroesophageal reflux disease (GERD) has become a common disorder in the USA and Western Europe in recent decades (1,2). Barrett's esophagus (BE) has been linked to a substantially increased risk of esophageal adenocarcinoma (EAC) and has been considered a consequence of long-standing acid-induced injury (3–6). However, a large population of patients with BE have been found to display periods with not only gastric acid but also duodenal juice exposure of the esophagus (7–12). Conversely, several investigators reported that duodeno-gastroesophageal reflux *per se* can induce BE to form EAC in rats (13–15).

To elucidate the carcinogenic effects of the duodenal contents, many researchers have addressed the factors bile acids and pancreatic juice (16,17) and others referred to the presence of bacterial flora in duodenal juice that are capable of catalyzing endogenous reactions to produce nitroso compounds (18,19). Recently, overexpression of inducible nitric oxide synthase (iNOS) protein has been reported in esophageal carcinoma of humans and animals (20–22) and reactive nitrogen species (RNS) such as nitric oxide (NO), peroxynitrite ($ONOO^-$) and nitroso compounds are suggested to play an important role in the esophageal neoplastic transformation process.

Thiazolidine-4-carboxylic acid (thioproline, TPRO) is a cyclic sulfur-containing amino acid and is a condensation product of cysteine and formaldehyde (23,24). TPRO is an effective nitrite-trapping agent both *in vitro* and in the human body and it is a very sensitive probe for evaluating nitrosating capacity (25–27). If RNS in the gastroduodenal reflux or nitrosative damage contribute to esophageal carcinogenesis, TPRO as a nitrite scavenger could inhibit the development of EAC.

Recently, we designed a new duodenal contents reflux model which retained normal stomach function (15). The model displays similarities to the situation in patients with duodeno-gastroesophageal reflux, inducing both duodenal contents and gastric juice reflux into the esophagus. To elucidate the causative factors underlying EAC, we investigated whether TPRO can inhibit EAC induced by duodeno-gastroesophageal reflux using this new rat model.

Materials and methods

Animal model

Forty-two male Wistar rats (8 weeks old, 200–250 g) were used in this experiment. Rats were housed two in each cage, under standard laboratory conditions (room temperature 22 ± 2°C, humidity 55 ± 5%, 12 h light/dark cycle). After 24 h fasting, a midline laparotomy incision was made under inhalation anesthesia with diethyl ether and the following procedure was performed according to previously reported methods (15). Briefly, an 1.5 cm incision was made at the esophago-gastric junction and a loop of jejunum, 3 cm distal to Treitz's ligament, was anastomosed side-by-side to the esophago-gastric junction (Figure 1). As a result, duodenal and gastric contents flowed back into the esophagus through the site of anastomosis and duodenal contents mixed with gastric acid in the reserved stomach repeat, to re-enter through the

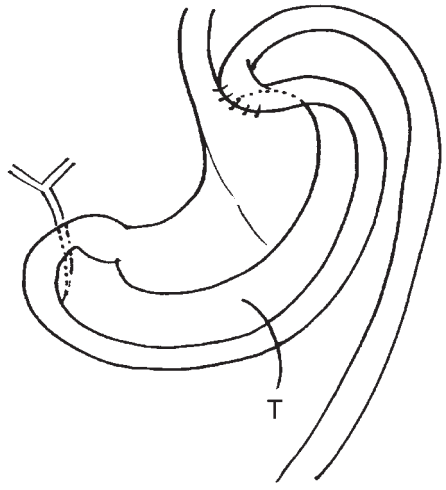

Fig. 1. Surgical procedure of gastric and duodenal contents reflux model. T, Treitz ligament. One of the advantages of this model compared with other models is normal stomach function and normal nutritional status is retained. Duodenal contents repeatedly flow back into the esophagus and the stomach through the site of esophagojejunostomy.

site of anastomosis. All sutures were made using interrupted 7-0 nylon sutures. Animals were allowed access to water at 12 h and to food at 36 h post-operatively. Animals were not treated with any known carcinogens and sequential morphological changes in the esophagus were studied. Post-operatively, surviving animals were divided into two groups according to diet. Animals belonging to the control group were given normal diet (CRF-1), while the TPRO group was provided food containing 0.5% TPRO. Animals were killed using an overdose of diethyl ether in post-operative week 70.

