

Ingestion of thioproline suppresses rat esophageal adenocarcinogenesis caused by duodenogastroesophageal reflux

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Abstract. Duodenogastroesophageal reflux causes esophageal adenocarcinoma in rats without the use of a carcinogen. This etiology is unclear, but may be associated with endogenous nitrosation in the gastrointestinal tract. Thioproline (TPRO) is an effective nitrite-trapping agent and blocks endogenous nitrosation. We investigated how ingested TPRO affected esophageal adenocarcinogenesis in rats with duodenogastroesophageal reflux (DGER) or gastroesophageal reflux (GER). A series of 200 male Fischer 344 rats received surgery to induce reflux of duodenogastric contents or gastric contents alone into the esophagus. The rats were separated into two divisions according to the surgical procedure employed (DGER or GER), and each division was further subdivided into two groups: one group was fed a special diet (CRF-1 containing 0.5% of TPRO); the other group was fed a standard diet (CRF-1). The rats were given no carcinogen and sacrificed at ten-week intervals from the 25th to the 45th week after surgery. Pathological examination was carried out using hematoxylin-eosin or immunohistochemical staining. Erosion, regenerative thickening, basal cell hyperplasia and columnar-lined epithelium (CLE) were found in both groups of the DGER rats. Adenocarcinoma (AC) appeared only in the DGER rats sacrificed at 35 and 45 weeks following surgery. The incidence of AC at the 45th week was significantly lower in

the group of rats fed the diet containing TPRO, as compared to those fed the standard diet, whereas the incidences of CLE were the same for both groups. iNOS protein and nitro-tyrosine protein were identified in the CLE and macrophages of the DGER group using immunohistochemical staining. There were no remarkable pathological changes in the esophagi of the rats which underwent the GER procedure. In conclusion, TPRO has an inhibitory effect on esophageal reflux-induced adenocarcinogenesis in rats in that it prevents the progression from CLE to AC.

Introduction

The incidence of esophageal adenocarcinoma (AC) has been increasing not only in Western countries (1,2) but also in East Asia (3). Esophageal AC occurs in the lower esophagus developing from the columnar-lined epithelium (CLE) known as Barrett's esophagus (4). Barrett's esophagus and esophageal AC are closely associated with duodenogastroesophageal (DGER) reflux (5-8). Rat experiments demonstrated that duodenal contents cause esophageal carcinoma without exposure to carcinogens (9-13), whereas gastric contents do not (9). Though the etiology of esophageal AC is unclear, Mirvish *et al* suggested that the human esophageal AC and Barrett's esophagus was initiated by nitrosoamine (14).

Endogenous *N*-nitroso compounds (NOCs) are produced by chemical reactions between amine or amide precursors and nitrites generated by nitrates (15,16). NOCs are produced by acid-mediated nitrosation at low pH levels in the stomach (17). They can also be produced at neutral pH levels by intestinal bacteria possessing nitrate reductase and nitrosating enzymes via nitric oxide formation (18-20). It has been shown that free radicals mediate reflux esophagitis and Barrett's esophagus (21-23). Increased expression of inducible nitric oxide synthase (iNOS) is observed in not only esophageal squamous cell carcinoma but also Barrett's esophagus and subsequent AC (24,25). Thus, nitric oxide might play an important role in esophageal carcinogenesis.

Thiazolidine-4-carboxylic acid (thioproline, TPRO) is a cyclic sulfur-containing amino acid that is a condensation product of cysteine and formaldehyde (26). TPRO rapidly

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Abbreviations: NOC, *N*-nitroso compound; TPRO, thioproline, (thiazolidine-4-carboxylic acid); iNOS, inducible nitric oxide synthase; DGER, duodenogastroesophageal reflux; GER, gastroesophageal reflux; CLE, columnar-lined epithelium; AC, adenocarcinoma

Key words: thioproline, duodenogastroesophageal reflux, columnar-lined epithelium, esophageal adenocarcinogenesis, *N*-nitroso compound

traps nitrites in the human body (27) and changes into *N*-nitrosothiazolidine-4-carboxylic acid (NTPRO) (Fig. 1). NTPRO is not carcinogenic and is excreted in the urine without further metabolism. Thus TPRO acts as a nitrite scavenger, rendering carcinogenic *N*-nitroso compounds inactive. Additionally, TPRO may act as an intracellular sulfhydryl antioxidant and as a scavenger of free radicals (28). Tahira *et al* have shown that TPRO suppresses carcinogenesis induced by *N*-benzylmethylamine and nitrites (29). Our previous investigation showed that the gastric carcinogenesis induced by duodenogastric reflux in rats was inhibited by ingested TPRO (30). Kumagai *et al*, our colleagues, reported the suppressing effect of TPRO on esophageal adenocarcinogenesis in rats (31).

