

Figure 4. *miR-142-5p* and *miR-155* suppress *TP53INP1* as their target. (A) Luciferase reporter constructs of wild-type (WT) and mutant (MUT) target sites in the 3' UTR of *TP53INP1* mRNA. The base pairings between *miR-142-5p* and *miR-155* and their putative target sites in the 3' UTR of *TP53INP1* mRNA are shown. Underlining indicates mutant sequences. (B) Firefly luciferase activity was normalized to the *Renilla* luciferase activity of *TP53INP1* wild-type (WT), mutant (MUT), and pGL3 control (empty vector) in AGS cells transfected with negative control (NC), *miR-142-5p*, and *miR-155*. The luciferase activities of the AGS cells transfected with the *TP53INP1*-WT construct were significantly lower after transfection of *miR-142-5p* and *miR-155*, whereas those transfected with the *TP53INP1*-MUT construct or the pGL3 control vector (empty vector) showed no significant differences. * $p < 0.05$ and ** $p < 0.005$, compared with negative control. (C) Western blot analysis of TP53INP1 in control and *H. heilmannii*-infected mice. β -actin was used as a loading control. The expression of TP53INP1 was suppressed in *H. heilmannii*-infected mice, compared with the expression level in control mice. (D) Immunohistochemistry for TP53INP1 in human gastric MALT lymphoma. Sections were counterstained with HE. TP53INP1 staining was markedly reduced in the MALT lymphoma lesions.
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human malignancies. The results of microarray analysis revealed a unique miRNA expression profile in gastric MALT lymphoma. Recent studies have demonstrated that the expression level of *miR-155* is significantly elevated in DLBCL, which is considered to represent high-grade transformation from MALT lymphoma [20,33]. *miR-142*, *miR-155* and *miR-223* have been reported to be hematopoiesis-specific miRNAs [24]. These findings are

consistent with our results, and in the present study we focused on *miR-142* and *miR-155*, because *miR-142* is the most up-regulated miRNA and *miR-155* plays a critical role in the pathogenesis of B-cell lymphoma. The levels of *miR-142-5p* and *miR-155* expression were associated with the clinical course of gastric MALT lymphoma, including the response to *H. pylori* eradication. The *API2-MALT1* fusion gene has been identified as a

potential predictor of resistance to *H. pylori* eradication therapy. In the present study, two cases of gastric MALT lymphoma resistant to *H. pylori* eradication lacked the *API2-MALT1* fusion gene (patients #7, #8). These cases showed increased expression of *miR-142-5p* and *miR-155*. Therefore, these miRNAs may be more useful markers than the *API2-MALT1* fusion gene in patients with gastric MALT lymphoma.

TP53INP1 is a proapoptotic stress-induced p53 target gene. p53 activates *TP53INP1* transcription, and overexpression of *TP53INP1* induces cell cycle arrest and apoptosis [34]. Gironella *et al.* [29] have shown that oncogenic *miR-155* is overexpressed in pancreatic ductal adenocarcinoma and suppresses its target, *TP53INP1*, resulting in cancer progression. In agreement with these findings, our results showed that *TP53INP1* was suppressed by both *miR-142-5p* and *miR-155*, possibly leading to inhibition of apoptosis and acceleration of MALT lymphoma cell proliferation. These findings suggest that overexpression of *miR-142-5p* and *miR-155* concomitant with suppression of *TP53INP1* reflect the increased proliferation of MALT lymphoma cells. The distinct connection between aberrant expression of *miR-142-5p* and *miR-155* and the progression of MALT lymphoma suggests that miRNAs could be potential therapeutic targets. A recent study has shown that chemically engineered oligonucleotides, termed

'antagomirs,' can work as specific inhibitors of endogenous miRNAs in mice [35], and might be potentially applicable to silence *miR-142-5p* and *miR-155* for the treatment of gastric MALT lymphomas that are resistant to *H. pylori* eradication therapy.

Conclusions

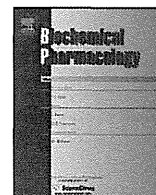
Overexpression of *miR-142-5p* and *miR-155* is presumed to play a critical role in the initiation and progression of gastric MALT lymphoma, suggesting that these miRNAs may be potentially useful as therapeutic targets and novel biomarkers for gastric MALT lymphomas. Inhibition of *miR-142-5p* and *miR-155* might be a novel approach for the prevention and treatment of gastric MALT lymphoma. Further studies involving more patients are warranted to explore the clinical use of *miR-142-5p* and *miR-155* for diagnosis and treatment of gastric MALT lymphoma.

Author Contributions

Conceived and designed the experiments: YS H. Suzuki TH. Performed the experiments: YS HT JM KH. Analyzed the data: YS H. Suzuki JM. Contributed reagents/materials/analysis tools: YS H. Suzuki HI JM KH NH MN MM H. Saito. Wrote the paper: YS H. Suzuki.

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Identification of a unique nsaid, fluoro-loxoprofen with gastroprotective activity^{☆,☆☆}

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ABSTRACT

We previously proposed that direct cytotoxicity of NSAIDs due to their membrane permeabilization activity, together with their ability to decrease gastric prostaglandin E₂, contributes to production of gastric lesions. Compared to loxoprofen (LOX), fluoro-loxoprofen (F-LOX) has much lower membrane permeabilization and gastric ulcerogenic activities but similar anti-inflammatory activity. In this study, we examined the mechanism for this low ulcerogenic activity in rats. Compared to LOX, the level of gastric mucosal cell death was lower following administration of F-LOX. However, the gastric level of prostaglandin E₂ was similar in response to treatment with the two NSAIDs. Oral pre-administration of F-LOX conferred protection against the formation of gastric lesions produced by subsequent administration of LOX and orally administered F-LOX resulted in a higher gastric pH value and mucus content. In the presence of a stimulant of gastric acid secretion, the difference in the ulcerogenic activity of F-LOX and LOX was less apparent. Furthermore, an increase in the mucus was observed in gastric cells cultured in the presence of F-LOX in a manner dependent of increase in the cellular level of cAMP. These results suggest that low ulcerogenic activity of F-LOX involves its both low direct cytotoxicity and protective effect against the development of gastric lesions. This protective effect seems to be mediated through an increase in a protective factor (mucus) and a decrease in an aggressive factor (acid).