Histological examination

Immediately after death the entire esophagus, site of anastomosis and a 5 mm length of jejunum were removed. The esophagus was longitudinally opened, spread on a cork plate mucosal side up for macroscopic examination and then fixed in 10% neutral buffered formalin in phosphate-buffered saline. After 4 h fixation the removed tissue was cut serially into 3 mm slices along the longitudinal axis. Three sections were made from each esophageal sample. Sections were embedded in paraffin and cut into 4 μm sections. All sections were stained with hematoxylin and eosin (HE).

Severe esophagitis was characterized as hyperplastic, when the squamous epithelium was thickened with normal maturation and hyperkeratosis, or regenerative, when the squamous epithelium showed increased height of the lamina papillae and basal cell hyperplasia (28). BE, squamous cell dysplasia, adenosquamous carcinoma, EAC and esophageal squamous cell carcinoma (ESCC) were classified according to the WHO classification (29). We examined the full length of esophagus in all cases. If some lesions mentioned above were partially detected, we counted them as positive cases.

Immunohistochemical analysis for iNOS

Serial sections of HE staining were deparaffinized and endogenous peroxidase activity was quenched by incubation in 0.3% H_2O_2 in methanol for 30 min at room temperature. Sections were then microwaved in citrate buffer, pH 6.1, for 40 min for antigen retrieval. Non-specific binding was blocked with 10% rabbit serum for 10 min. After blocking, sections were incubated with mouse monoclonal antibodies to iNOS (NOS2, C-11, 1:100 dilution; Santa Cruz Biotechnology, Santa Cruz, CA) overnight at 4°C. Normal mouse IgG served as a negative control. The next day, immunoreactivity was detected by incubation with biotinylated rabbit anti-mouse antibody and streptavidin–biotin–peroxidase complex [Histofine SAB-PO (M) Kit; Nichirei, Tokyo, Japan], for 20 min each. The color was then developed using 3,3′-diaminobenzidine. Nuclei were lightly counterstained with hematoxylin.

Statistical analyses

Statistical evaluation was performed using Fisher's exact test. Values of $P < 0.05$ were considered statistically significant.

Table I. Histopathological findings in both groups and statistical evaluation in every lesion

Finding	Positive cases/total at 70 weeks (%)		*P*
	TPRO	Control	
Severe esophagitis	10/13 (76.9)	18/18 (100)	0.064
Squamous cell dysplasia (severe)	4/13 (30.8)	12/18 (66.7)	0.117
Esophageal ulcer	6/13 (46.2)	15/18 (83.3)	0.036
Specialized columnar epithelium	6/13 (46.2)	14/18 (77.8)	0.076
Esophageal adenocarcinoma	0/13 (0)	7/18 (38.9)	0.012
Adenosquamous carcinoma	0/13 (0)	3/18 (16.7)	0.182
Squamous cell carcinoma	1/13 (7.7)	1/18 (5.6)	0.671

Fisher's exact test was used for statistical evaluation. *P* values <0.05 were considered significant. Esophageal ulcer and EAC in the TPRO group were significantly less than in the control group. However, the incidence of ESCC was not significant.

Results

Five and six rats died as a result of complications of duodenal contents reflux after surgery in the control and TPRO groups, respectively. The other 31 animals survived for the 70 week experimental period and comprised a control group ($n = 18$) and a TPRO group ($n = 13$). There was no significant difference in body weight of rats between the control and the TPRO groups.

Macroscopically, deep ulceration in the lower portion and an uneven surface with erosion in the middle portion was detected at the oral side away from the site of anastomosis in most rats of the control group. Although a small number of rats in the TPRO group displayed similar mucosal changes to the control group, mucosal changes in most animals of the TPRO group were mild compared with those in the control group.