Progression of esophageal adenocarcinogenesis is divided into 3 parts: esophagitis, CLE, and AC (32). The aim of this study is to investigate which component of the esophageal metaplasia-adenocarcinoma sequence in rats is suppressed by TPRO.

Materials and methods

Animals and experimental environment. Two hundred male Fischer 344 rats weighing ~180 g each were housed three to a cage and maintained under conditions of $22 \pm 3\%$ room temperature and $55 \pm 5\%$ humidity with a 12-h light-dark cycle.

Chemicals and diets. TPRO was purchased from ICN Biochemicals Inc. (OH, USA). CRF-1 (Charles River Co., Tokyo, Japan) was used as the standard diet. TPRO was added at the concentration of 0.5% (W/W) to the CRF-1.

Surgical procedure. After fasting for 24 h, the rats received upper abdominal incisions under diethyl-ether inhalation anesthesia (Fig. 2).

Duodenogastroesophageal reflux (DGER). After both vagus nerves were preserved, the abdominal esophagus was transected under the diaphragm, and the distal cut end was closed with sutures. The esophageal stump was then anastomosed end-to-side to a loop of jejunum 4 cm distal to the ligament of Treitz in an ante-colic manner. This procedure allowed duodenogastric contents to flow back into the esophagus.

Gastroesophageal reflux (GER). The duodenum was cut off near the pylorus, and the distal duodenal stump was closed. The jejunum was transected ~4 cm distal to the ligament of Treitz, and the distal, cut end was closed with sutures. The proximal duodenal stump was anastomosed end-to-side with the distal jejunum near the sutures. After both vagus nerves were preserved, the abdominal esophagus was transected below the diaphragm, and the distal cut end was closed with sutures. The esophageal stump was anastomosed end-to-side to the distal jejunum ~1 cm distal to the gastro-jejunostoma. Finally, the proximal jejunal cut end was anastomosed end-to-side with the jejunal loop ~4 cm distal to the esophago-jejunostoma. This surgery permitted only gastric contents to flow back into the esophagus.

Every intestinal anastomosis was carried out with 4 interrupted stitches through all the intestinal layers in a single

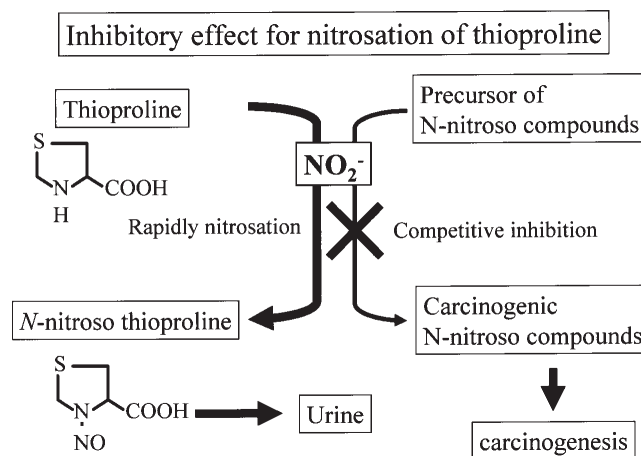


Figure 1. Inhibitory effect of thioproline (TPRO). TPRO is rapidly nitrosated and prevents the formation of NOC by trapping nitrites in the human body. TPRO is nitrosated to nitrosothioproline, which is non-mutagenic, and excreted in the urine.

line using 7-0 polypropylen-monofilament sutures. After the operation, the animals were allowed to drink immediately but continued fasting for 1 day. The rats were weighed every 4 weeks throughout the experiment. They were given no carcinogen, and sub-groups were sacrificed every 10 weeks from the 25th week until the 45th week after surgery.

Experimental groups. The animals were divided into 2 divisions according to the surgical procedure employed - the DGER division and the GER division - and each division was subdivided into 2 groups according to the diets administered as follows:

DGER division. Group A: animals fed a special diet (CRF-1 containing 0.5% TPRO) following the DGER procedure (40 animals); Group B: animals fed a standard diet (CRF-1) following the DGER procedure (60 animals).