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Abbreviations: ABTS, 2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid)-2NH₄; BAPTA-AM, 1,2-bis(2-aminophenoxy)ethane-*N,N,N',N'*-tetraacetic acid; COX, cyclooxygenase; DAPI, 4',6-diamidino-2-phenylindole dihydrochloride; EIA, enzyme immunoassay; ELLA, enzyme-linked lectin-binding assay; F-LOX, fluoro-loxoprofen; FBS, fetal bovine serum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; H & E, hematoxylin and eosin; IBMX, 3-isobutyl-1-methylxanthine; LOX, loxoprofen; NSAID, non-steroidal anti-inflammatory drug; PG, prostaglandin; RGM1, rat normal gastric epithelial cell line; SD, Sprague-Dawley; SBA, soybean agglutinin; TCA, trichloroacetic acid; TUNEL, terminal deoxynucleotidyl transferase-mediated biotinylated UTP nick end labeling.

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1. Introduction

The balance between aggressive and defensive factors determines the development of gastric lesions, with either a relative increase in aggressive factors or a decrease in protective factors resulting in lesions. The gastric mucosa can be challenged by a variety of both endogenous and exogenous factors, including gastric acid, reactive oxygen species, ethanol, *Helicobacter pylori* and non-steroidal anti-inflammatory drugs (NSAIDs) [1]. In order to protect the mucosa, the body relies on defence systems such as the production of surface mucus and bicarbonate, and the regulation of gastric mucosal blood flow. Prostaglandin E₂ (PGE₂) also exerts a strong protective effect, inhibiting the secretion of gastric acid and stimulating the production of mucus [2].

NSAIDs, such as indomethacin, comprise a therapeutically valuable family of drugs [3]. An inhibitory effect of NSAIDs on cyclooxygenase (COX) activity is responsible for their anti-inflammatory actions, COX being an enzyme that is essential for

the synthesis of prostaglandins, which have a strong capacity to induce inflammation. However, as described above, NSAID use is also associated with gastrointestinal complications [4–7], which was thought to result from the inhibition of COX and a decrease in gastric PGE₂ level. In fact, NSAIDs have been reported to stimulate the secretion of gastric acid and inhibit the production of mucus through decreasing gastric PGE₂ level [8,9]. However, it is now believed that the production of gastric lesions by NSAIDs involves additional mechanisms, given that the increased incidence of gastric lesions and the decrease in PGE₂ levels induced by NSAIDs do not always occur in parallel [10,11]. We have recently demonstrated that NSAIDs induce cell death (apoptosis) in cultured gastric mucosal cells and at the gastric mucosa in a manner independent of COX inhibition [12–16]. With regards to the molecular mechanism governing this apoptosis, we have proposed the following pathway. Permeabilization of cytoplasmic membranes by NSAIDs stimulates Ca²⁺ influx and increases intracellular Ca²⁺ levels, which in turn induces the endoplasmic reticulum stress response [12,17,18]. During the course of this response, an apoptosis-inducing transcription factor, C/EBP homologous transcription factor, is induced and, as we have previously shown, this protein is essential for NSAID-induced apoptosis [13,19]. Furthermore, we have proposed that both COX inhibition and gastric mucosal cell death are important for the formation of NSAID-induced gastric lesions *in vivo* [16,20].

In 1991, two subtypes of COX, COX-1 and COX-2, which are responsible for the majority of COX activity at the gastrointestinal mucosa and in tissues subject to inflammation, respectively, were identified [21]. It is therefore not surprising that a reduced incidence of gastroduodenal lesions has been reported following treatment with selective COX-2 inhibitors [22–24]. However, a recently raised issue concerning the use of selective COX-2 inhibitors is the potential risk of cardiovascular thrombotic events [25,26]. This may be due to the fact that prostacyclin, a potent anti-aggregator of platelets and a vasodilator, is mainly produced by COX-2 [27–29]. Therefore, in order to minimize clinical complications, gastric safe NSAIDs other than selective COX-2 inhibitors need to be developed. Based on the hypothesis outlined above, we believe that NSAIDs with lower membrane permeabilization activity would represent an efficacious alternative, even if they had no selectivity for COX-2 [14].

In order to investigate this possibility, we screened for such compounds from a range of clinically used NSAIDs without COX-2 selectivity, and found that the membrane permeabilization activity and direct cytotoxicity of loxoprofen (LOX) (Fig. 1A) was relatively lower than that of the other NSAIDs tested [30]. LOX is a leading NSAID on the Japanese market, being widely used because clinical studies have suggested that it is safer than other traditional (non-selective) NSAIDs [31,32]. LOX is a pro-drug, which is converted (by reduction of the cyclopentanone moiety) to its active metabolite (the *trans*-alcohol metabolite, LOX-OH) by aromatic aldehyde-ketone reductase (Fig. 1A) [33]. We therefore synthesized a series of LOX derivatives, demonstrating that fluoro-loxoprofen (F-LOX) (Fig. 1A) has much lower membrane permeabilization and gastric ulcerogenic activities than LOX, but similar anti-inflammatory activity, suggesting that it is likely to be a therapeutically viable drug [34]. We suggested that F-LOX is also a pro-drug, which is converted to its active metabolite (the *trans*-alcohol metabolite, F-LOX-OH (Fig. 1A)), because the inhibitory effect of F-LOX-OH on COX is much more potent than that of F-LOX *in vitro* [34]. Although we concluded that the low membrane permeabilization activity of F-LOX is responsible for its low ulcerogenic activity [34], it remained possible that other mechanisms could also be involved. In this study, we therefore examined the mechanism governing the low ulcerogenic activity of F-LOX. Our results suggest that this effect is mediated not only by the low

direct cytotoxicity due to its low membrane permeabilization activity, but also by protection of the gastric mucosa. In contrast to LOX and other NSAIDs, oral administration of F-LOX led to an increase in gastric pH value and mucus, suggesting that these effects are also involved in the low ulcerogenic activity of this drug.