All histopathological findings are summarized in Table I. Severe esophagitis with basal cell hyperplasia were recognized in all rats of the control group. Severe esophageal dysplasia (66.7%), esophageal ulceration (83.3%), specialized BE columnar epithelium (77.8%) (Figure 2A), EAC (38.9%) (Figure 2B), adenosquamous carcinoma (16.7%) (Figure 2C) and ESCC (5.6%) (Figure 2D) were also observed in the control group. Almost all of these lesions were detected in the TPRO group. Although esophagitis was detected in three rats of the TPRO group, the degree of inflammation was mild compared with the control group. Severe esophagitis developed in all other rats of the TPRO group. EACs and adenosquamous carcinomas were not observed in the TPRO group and the incidence of esophageal ulcer and EACs were significantly less in the TPRO group than in the control group ($P < 0.05$). No clear significances were seen between the TPRO and control groups. However, the incidence of BE (46.2%) in the TPRO group was less than in the control group (77.8%) ($P = 0.076$), and most lesions except for ESCC were decreased in the TPRO group compared with the control group.

Conversely, ESCC was detected in 1 of 18 rats (5.6%) of the control group and in 1 of 13 rats (7.7%) of the TPRO group. Severe inflammation was detected in tissue adjacent to ESCC. The incidence of ESCC was not significantly different between the two groups ($P = 0.671$).

There was no iNOS protein expression in adjacent normal or squamous cell epithelium with basal cell hyperplasia compared with intense staining of BE in both the control and