GER division. Group C: animals fed a special diet (CRF-1 containing 0.5% of TPRO) following the GER procedure (40 animals); Group D: animals fed a standard diet (CRF-1) following the GER procedure (60 animals).

Pathological evaluation. The rats were sacrificed using diethyl-ether inhalation and their abdomens were opened. The afferent and efferent jejunal loops were cut off and the esophagus was transected at the level of the thyroid cartilage. Then the esophagus and anastomosed jejunum were removed.

The esophagus was opened longitudinally and spread on a cork plate with the mucosal side up. After having been fixed with 10% formalin solution for 24 h, the esophagus was cut at 3-mm intervals along the longitudinal section and embedded in paraffin. Sections of each block (4 μ m) were prepared for pathological assessment with hematoxylin-eosin and immunohistochemical staining.

Definition of the pathological findings. The esophageal histological findings were classified in accordance with the descriptions by Miwa *et al* (10).

Erosion. Defect of the epithelium with inflammatory cell infiltration.

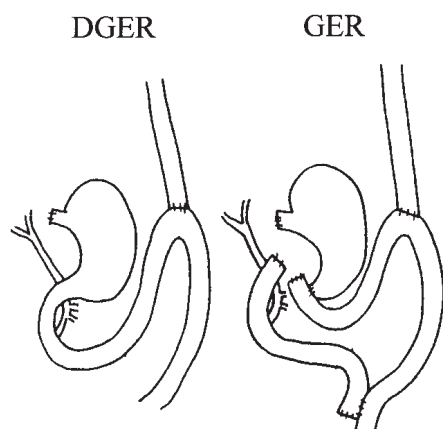


Figure 2. A surgical model for esophageal adenocarcinoma through reflux. In the duodenogastroesophageal reflux (DGER) case, end-to-side esophagojejunostomy to the jejunum 4 cm distal to the ligament of Treitz was performed to induce the return flow of gastroduodenal contents back into the esophagus. In the gastroesophageal reflux (GER) case, the end-to-side esophagojejunostomy to the jejunum 1 cm distal to the gastrojejunostoma and end-to-side anastomosis to the jejunal loop 4 cm distal to the esophagojejunostoma was made to induce the flow of gastric contents back into the esophagus.

Regenerative thickening. This condition is marked by esophageal epithelial thickening more than double the thickness of the normal epithelium with acanthosis, abnormal extension of papilla towards the mucosal surface and parakeratosis. The stratified structure of the epithelium is not disturbed.

Basal-cell hyperplasia. In this condition the basal layer in the squamous epithelium occupies >15% of the full thickness of the squamous epithelial layer. It may contain intramural cysts. The stratified structure of the epithelium is preserved.

CLE. The esophageal squamous epithelium is replaced by columnar-lined epithelium with brush borders and goblet cells.

AC. Carcinoma is defined as an epithelial growth with cellular and structural atypism, invading into the submucosal layer. AC is a dysplastic glandular cell growth with both atypism and invasiveness, and is composed of tubular or papillary carcinoma or mucinous carcinoma.

Immunohistochemical analysis for iNOS and nitrotyrosine. Immunohistochemical staining for iNOS and nitrotyrosine in the DGER division were performed on the esophageal sections of the rats sacrificed at 45 weeks after surgery, using a Dako EnVision™ system (Dako, Tokyo, Japan). The sections were deparaffinized and incubated with 2N HCl for 30 min and neutralized with 1N sodium borate for DNA denaturation. Then all the sections were heated with microwaves in a 0.01 mol/l citrate buffer for 10 min. The endogenous peroxidase activity in the tissue was quenched in methanol containing 0.3% hydrogen peroxide for 20 min, and then each section was placed into protein block serum-free (Dako) for blocking the sections. The sections were incubated with mouse monoclonal antibodies to iNOS (Santa Cruz, CA, USA) and nitrotyrosine (EMD Bioscience Inc., USA) overnight at 4°C. Dako EnVision labeled polymer (Dako), which is the secondary antibody to mouse and rabbit immunoglobulin combined with dextran marked with peroxidase, was used to detect the immunoreactivity. The antibody complexes were visualized by incubation with 3,3'-diaminobenzidine tetrahydrochloride. Sections were counterstained with hematoxylin staining.