2. Experimental procedures

2.1. Chemicals and animals

LOX, LOX-OH, F-LOX and F-LOX-OH (Fig. 1A) were synthesized in our laboratory as previously described [34]. Methylcellulose and RPMI1640 were obtained from Wako Pure Chemical Industries (Osaka, Japan). Formaldehyde, paraformaldehyde, Alcian blue 8GX, mucin, cycloheximide, histamine, fetal bovine serum (FBS), Dulbecco's modified Eagle's medium nutrient mixture F-12 Ham, 2-methyl-8-(phenylmethoxy)imidazo[1,2-*a*]pyridine-3-acetonitrile (SCH 28080; an inhibitor of gastric H⁺,K⁺-ATPase), 3-isobutyl-1-methylxanthine (IBMX), omeprazole, forskolin and bovine serum albumin (BSA) were purchased from Sigma (St. Louis, MO). Mayer's hematoxylin, 1% eosin alcohol solution and mounting medium for histochemical analysis (malinol) were from MUTO Pure Chemicals (Tokyo, Japan). Terminal transferase was obtained from Roche Diagnostics (Mannheim, Germany). Biotin 14-ATP and streptavidin-conjugated Alexa Fluor 488 were purchased from Invitrogen (Carlsbad, CA). Mounting medium for the TdT-mediated biotinylated UTP nick end labeling (TUNEL) assay (VECTASHIELD) was from Vector Laboratories, Inc. (Burlingame, CA). 4',6'-diamidino-2-phenylindole dihydrochloride (DAPI) and 1,2-bis(2-aminophenoxy)ethane-*N,N,N',N'*-tetraacetic acid (BAPTA-AM) were obtained from Dojindo (Kumamoto, Japan). SQ22536 was from CALBIOCHEM (San Diego, CA). ONO-8711 and ONO-AE2-227 were from our laboratory stocks. The prostaglandin E₂ enzyme immunoassay (EIA) kit was purchased from Cayman Chemical (Ann Arbor, MI). cAMP complete ELISA kit was from Enzo Life Sciences (Farmingdale, NY). Horseradish peroxidase-labeled soybean agglutinin (SBA-HRP) was from Seikagaku Biobusiness Co. (Tokyo, Japan). 2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid)-2NH₄ (ABTS) was obtained from KPL (Gaithersburg, MD). The RNeasy Mini kit was from QIAGEN (Valencia, CA), the first-strand cDNA synthesis kit was obtained from Takara (Kyoto, Japan), and iQ SsoFast EvaGreen Supermix was purchased from Bio-Rad (Hercules, CA). Wistar rats (3-week-old males) and Sprague-Dawley (SD) rats (6-week-old males) were obtained from Charles River Laboratories Japan (Yokohama, Japan). Guinea pigs (3-week-old males) were obtained from Japan SLC (Shizuoka, Japan). Animals were housed under conditions of controlled temperature (22–24 °C) and illumination (12 h light cycle starting at 8:00 AM) for 1 week before experiments. The experiments and procedures described here were performed in accordance with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the National Institutes of Health, and were approved by the Animal Care Committee of Keio University and Kumamoto University. Totally, we used 287 Wistar rats, 182 SD rats and 4 guinea pigs for all experiments in this study.

2.2. Gastric and small intestinal damage assay

The gastric ulcerogenic response was examined as described previously [20,35] with some modifications. Wistar or SD rats fasted for 18 h were orally administered each NSAID and, after 8 h or 4 h, respectively, the animals were sacrificed, their stomachs were removed, and the gastric mucosal lesion area measured by an observer unaware of the treatment that the animals had received. For Wistar rats, calculation of the scores involved measuring the area of all the lesions in square millimeters and summing the

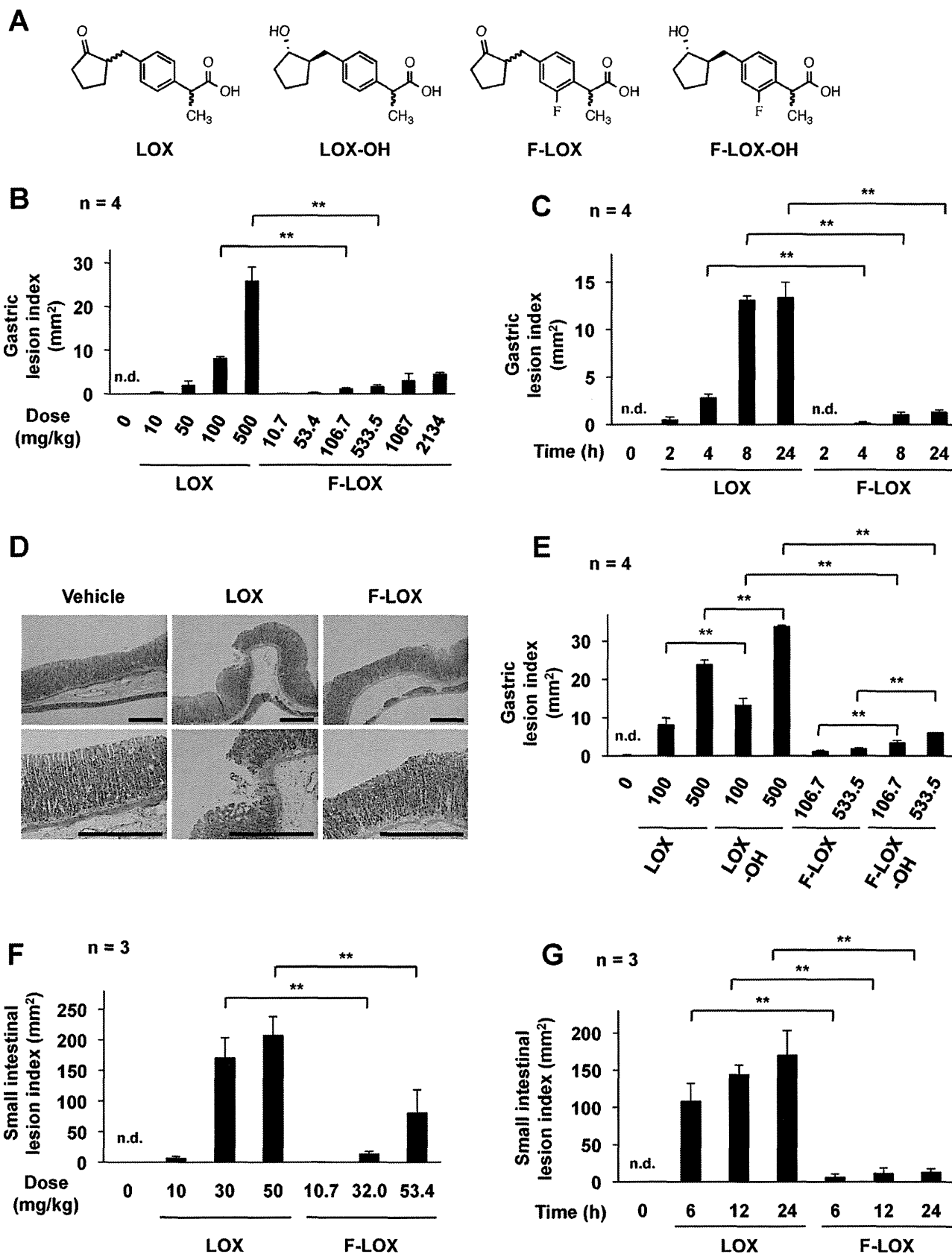


Fig. 1. Production of lesions in the stomach and small intestine by oral administration of F-LOX or LOX. Structures of drugs used in this study were shown (A). Wistar rats were orally administered either the indicated doses (B, E, –F), 200 or 213.4 mg/kg (C and D), or 30 or 32.0 mg/kg (G) of LOX or F-LOX, respectively. Rats were similarly administered LOX-OH or F-LOX-OH (E). Their stomachs (B–E) or small intestines (F, G) were then removed either 8 h later (B, D, E), at the indicated time-points (C, G), or 24 h later (F). The stomach and small intestine were scored for damage (B, C, E, F and G) and sections of gastric tissues were prepared and subjected to H & E staining. Images are magnified 2.5 times (lower panels) (D). Values are mean ± S.E.M. ***P* < 0.01; n.d., not detected. Scale bar, 50 μm.