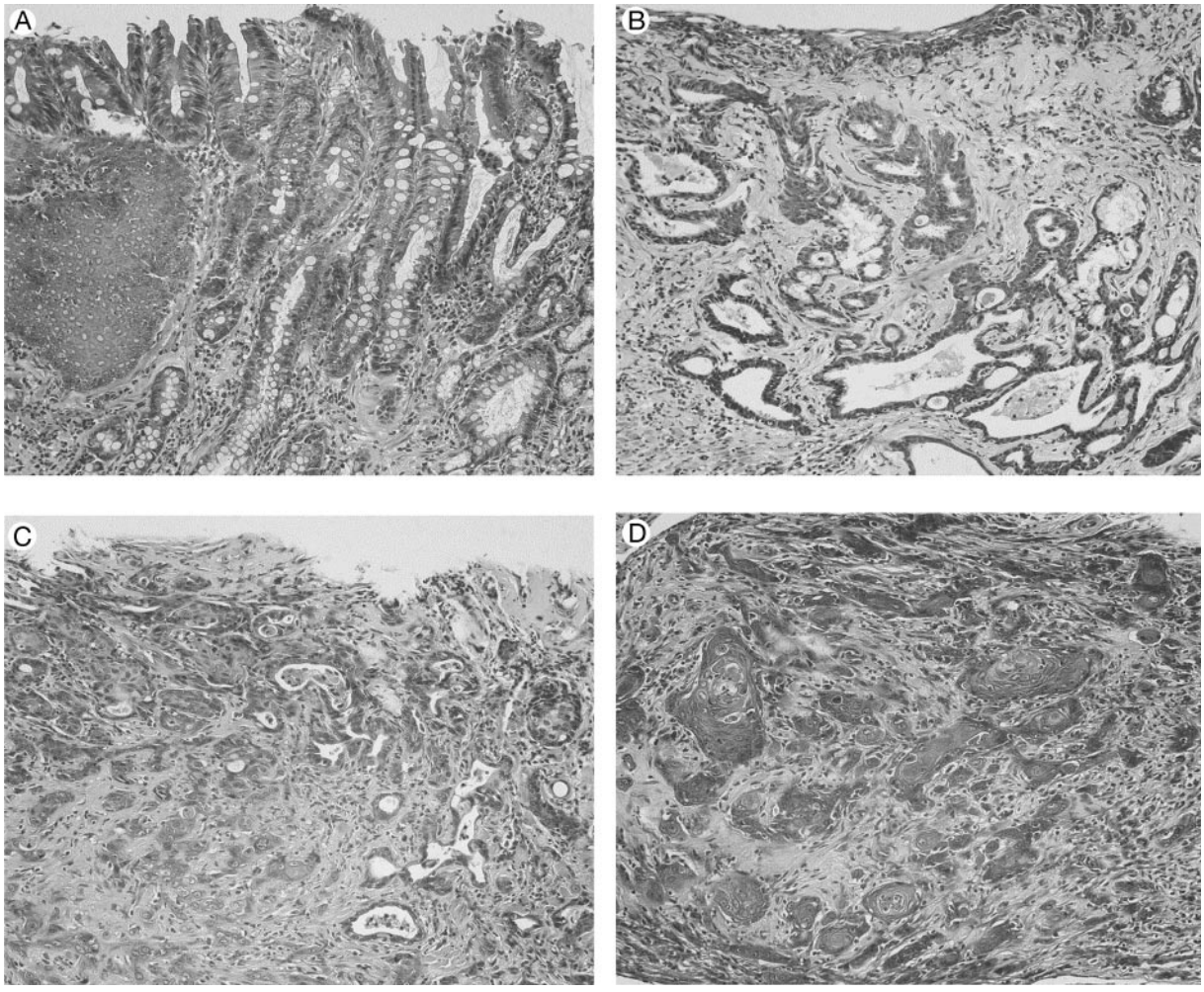

Fig. 2. Histological findings in the rat reflux model. HE staining, ×200. Various lesions developed in the reflux model. (**A**) Specialized columnar epithelium; (**B**) esophageal adenocarcinoma; (**C**) adenosquamous carcinoma; (**D**) squamous cell carcinoma.

TPRO groups (Figure 3A and B). iNOS protein was slightly expressed in EACs and adenosquamous carcinomas in the control group and ESCCs in both groups (Figure 3C and D).

Discussion

Despite progress in understanding the relationships between EAC and GERD, few studies have explored the underlying causes. Several animal models using surgical methods have been developed to study BE and EAC (13–15,30–32). Among these, only duodenal contents were shown to induce EAC in a model of esophagojejunostomy with gastrectomy (32). Duodenal contents must therefore play a crucial role in carcinogenesis leading to EAC.

N-nitroso compounds have been postulated as one of the causative factors for malignancies of the digestive tract. There is experimental data that two nitrosated cholic acids, *N*-nitroso-glycocholic acid (GCA) and *N*-nitroso-taurocholic acid (TCA), cause gastric carcinogenesis in rats (16). On the other hand, Correa *et al*. suggested that achlorhydria induced by atrophic gastritis or gastric surgery allows overgrowth of the microflora by nitrate-reducing bacteria which convert dietary nitrate to nitrite. It seems probable that nitrite is produced by bacterial flora in the duodenum after the reflux operation, which combines with amines and amides in foods to form *N*-nitroso compounds (33), causing EAC carcinogenesis.

On the other hand, it has also been reported that iNOS is overexpressed in esophageal cancer of humans and animals (20–22), and it is well known that RNS such as NO, $ONOO^-$ and nitroso compounds are implicated in the pathophysiology of inflammation and carcinogenesis (34). However, a recent study failed to demonstrate the presence of any TCA or GCA and no other nitroso derivatives could be detected in any samples of reflux animal models (28). The reason for these discrepancies must be that RNS are unstable and occur in very small amounts in esophagus of rat reflux models. To elucidate the effects of RNS derived from bile acid and nitroso compounds produced by bacterial flora in the reflux of gastric and duodenal contents' we evaluated the inhibitory effect of TPRO as a nitrite scavenger and as a sensitive probe to detect RNS indirectly.