Statistical analysis. The Fisher's exact test was used for statistical analysis of the incidence of pathological findings. P-values of <0.01 were considered significant.

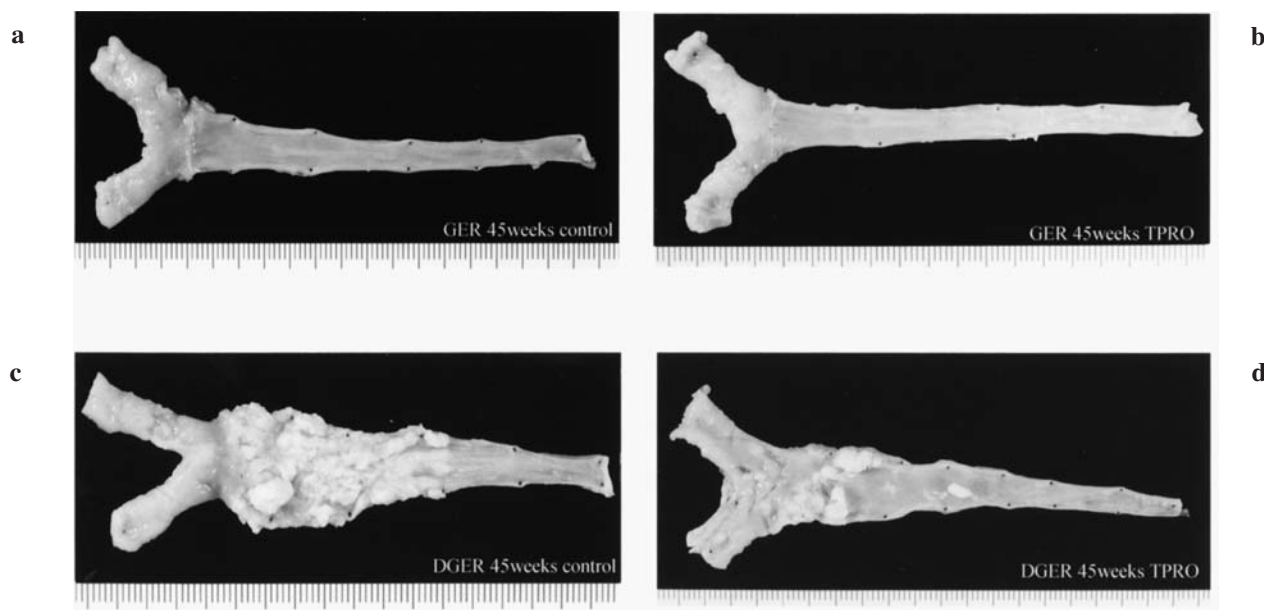


Figure 3. Macroscopic findings. There was very little erosion and only slight wall thickening of the esophagi of rats in both groups of the GER division at 45 weeks after operation (a and b). In both groups A and B, the esophageal walls of all animals revealed uneven surfaces with thickening of the wall and upper and middle esophageal dilatation (c and d). Most of the rats exhibited stenosis and elevated lesions in the lower or middle esophagus due to ulceration or carcinoma in both groups of the DGER division at all observation weeks after operation (c and d). These findings were more obvious in group A than in group B (c and d), and these findings increased in later weeks for both groups.

Table I. The incidence of pathological findings for rats with duodenogastroesophageal reflux.

Pathological findings	DGER					
	25 weeks		35 weeks		45 weeks	
	TPRO (n=5)	Control (n=10)	TPRO (n=6)	Control (n=9)	TPRO (n=20)	Control (n=27)
ERO	5 (100%)	10 (100%)	6 (100%)	9 (100%)	20 (100%)	27 (100%)
RT	5 (100%)	10 (100%)	6 (100%)	9 (100%)	20 (100%)	27 (100%)
Basal cell hyperplasia	5 (100%)	10 (100%)	6 (100%)	9 (100%)	20 (100%)	27 (100%)
CLE	2 (40%)	5 (50%)	5 (83%)	8 (89%)	17 (85%)	24 (89%)
AC	0 (0%)	0 (0%)	0 (0%)	1 (11%)	1 ^a (5%)	12 (44%)

^aP<0.01 for adenocarcinoma in rats fed thioproline vs. the control group rats sacrificed at 45 weeks.