values to give an overall lesion index. For SD rats, calculation of the scores involved measuring the length of all the lesions in millimeters and summing the values to give an overall gastric lesion index.

The gastric PGE₂ level was determined by EIA according to the manufacturer's instructions.

The intestinal ulcerogenic response was examined as described previously [36,37], with some modifications. LOX or F-LOX was orally administered to unfasted rats and the animals were sacrificed 24 h later. Both the jejunum and ileum were removed and treated with formalin for fixation. Samples were opened along the antimesenteric attachment and the areas of the small intestinal lesions were measured by an observer unaware of the treatment that the animals had received. Calculation of the scores involved measuring the area of all the lesions in square millimeters and summing the values to give an overall small intestinal lesion index.

2.3. Histochemical analysis and TUNEL assay

Wistar rats which had been fasted for 18 h were orally administered LOX or F-LOX and, 8 h later, the animals were sacrificed and their stomachs were removed. Samples were fixed in 4% buffered paraformaldehyde and embedded in paraffin before being cut into 4- μ m sections.

For histological examination (hematoxylin and eosin (H&E) staining), the sections were stained first with Mayer's hematoxylin and then with 1% eosin alcohol solution. The samples were mounted with malinol and inspected with the aid of an Olympus BX51 microscope (Tokyo, Japan).

For the TUNEL assay, the sections were incubated first with proteinase K (20 μ g/ml) for 15 min at 37 °C, then with TdT and biotin 14-ATP for 1 h at 37 °C, and finally with streptavidin-conjugated Alexa Fluor 488 for 1 h. Samples were mounted with VECTASHIELD and inspected using fluorescence microscopy (KEYENCE BIOREVO, Osaka, Japan).

2.4. Measurement of gastric content volume, gastric pH value and contents of gastric mucin

The gastric pH value and mucus level were measured as previously described [38–40]. SD rats which had been fasted for 18 h were orally administered LOX or F-LOX and, 1 h later, the abdomen was opened and the pylorus was ligated under ether anesthesia. Three hours later, the animals were killed by deep ether anesthesia, the stomach was removed, the gastric contents were collected and its volume was determined. The gastric contents were titrated with 10 mM NaOH to pH 7.0 using Twin pH (Horiba, Kyoto, Japan). The gastric acid output was calculated based on the volume of 10 mM NaOH required for neutralization and the gastric content volume.

For determination of mucus content, the gastric contents were incubated with 0.4 mg/ml (final concentration) Alcian blue 8GX for 24 h at 20 °C and then centrifuged. The concentration of Alcian blue in the supernatant was estimated by measuring the optical density at 615 nm. The amount of mucus adhering to the gastric mucosa was also determined, and the sum of the two values used as a measure of the total mucus content.

2.5. Real-time RT-PCR analysis

Total RNA was extracted from gastric tissues and cells using an RNeasy kit according to the manufacturer's protocol. Samples (2.5 μ g of RNA) were reverse-transcribed using a first-strand cDNA synthesis kit according to the manufacturer's instructions. Synthesized cDNA was used in real-time RT-PCR (Bio-Rad Chromo 4 system) experiments using SsoFast EvaGreen Supermix, and

analyzed with Opticon Monitor software according to the manufacturer's instructions. Specificity was confirmed by electrophoretic analysis of the reaction products and by inclusion of template- or reverse transcriptase-free controls. To normalize the amount of total RNA present in each reaction, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) or actin cDNA was used as an internal standard.

Primers were designed using the Primer3 website. The primers used were (name, forward primer and reverse primer): *muc1*, 5'-agagaccgctactgccattg-3' and 5'-cagctggacctttccaac-3'; *muc5ac*, 5'-aactctgcccaccacaagc-3' and 5'-tgccatctatccaatcagtcctcaat-3'; *muc6*, 5'-tgctgtctccagcacaacaac-3' and 5'-tcagaagtctgctgctactgc-3'; *gapdh*, 5'-atgtatccgttggtgatctgac-3' and 5'-cctgcttcaccacctctctg-3'; *actin*, 5'-gtcgtaccactggcattgtg-3' and 5'-gctgatcttgccttgagac-3'.

2.6. Cell culture

A rat normal gastric epithelial cell line (RGM1) [41] was provided by the Riken Cell Bank (Tsukuba, Japan). Cells were cultured in Dulbecco's modified Eagle's medium nutrient mixture F-12 Ham containing 20% FBS, 100 U/ml penicillin, and 100 μ g/ml streptomycin on plastic culture plates without collagen coating. Cells were exposed to LOX or F-LOX by replacement of the entire bathing medium. The cAMP level in cells was measured by EIA according to the manufacturer's instructions.

2.7. Determination of mucin level in culture medium

The amount of mucin in the culture medium was determined by an enzyme-linked lectin-binding assay (ELLA) as described previously [42]. The culture medium was loaded on polystyrene 96-well ELISA plates (Iwaki) and incubated at 4 °C for 12 h. After washing, each well was incubated with blocking buffer (1% BSA in phosphate-buffered saline) for 2 h at 37 °C. After washing, each well was incubated with SBA solution (1 μ g/ml SBA-HRP (lectin) in phosphate-buffered saline) for 1 h at 37 °C. After further washing, each well was finally incubated with 100 μ l ABTS solution (1 mM ABTS, 0.1 M citrate buffer (pH 4.0) and 0.03% hydrogen peroxide) for 15 min at room temperature. The optical density at 405 nm was then measured.