The ubiquitous signaling molecule NO is synthesized by the nitric oxide synthase enzymes: iNOS is the primary nitric oxide synthase responsible for heightened NO production during inflammation. NO has been linked to early events in the tumorigenic process, including DNA damage, lipid peroxidation and regulation of inflammation (35,36), all of which can

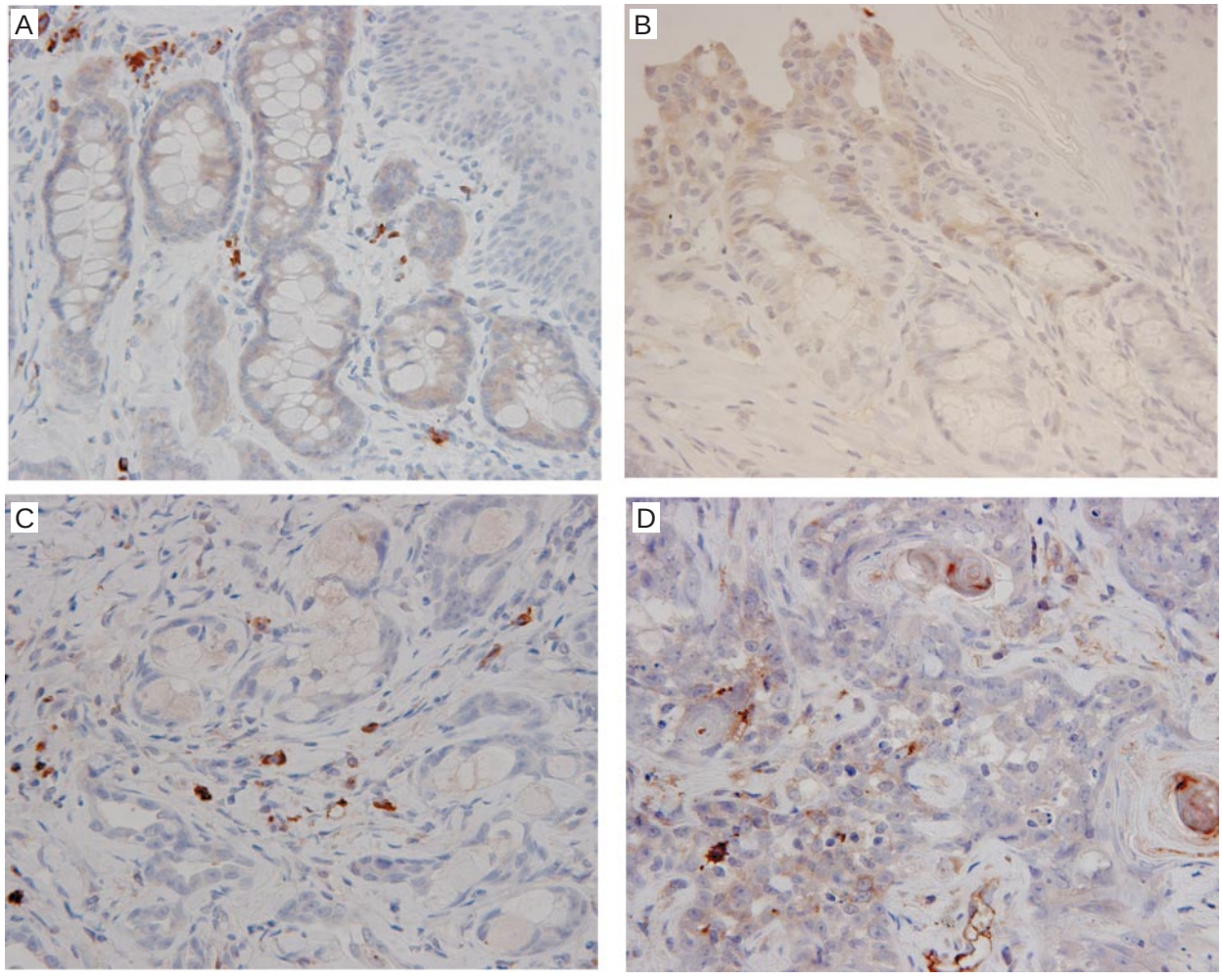

Fig. 3. Immunohistochemistry of iNOS in various lesions. INOS, ×400. Although iNOS protein is not expressed in normal esophageal squamous epithelium, it is strongly expressed in most BE of both control (**A**) and TPRO groups (**B**). iNOS protein is slightly expressed in EAC (**C**) and ESCC (**D**) in the control group. INOS was also overexpressed in macrophages of all sections.