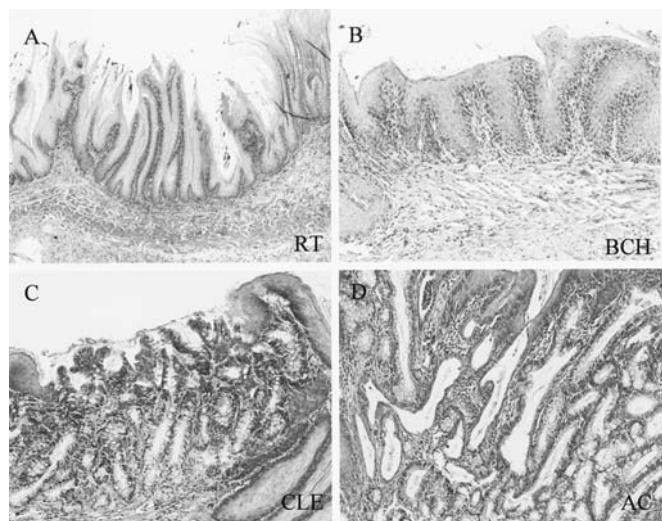


Figure 4. Histological findings. Regenerative thickening (A) and basal cell hyperplasia (B) were present in all groups. Columnar-lined epithelium (C) and adenocarcinoma (D) were found in the lower third of the esophagi in both groups of the DGER division.

Results

General observations. The number of examined rats sacrificed at the 25th, 35th and 45th weeks after surgery was: 5, 6 and 20 from group A; 10, 9 and 27 from group B; 5, 6 and 17 from group C; and 7, 10 and 30 from group D, respectively. Twenty-three rats in the DGER division died of malnutrition and esophageal stenosis complicated by reflux esophagitis. Twenty-five rats in the GER division died within a week after surgery.

There were no significant differences in pre-operative average body weight (g) (mean \pm SD) among the groups. The

average body weights (g) (mean \pm SD) at the 45th week after surgery exhibited no significant differences between the groups A (229 \pm 58) and B (229 \pm 42) or between the groups C (320 \pm 35) and D (319 \pm 29), respectively. However, the body weights (g) in the groups A and B were significantly lower than the groups C and D, respectively.

Macroscopic findings. There were no remarkable macroscopic changes in the esophagi of the rats in the GER division at any examined week. On the other hand, in the DGER division, the lower portion of the esophagi revealed uneven surfaces and wall thickening whereas the middle and upper portions were dilated in all rats at observed week.

Mucosal lesions such as ulceration and protruded lesions in the lower esophagi in groups A and B were found with incidences of 50 and 40% in the 25th week, 89 and 83% in the 35th week, and 92 and 80% in the 45th week, respectively. The incidence of the lesions increased in the later weeks but was almost the same for both groups in each week observed (Fig. 3).

Histological changes. There were slight histological changes including erosion and regenerative thickening in the GER division. Histological changes in the DGER division are shown in Table I. Erosion, regenerative thickening and basal cell hyperplasia were present in all rats at each examined week (Fig. 4). CLE was observed in both groups A and B, with incidences of 40 and 50% in the 25th week, 83 and 89% in the 35th week, and 85 and 89% in the 45th week after surgery, respectively (Fig. 4). Observations during the same weeks revealed the incidence of CLE was almost the same between both groups. AC appeared in the lower esophagus in groups A and B, and the incidence was 0 and 0% in the 25th week, 0 and 11% in the 35th week, and 5 and 44% in the 45th week, respectively (Fig. 4). The occurrence of AC in group A was