2.8. Determination of activities of H⁺,K⁺-ATPase and adenylate cyclase in membrane fraction

Cytoplasmic membrane fractions were prepared from guinea pig gastric mucosa as described previously [43]. Briefly, the fundic region of the mucosa was scraped and homogenized in 5 mM Tris-HCl (pH 7.4) buffer containing 250 mM sucrose and 1 mM EGTA. The suspension was centrifuged at 800 \times g for 10 min, and the resultant supernatant was further centrifuged at 100,000 \times g for 90 min. The pellet was re-suspended in PBS and used for the assay as membrane fraction.

H⁺,K⁺-ATPase activity was measured as described previously [44]. Briefly, membrane fraction (100 μ g protein) was diluted with 40 mM Tris-HCl (pH 6.8) buffer containing 3 mM MgSO₄, 15 mM KCl, 5 mM Na₂N₃ and 2 mM ouabain and pre-incubated with each tested chemical in the presence or absence of 50 μ M SCH 28080 for 30 min at 37 °C. Omeprazole was activated by treatment with Tris-PIPES (pH 5.7) buffer before this incubation. Then, ATP solution (1 mM at the final concentration) was added, incubated for 10 min at 37 °C and the reaction was terminated by the addition of ice-cold stop solution (12% perchloric acid and 3.6% ammonium molybdate). Inorganic phosphate released was measured as previously described [45]. The H⁺,K⁺-ATPase activity was calculated as the difference between the activities in the presence and absence of SCH 28080.

Adenylate cyclase activity was measured as described previously [46]. Briefly, membrane fraction (100 μ g protein, 75 μ l) was pre-incubated with each tested chemical in 96 well plates for 5 min at room temperature. Then 25 μ l adenylate cyclase assay buffer (final concentrations; 50 mM Tris-HCl (pH 7.4), 100 mM KCl, 10 mM MgCl₂, 0.25 mM ATP and 1 mM IBMX) was added, incubated for 30 min at 37 °C and the reaction was terminated by the addition of 100 μ l 0.2 N HCl. The cAMP level was measured by EIA according to the manufacture's instructions.

2.9. Statistical analysis

All values are expressed as the mean \pm S.E.M. Two-way ANOVA followed by the Tukey test or the Student's *t*-test for unpaired results was used to evaluate differences between more than two groups or between two groups, respectively. Differences were considered to be significant for values of $P < 0.05$.

3. Results

3.1. Comparison between the ulcerogenic response of F-LOX and LOX

The development of gastric lesions following oral administration of F-LOX and LOX to Wistar rats was compared. LOX produced gastric lesions in a dose-dependent manner but F-LOX produced fewer lesions (Fig. 1B). The level of gastric lesions caused by 2134 mg/kg F-LOX (corresponding to 2000 mg/kg LOX, in terms of number of molecules) was lower than that observed with 100 mg/kg LOX, demonstrating that the ulcerogenic activity of F-LOX was less than one-twentieth that of LOX. The time-course for the effect of the two NSAIDs was similar (Fig. 1C), showing that the difference in ulcerogenic activity illustrated in Fig. 1B was not a function of time. Histochemical analysis of gastric sections also revealed that F-LOX caused less gastric mucosal damage than LOX (Fig. 1D). We also examined the ulcerogenic activity of LOX-OH and F-LOX-OH. As shown in Fig. 1E, the ulcerogenic activity of F-LOX-OH was

much lower than that of LOX-OH and the activity of LOX-OH or F-LOX-OH was a little more potent than that of LOX or F-LOX, respectively.

We also compared the production of lesions in the small intestine. As shown in Fig. 1F and G, oral administration of F-LOX produced fewer lesions than LOX at all examined time-points. However, the difference between the effect of the two drugs in this case was not as marked as that seen for gastric lesions (Fig. 1B and F).

Both gastric mucosal cell death and a decrease in the gastric level of PGE₂ have been shown to play an important role in the production of gastric lesions by NSAIDs, leading us to compare these processes after oral administration of F-LOX and LOX. The number of TUNEL-positive gastric mucosal cells (level of cell death) was lower in the F-LOX-treated rats (Fig. 2A and B). However, both the dose-response and time-course profiles of the gastric PGE₂ level were similar between the two treatment groups (Fig. 3A and B), suggesting that mucosal cell death rather than gastric PGE₂ level is involved in the lower ulcerogenic activity of F-LOX.

To test this idea, we compared the production of gastric lesions between F-LOX-treated and LOX-treated rats in which the gastric PGE₂ level had been lowered by pre-administration of aspirin (10 mg/kg). This regimen reduced PGE₂ to a negligible level, with subsequent administration of F-LOX or LOX having no effect (Fig. 3C). As shown in Fig. 3D, the production of gastric lesions in the two treatment groups of rats pre-administered with aspirin was similar to that of control rats, supporting the idea that gastric PGE₂ level is not responsible for the lower ulcerogenic activity of F-LOX compared with LOX.

3.2. Protective effect of orally administered F-LOX on the gastric mucosa

The lower ulcerogenic activity of F-LOX was also observed in SD rats (Fig. 4A), which we used in the following experiments due to