cause neoplastic growth. In the present study, TPRO inhibited the development of EAC and iNOS protein was overexpressed in BE of both the control and TPRO groups. TPRO could not suppress the overexpression of iNOS, and our results suggested that the mechanism by which TPRO inhibits esophageal carcinogenesis induced by duodenogastric reflux could be summarized as follows. TPRO, a nitrite-trapping agent by virtue of its nitrosating capacity, inhibits not only the production of nitroso compounds by nitrite-reducing bacteria but also RNS, such as NO, $ONOO^-$ and *N*-nitroso compounds, derived from reflux of duodenal contents. Our present study was unable to detect any RNS or nitroso compounds. However, it is possible to speculate that RNS derived from duodenal contents and nitroso compounds produced by bacterial flora play an important role in the development of EAC.

It is widely accepted that ESCC is associated with smoking and alcohol (37–40). A risk of ESCC is reported not to be associated with gastroesophageal reflux. Individuals with long-standing and severe symptoms of reflux display odds ratios of 43.5 for esophageal adenocarcinoma and 1.1 for ESCC (3). The present results suggest that EAC are associated with RNS derived from bile acids in the esophagus. Some previous investigations, in addition to the present study, have suggested that ESCC can be induced by reflux of duodenal contents alone. However, despite the decreased incidence of EAC, the incidence of ESCC did not significantly change in the TPRO group. These results suggest that the primary causative factors differ between ESCC and EAC.

References

1. Thompson,W.G. and Heaton,K.W. (1982) Heartburn and globus in apparently healthy people. *Can. Med. Assoc. J.*, **126**, 46–48.
2. Locke,G.R.,III, Talley,N.J., Fett,S.L., Zinsmeister,A.R. and Melton,L.J.,III (1997) Prevalence and clinical spectrum of gastroesophageal reflux: a population-based study in Olmsted County, Minnesota. *Gastroenterology*, **112**, 1448–1456.
3. Lagergren,J., Bergstrom,R., Lindgren,A. and Nyren,O. (1999) Symptomatic gastroesophageal reflux as a risk factor for esophageal adenocarcinoma. *N. Engl. J. Med.*, **340**, 825–831.
4. Winters,C.,Jr, Spurling,T.J., Chobanian,S.J. *et al.* (1987) Barrett's esophagus. A prevalent, occult complication of gastroesophageal reflux disease. *Gastroenterology*, **92**, 118–124.
5. Dixon,M.F., Neville,P.M., Mapstone,N.P., Moayyedi,P. and Axon,A.T. (2001) Bile reflux gastritis and Barrett's oesophagus: further evidence of a role for duodenogastro-oesophageal reflux? *Gut*, **49**, 359–363.
6. Cameron,A.J., Lomboy,C.T., Pera,M. and Carpenter,H.A. (2001) Adenocarcinoma of the esophagogastric junction and Barrett's esophagus. *Gastroenterology*, **109**, 1541–1546.
7. Fein,M., Ireland,A.P., Ritter,M.P., Peters,J.H., Hagen,J.A., Bremner,C.G. and DeMeester,T.R. (1997) Duodenogastric reflux potentiates the injurious effects of gastroesophageal reflux. *J. Gastrointest. Surg.*, **1**, 27–33.