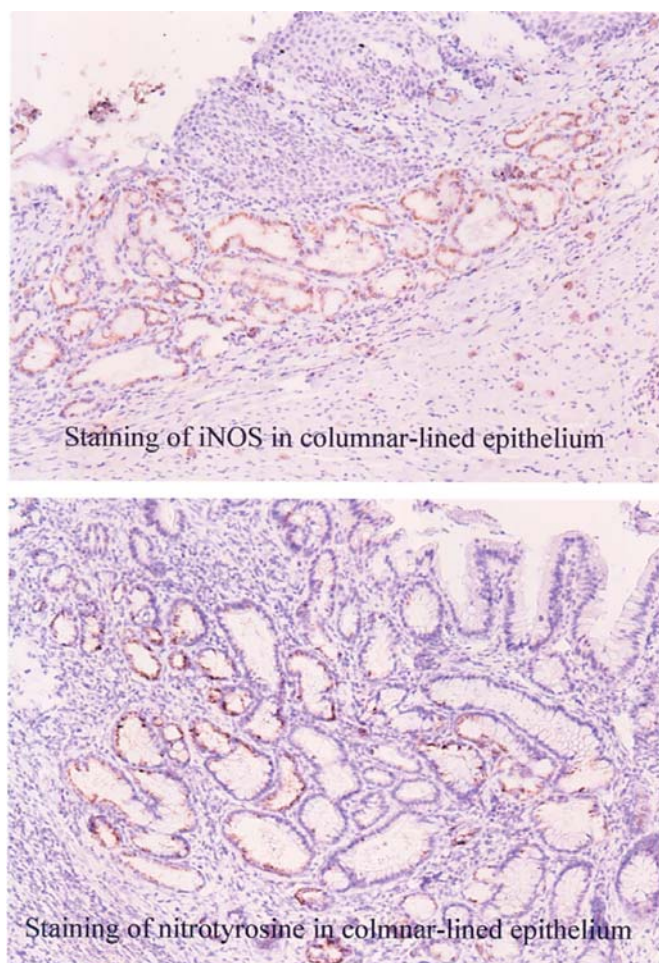


Figure 5. Immunohistochemical staining. Immunohistochemical staining for iNOS and nitrotyrosine were performed on esophageal sections from rats in the DGER division sacrificed at 45 weeks after surgery. Staining of iNOS and nitrotyrosine were seen in the CLE and stromal macrophages.

significantly lower than in group B at 45 weeks after surgery ($p < 0.01$).

Immunohistochemical staining. Though iNOS and nitrotyrosine proteins were not detected in AC, they were overexpressed in CLE and its stromal macrophages (Fig. 5). No difference was found in the staining between groups A and B.

Discussion

The present investigation reconfirmed that esophageal AC developed in rats with DGER, but not in those with GER. In addition, it was shown that duodeno-esophageal reflux alone can develop AC in rat esophagi (32). These findings suggest that regurgitated duodenal contents probably contain a carcinogenic substance which induces this carcinogenesis. A few reports suggest that duodenal juice might be carcinogenic. Mirvish (33) hypothesized that the intragastric formation of nitrosamides by the acid-catalyzed reaction of amides with nitrites is a plausible etiological factor in the development of human gastric cancer. Calmels *et al* described that endogenous nitrosation may be caused by bacteria that can carry out

denitrification in rat stomach with achlorhydria (18). Busby *et al* indicated that two nitrosated bile acid conjugates, *N*-nitrosoglycocholic acid and *N*-nitrosotaurocholic acid, were hepatocarcinogenic in rats (34).

In 1996, Nakai reported from our laboratory an inhibitory effect of TPRO on gastric carcinogenesis induced by duodenogastric reflux (30). He made a rat model of duodenogastric reflux and administered a commercial diet containing 0.5% TPRO (TPRO group) and a commercial diet without additives (control group). At the 50th week after surgery the incidence of glandular stomach carcinoma of rats was 0% in the TPRO group, whereas it was 36% in the control group. The daily urinary excretion of NTPRO in rats was $2.0 \mu\text{g}$ in the TPRO group and $<0.05 \mu\text{g}$ in the control group. The results demonstrated that TPRO acts as a scavenger of nitrites and prevents carcinogenesis. This evidence led us to our present speculation that esophageal carcinogenesis may also be caused by endogenous nitrosation.

The sequential histological study disclosed 3 steps in the present carcinogenesis: firstly esophagitis; secondly CLE; and lastly AC. In the DGER control rats, the lower part of the esophagi exhibited the esophagitis featuring erosion, regenerative thickening and basal cell hyperplasia at the 25th week after surgery. The CLE and AC were first observed at the 25th and 35th week, respectively. The incidence of CLE and AC sequentially increased after surgery and reached 89 and 44% at the 45th week, respectively. This suggested the esophageal carcinogenesis as an inflammation-metaplasia-adenocarcinoma sequence.

It is not clearly understood which step of the experimental esophageal carcinogenesis can be prevented. Our laboratory has presented two studies on how to prevent this carcinogenesis. Nishijima *et al* made it clear that a switch procedure from the duodeno-esophageal reflux model into the Roux-en-Y type model can stop the development of esophageal AC from CLE (35). Oyama *et al* reported that a COX-2 inhibitor, celecoxib, alleviates esophagitis to result in the inhibition of CLE and AC occurrence in rats (36).