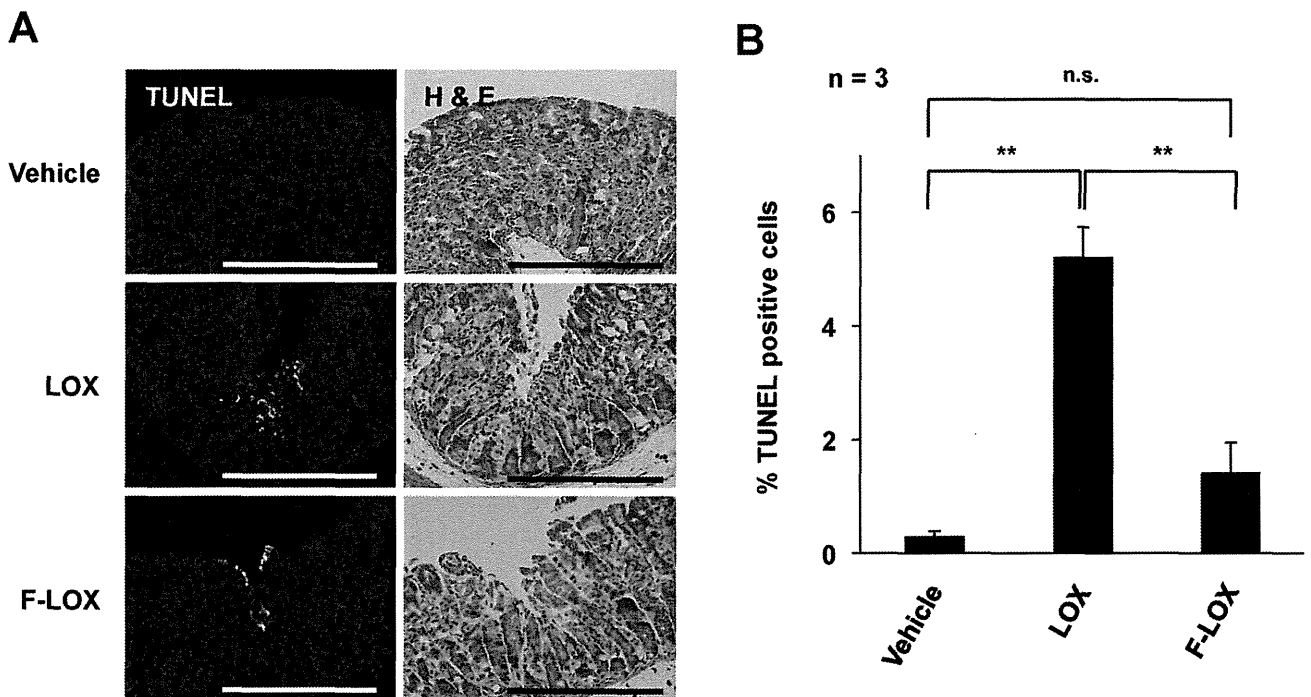


Fig. 2. Gastric mucosal cell death following oral administration of F-LOX or LOX. Wistar rats were orally administered 100 or 106.7 mg/kg of LOX or F-LOX, respectively, and their stomachs were removed 8 h later. A, sections of gastric tissues were prepared and subjected to TUNEL assay, DAPI staining and H & E staining. B, the ratio of TUNEL-positive cells to total cells was determined. Values are mean \pm S.E.M. ** $P < 0.01$; n.s., not significant. Scale bar, 50 μ m.

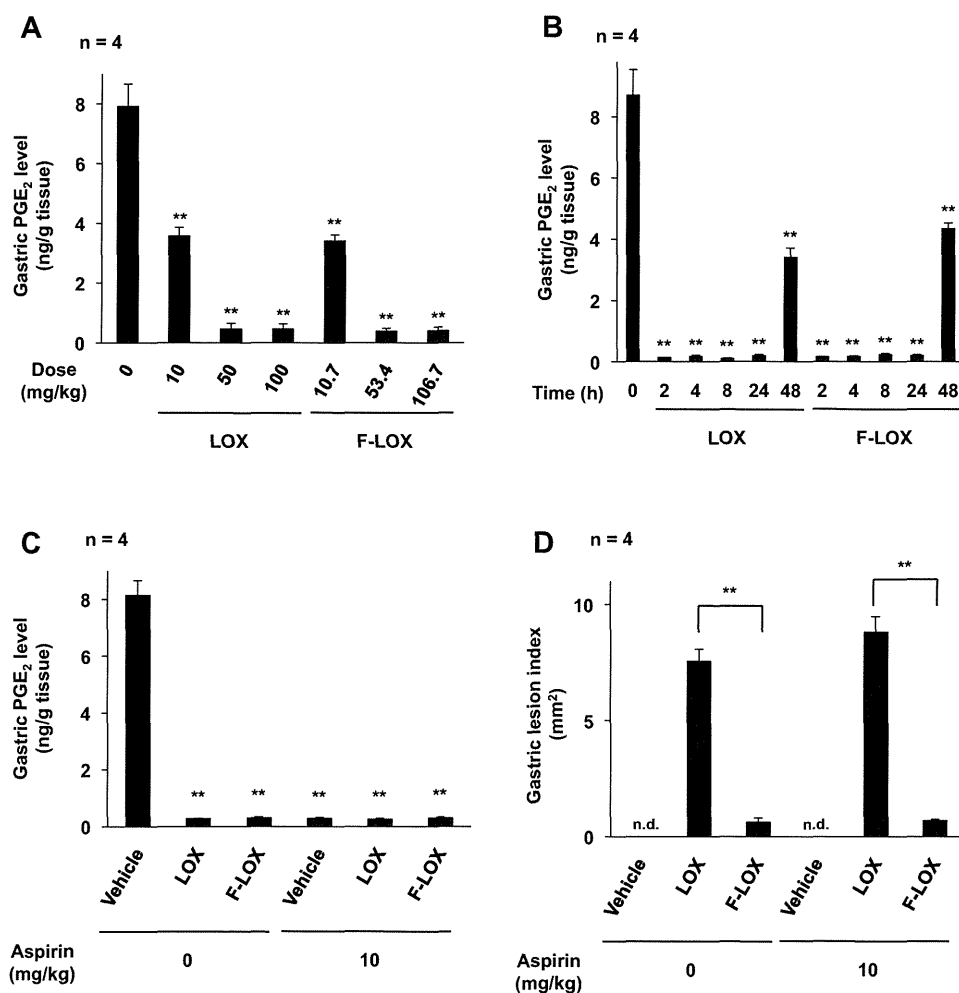


Fig. 3. Decrease in gastric PGE₂ level in response to oral administration of F-LOX or LOX. Wistar rats were orally administered either the indicated doses (A), 200 or 213.4 mg/kg (B), or 100 or 106.7 mg/kg (C, D) of LOX or F-LOX, respectively. Rats were orally pre-administered the indicated dose of aspirin, 1 h before NSAID administration (C, D). The stomach was removed either 8 h (A, C, D) or at the indicated time-points (B) after the administration of LOX or F-LOX. A–C, the gastric PGE₂ level was determined by EIA. D, the stomach was scored for damage. Values are mean ± S.E.M. ***P* < 0.01; n.d., not detected.

the availability of established protocols for monitoring gastric pH and mucus level in this strain.

We found that there was no significant difference in the gastric lesions produced by subcutaneous administration of F-LOX and LOX (Fig. 4B). In contrast to treatment with LOX, subcutaneous administration of F-LOX produced more gastric lesions than oral administration (Fig. 4B), although the gastric PGE₂ level in both cases was similar to that obtained following oral administration of the drugs (Fig. 4C). Surprisingly, we found that oral pre-administration of F-LOX suppressed the production of gastric lesions induced by subsequent oral administration of LOX (Fig. 4D). A similar protective effect of F-LOX was observed for indomethacin-induced gastric lesions (data not shown). As shown in Fig. 4E and F-LOX-OH also showed such a protective effect against LOX-induced gastric lesions. We also found that this protective effect of F-LOX did not occur following its subcutaneous administration (data not shown). These results suggest that the direct interaction of F-LOX (at high concentrations) with the gastric mucosa is somehow protective against NSAID-induced gastric lesions.