8. Vaezi,M.F. and Richter,J.E. (1995) Synergism of acid and duodenogastroesophageal reflux in complicated Barrett's esophagus. *Surgery*, **117**, 699–704.
9. Caldwell,M.T., Lawlor,P., Byrne,P.J., Walsh,T.N. and Hennessy,T.P. (1995) Ambulatory oesophageal bile reflux monitoring in Barrett's oesophagus. *Br. J. Surg*., **82**, 657–660.
10. Kauer,W.K., Burdiles,P., Ireland,A.P., Clark,G.W., Peters,J.H., Bremner,C.G. and DeMeester,T.R. (1995) Does duodenal juice reflux into the esophagus of patients with complicated GERD? Evaluation of a fiberoptic sensor for bilirubin. *Am. J. Surg*., **169**, 98–103, discussion 103–104.
11. Liron,R., Parrilla,P., Martinez,L.F., Ortiz,A., Robles,R., Lujan,J.A., Fuente,T. and Andres,B. (1997) Quantification of duodenogastric reflux in Barrett's esophagus. *Am. J. Gastroenterol*., **92**, 32–36.
12. Stein,H.J., Kauer,W.K., Feussner,H. and Siewert,J.R. (1998) Bile reflux in benign and malignant Barrett's esophagus: effect of medical acid suppression and nissen fundoplication. *J. Gastrointest. Surg*., **2**, 333–341.
13. Chen,X., Ding,Y.W., Yang,G., Bondoc,F., Lee,M.J. and Yang,C.S. (2000) Oxidative damage in an esophageal adenocarcinoma model with rats. *Carcinogenesis*, **21**, 257–263.
14. Chen,X. and Yang,C.S. (2001) Esophageal adenocarcinoma: a review and perspectives on the mechanism of carcinogenesis and chemoprevention. *Carcinogenesis*, **22**, 1119–1129.
15. Kumagai,H., Mukaisho,K., Sugihara,H., Bamba,M., Miyashita,T., Miwa,K. and Hattori,T. (2003) Cell kinetic study on histogenesis of Barrett's esophagus using rat reflux model. *Scand. J. Gastroenterol*., **38**, 687–692.
16. Busby,W.F,Jr, Shuker,D.E., Charnley,G., Newberne,P.M., Tannenbaum,S.R. and Wogan,G.N. (1985) Carcinogenicity in rats of the nitrosated bile acid conjugates N-nitrosoglycocholic acid and N-nitrosotaurocholic acid. *Cancer Res*., **45**, 1367–1371.
17. Kaibara,K., Takebayashi,M. and Koga,H. (1988) Deoxycholic acids as a promoter in the development of gastric carcinoma induced by N-methyl-N′-nitrosoguanidine in rats. *Cancer J*., **2**, 14–16.
18. Domellof,L., Reddy,B.S. and Weisburger,J.H. (1980) Microflora and deconjugation of bile acids in alkaline reflux after partial gastrectomy. *Am. J. Surg*., **140**, 291–295.
19. Muscroft,T.J., Deane,S.A., Youngs,D., Burdon,D.W. and Keighley,M.R. (1981) The microflora of the postoperative stomach. *Br. J. Surg*., **68**, 560–564.
20. Tanaka,H., Kijima,H., Tokunaga,T. *et al*. (1999) Frequent expression of inducible nitric oxide synthase in esophageal squamous cell carcinomas. *Int. J. Oncol*., **14**, 1069–1073.
21. Wilson,K.T., Fu,S., Ramanujam,K.S. and Meltzer,S.J. (1998) Increased expression of inducible nitric oxide synthase and cyclooxygenase-2 in Barrett's esophagus and associated adenocarcinomas. *Cancer Res*., **58**, 2929–2934.
22. Goldstein,S.R., Yang,G.Y., Chen,X., Curtis,S.K. and Yang,C.S. (1998) Studies of iron deposits, inducible nitric oxide synthase and nitrotyrosine in a rat model for esophageal adenocarcinoma. *Carcinogenesis*, **19**, 1445–1449.
23. Schubert,M.P. (1936) Compounds of thiol acids with aldehydes. *J. Biol. Chem*., **114**, 341–350.