Present sequential observation demonstrated erosion, regenerative thickening and basal cell hyperplasia in all of the rats at each periodic examination after surgery and did not show any difference in the incidence between the control and the TPRO groups. The occurrence of CLE was not different between the TPRO and the control groups. The incidence of AC was significantly depressed in the TPRO group at the 45th week after surgery as compared with the control group, though that of CLE was not different. This implied that TPRO prevented the progression from CLE to esophageal AC. Contrarily, Kumagai *et al* (31), have reported that TPRO reduced the occurrence of esophageal adenocarcinoma in rats which had undergone a side-to-side anastomosis between the jejunum and the esophagogastric junction 70 weeks prior to being sacrificed. In this rat model of duodeno-esophageal reflux, the incidence of associated lesions such as esophageal ulcers and specialized columnar epithelium were lower in the TPRO group than in the control group, though there was no statistically significant difference. The occurrence of every pathological finding in the respective control groups was higher in our case than in the case of Kumagai, though the observation period for our case was

shorter than in the case of Kumagai (31). This discrepancy can not be clearly explained, but we believe it may be attributable to different levels of intensity of DGER, said difference in levels perhaps resulting from the different methods of surgical intervention employed.

The question then arises whether TPRO may be applied to prevention of AC in humans. Goldstein *et al* described the pathological features of adenocarcinogenesis in rat esophagi as being similar to that in humans (11). We hypothesize that TPRO also may inhibit the formation of carcinogenic NOC in humans, and may also have effects on CLE (Barrett's esophagus) patients.

TPRO has been marketed in France since 1964 for the treatment of hepatic and biliary disorders (37) and has been administered to patients with advanced squamous cell carcinoma in the head and neck (38). However, due to suspected side-effects and toxicity of this drug (39), we have never been able to use it due to government restrictions. Though the clinical use of TPRO itself cannot be put into practice, suggestions for the further development of drugs suppressing the production of NOC are offered.

In immunohistochemical staining, iNOS and nitrotyrosine staining were positive in CLE in the DGER division. It is probable that nitric oxide is also related with carcinogenesis of esophageal AC, because there are some reports that the expression level of iNOS is higher in esophageal AC than in non-neoplastic tissue in humans and rats (24,25). Nitrites also promote tyrosine nitration through formation of nitryl chloride and nitrogen dioxide by myeloperoxidase (40). Since nitrotyrosine is a stable nitration product of tyrosine residue, it can be used as a marker for peroxynitrite and other nitrating species (41). This study showed that the stain of iNOS and nitrotyrosine were not suppressed by TPRO. Though reactive nitrogen substances could be associated with adenocarcinogenesis of the esophagus, more studies are necessary to investigate whether TPRO suppresses nitrating or not.

Miwa *et al* reported that bile was a more important component for the development of gastric carcinoma than pancreatoduodenal secretion (42). On the other hand, some groups of investigators concluded pancreatic exocrine secretion is the factor responsible for gastric (43) and esophageal (44) carcinogenesis. Pera *et al* reported that esophageal carcinomas were induced only when pancreatic secretion was present in the duodenal-content reflux together with low dose carcinogens (45), and that both pancreatobiliary and pancreatic secretion stimulated an expansion of the proliferative compartment of the esophageal squamous epithelium in rats (46). Nevertheless, we have found no reports that pancreatic juice is carcinogenic or mutagenic. Our results lead us to suppose that NOCs, such as *N*-nitrosoglycocholic acid and *N*-nitrosotaurocholic acid, may be related to the carcinogens of esophageal AC. Although no presence of NOCs including *N*-nitrosoglycocholic acid and *N*-nitrosotaurocholic acid could be confirmed in the duodenal juice of the rats with esophagojejunostomy (47), the unstable nature of *N*-nitroso compounds may account for the difficulty in detecting NOCs.

In conclusion, AC developed in rats with duodenogastroesophageal reflux and did not in those with gastroesophageal reflux. The oral administration of 0.5% TPRO inhibited the process of development from CLE to AC in this esophageal

carcinogenesis. This implies that the carcinogenesis is related to endogenous duodenal nitrosation, and TPRO has been shown to exhibit a potent role in preventing reflux-induced esophageal adenocarcinogenesis in rats.

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