3.3. Mechanism for the protective effect of F-LOX on the gastric mucosa

To understand the mechanism responsible for the protective effect of F-LOX, we examined the outcome of oral administration of

F-LOX or LOX on gastric levels of aggressive (acid) and defensive (mucus) factors. LOX lowered the gastric pH and mucus content (Fig. 5A), phenomena that have been reported with various NSAIDs [8,9]. Surprisingly, oral administration of F-LOX shifted these indexes in the opposite direction, elevating the gastric pH value and mucus content (Fig. 5A). However, subcutaneous administration of F-LOX produced no such effects; subcutaneous administration of both LOX and F-LOX lowered the gastric pH value and mucus content (Fig. 5B).

To understand the mechanism responsible for the increase in the gastric pH value after oral administration of F-LOX, we measured the gastric content volume and determined the gastric acid output. As shown in Fig. 5A, oral administration of F-LOX but not that of LOX increased the gastric content volume, however, both of these drugs similarly increased the gastric acid output. On the other hand, subcutaneous administration of both LOX and F-LOX increased the gastric acid output but did not affect the gastric content volume (Fig. 5B). These results suggest that oral administration of F-LOX increases the gastric pH value through increasing the gastric content volume rather than decreasing the gastric acid output. Supporting this notion, we found that neither LOX nor F-LOX affected the H⁺,K⁺-ATPase activity in membrane fraction prepared from guinea pig gastric mucosa (Fig. 5C).

To test the contribution of the F-LOX-dependent increase in gastric pH value to the low ulcerogenic activity of this NSAID, we

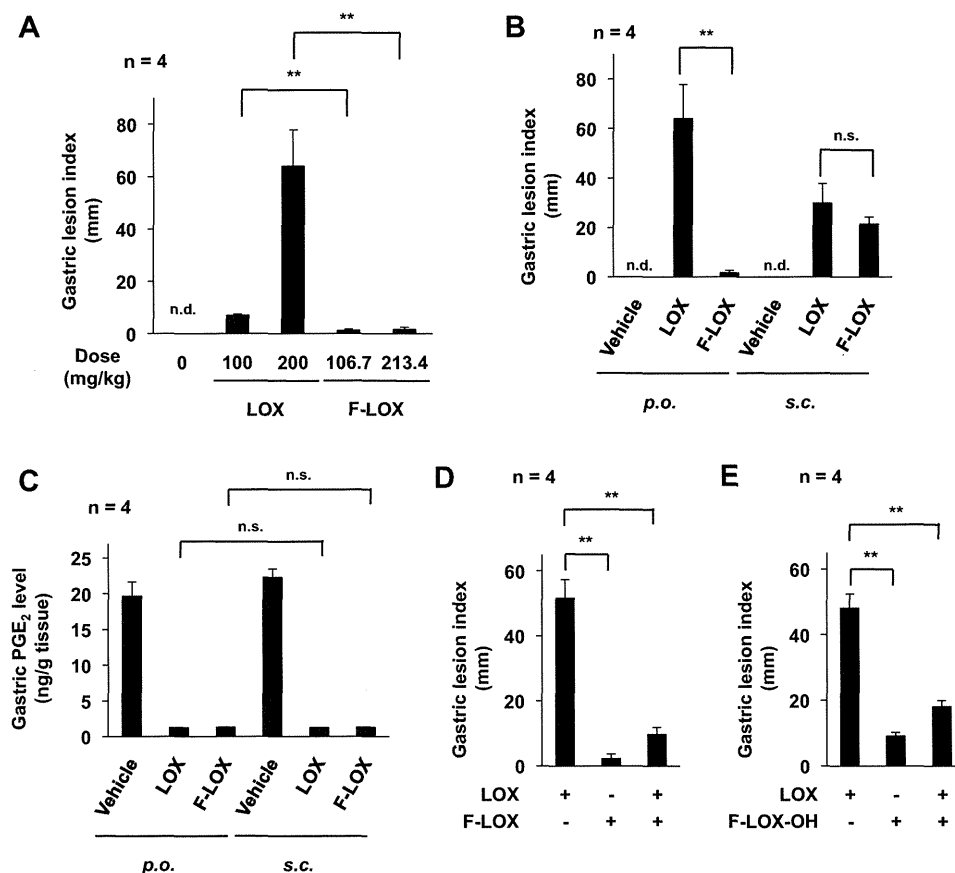


Fig. 4. Protective effect of orally administered F-LOX on the gastric mucosa. (A) SD rats were orally administered the indicated doses of LOX or F-LOX. (B and C) SD rats were orally (*p.o.*) or subcutaneously (*s.c.*) administered 200 or 213.4 mg/kg of LOX or F-LOX, respectively. (D and E) SD rats were orally pre-administered 106.7 mg/kg F-LOX (D) or F-LOX-OH (E), 1 h after which they were orally administered 200 mg/kg of LOX. A, B, D and E, the stomach was removed 4 h after the final administration of the NSAID and scored for damage. C, the gastric PGE₂ level was determined by EIA 4 h after the administration of LOX. Values are mean \pm S.E.M. * $P < 0.01$; n.s., not significant; n.d., not detected.

examined the effect of a stimulator of gastric acid secretion (histamine). Pre-administration of 5 mg/kg histamine decreased the pH value in both the presence and absence of subsequent oral administration of F-LOX or LOX (Fig. 6A), as a result of which the extent of the difference in the gastric pH value between the F-LOX- and LOX-treated groups became less apparent (Fig. 6A). As shown in Fig. 6B, F-LOX produced fewer gastric lesions than LOX, even following pre-administration of histamine; however, the extent of this difference was less marked than in the absence of histamine treatment. These results suggest that the higher gastric pH value observed after oral administration of F-LOX is partially responsible for the lower ulcerogenic activity of this NSAID compared with LOX.