24. Ratner,S. and Clarke,H.T. (1937) The action of formaldehyde upon cysteine. *J. Am. Chem. Soc*., **59**, 200–206.
25. Tahira,T., Suda,M., Wakabayashi,K., Nagao,M. and Sugimura,T. (1984) Kinetics of nitrosation of thioproline, the precursor of a major nitroso compound in human urine and its role as a nitrite scavenger. *Gann*, **75**, 889–894.
26. Brugarolas,A. and Gosalvez,M. (1980) Treatment of cancer by an inducer of reverse transformation. *Lancet*, **12**, 68–70.
27. Tsuda,M. and Kurashima,Y. (1991) Nitrite-trapping capacity of thioproline in the human body. *IARC Sci. Publ*., **105**, 123–128.
28. Fein,M., Fuchs,K.H., Stopper,H., Diem,S. and Herderich,M. (2000) Duodenogastric reflux and foregut carcinogenesis: analysis of duodenal juice in a rodent model of cancer. *Carcinogenesis*, **21**, 2079–2084.
29. Stanley,R. and Lauri,A. (1999) *The WHO Classification of Tumors of the Digestive System*. IARC, Lyon, France.
30. Miwa,K., Sahara,H., Segawa,M., Kinami,S., Sato,T., Miyazaki,I. and Hattori,T. (1996) Reflux of duodenal or gastro-duodenal contents induces esophageal carcinoma in rats. *Int. J. Cancer*, **17**, 269–274.
31. Mirvish,S.S. (1997) Studies on experimental animals involving surgical procedures and/or nitrosamine treatment related to the etiology of esophageal adenocarcinoma. *Cancer Lett*., **117**, 161–174.
32. Sato,T., Miwa,K., Sahara,H., Segawa,M. and Hattori,T. (2002) The sequential model of Barrett's esophagus and adenocarcinoma induced by duodeno-esophageal reflux without exogenous carcinogens. *Anticancer Res*., **22**, 39–44.
33. Correa,P., Haenszel,W., Cuello,C., Tannenbaum,S. and Archer,M. (1975) A model for gastric cancer epidemiology. *Lancet*, **12**, 58–60.
34. Fernandes,E., Toste,S.A., Lima,J.L. and Reis,S. (2003) The metabolism of sulindac enhances its scavenging activity against reactive oxygen and nitrogen species. *Free Radic. Biol. Med*., **35**, 1008–1017.
35. Ohshima,H. and Bartsch,H. (1994) Chronic infections and inflammatory processes as cancer risk factors: possible role of nitric oxide in carcinogenesis. *Mutat. Res*., **305**, 253–264.
36. Liu,R.H. and Hotchkiss,J.H. (1995) Potential genotoxicity of chronically elevated nitric oxide: a review. *Mutat. Res*., **339**, 73–89.
37. Nozoe,T., Korenaga,D., Kabashima,A. and Sugimachi,K. (2002) Smoking-related increase of O(6)-methylguanine-DNA methyltransferase expression in squamous cell carcinoma of the esophagus. *Cancer Lett*., **8**, 49–55.
38. Devesa,S., Blot,W.J. and Fraumeni,J.F.,Jr (1998) Changing patterns in the incidence of esophageal and gastric carcinoma in the United States. *Cancer*, **83**, 2049–2053.
39. Yokoyama,A., Ohmori,T., Muramatsu,T. *et al*. (1996) Cancer screening of upper aerodigestive tract in Japanese alcoholics with reference to drinking and smoking habits and aldehyde dehydrogenase-2 genotype. *Int. J. Cancer*, **68**, 313–316.
40. Ke,L., Yu,P. and Zhang,Z.X. (2002) Novel epidemiologic evidence for the association between fermented fish sauce and esophageal cancer in South China. *Int. J. Cancer*, **20**, 424–426.

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