3.4. Mechanism for the stimulative effect of F-LOX on the production of mucus

We next examined the effect of oral administration of F-LOX or LOX on the expression levels of mRNAs corresponding to mucin proteins. As shown in Fig. 7A, oral administration of F-LOX or LOX either up-regulated or down-regulated, respectively, the mRNA expression of the *muc1*, *muc5ac* and *muc6* genes (although the down-regulation of *muc6* mRNA by LOX was not statistically significant). We also examined the effect of the two NSAIDs on the production and secretion of mucin *in vitro*, using a rat normal gastric epithelial cell line (RGM1 cells). Treatment of these cells with F-LOX or LOX increased or decreased, respectively, the mucin content in the culture medium (Fig. 7B). Treatment of cells with F-LOX-OH also increased the mucin content in the culture

medium (data not shown). To detect the secretion of pre-produced mucin, we examined the effect of F-LOX on the mucin secretion from cells whose protein synthesis was inhibited. Even in this situation, an increase in the mucin content in the culture medium was observed following pre-treatment with a protein synthesis inhibitor, cycloheximide (Fig. 7C), suggesting that the secretion of mucin was stimulated by F-LOX treatment of the cells. On the other hand, treatment of the cells with F-LOX up-regulated the expression levels of mRNAs corresponding to mucin proteins (Fig. 7D). Although there was a trend towards down-regulation of the mRNA expression of these genes following LOX treatment, this effect was not statistically significant (Fig. 7D). Taken together, these results suggest that direct interaction of F-LOX with the gastric mucosa directly stimulates the production and secretion of mucin.

Finally, we addressed the molecular mechanism governing these phenomena. PGE₂ stimulates the production of mucus through both EP₁ and EP₄ receptors [47]. As shown in Fig. 8A, pre-treatment of RGM1 cells with antagonists for EP₁ and EP₄ receptors suppressed PGE₂-induced production of mucin but not F-LOX-induced one (Fig. 8A). EP receptor subtypes are coupled to different intracellular signaling pathways. The EP₁ receptor is coupled to Ca²⁺ mobilization and activation of EP₄ receptor causes activation of adenylate cyclase activity and an increase in the cellular level of cAMP [48]. Thus, we examined the effect of an intracellular Ca²⁺ chelator that is permeable for cytoplasmic membranes (BAPTA-AM) or an inhibitor of adenylate cyclase (SQ22536) on F-LOX-induced production of mucin. As shown in Fig. 8B, pre-treatment of RGM1 cells with SQ22536 but not with BAPTA-AM

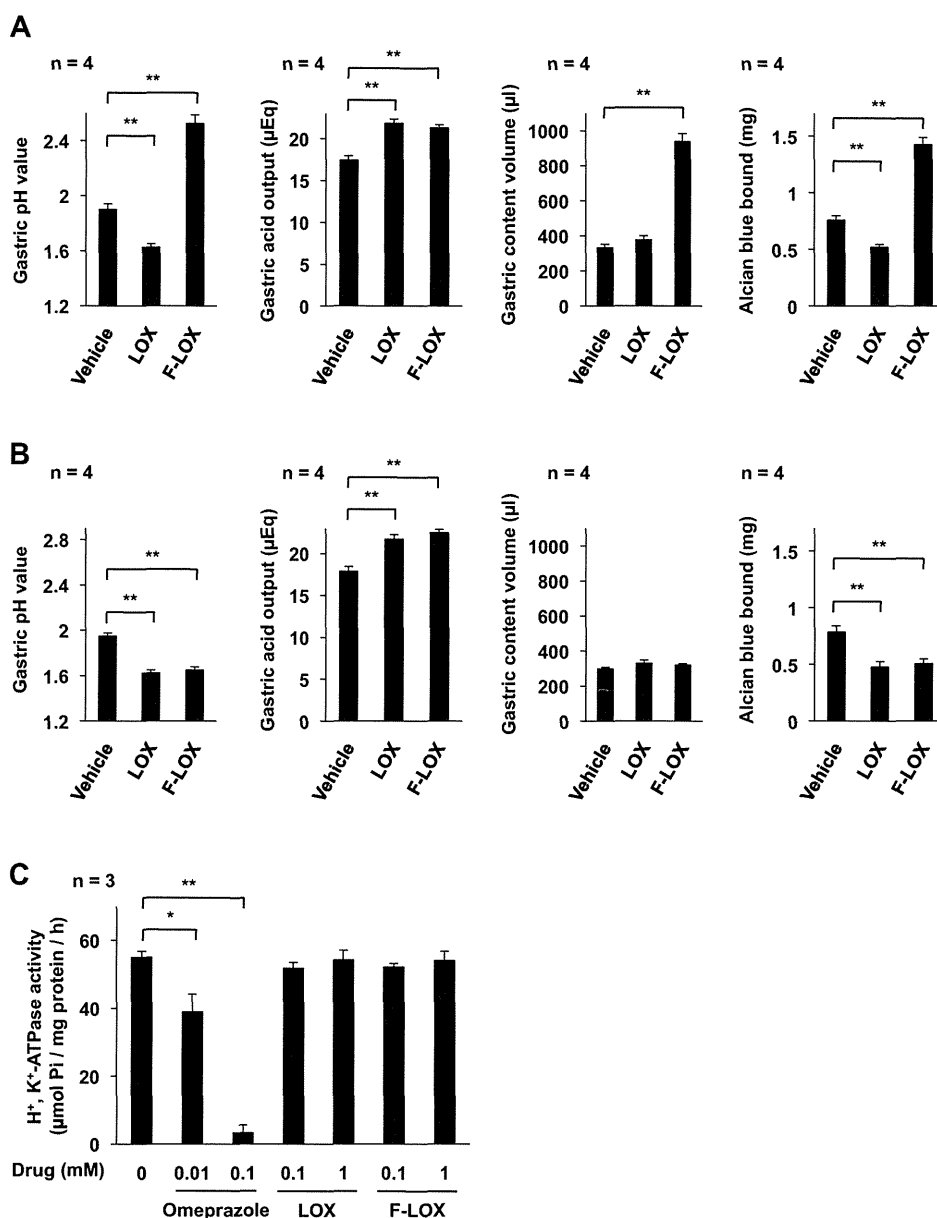


Fig. 5. Effect of F-LOX and LOX on gastric pH and mucus content. SD rats were orally (A) or subcutaneously (B) administered 100 or 106.7 mg/kg of LOX or F-LOX, respectively, 1 h after which the pylorus was ligated and the rats were maintained for a further 3 h. The gastric pH value, gastric acid output, gastric content volume and the amount of mucus in the gastric contents were measured as described in the experimental procedures. (C) Activity of H⁺,K⁺-ATPase in membrane fraction prepared from guinea pig gastric mucosa was measured in the presence of indicated concentrations of omeprazole (an inhibitor of H⁺,K⁺-ATPase), LOX or F-LOX as described in the experimental procedures. Values are mean ± S.E.M. **P* < 0.01; ***P* < 0.05.

suppressed F-LOX-induced production of mucin. We also found that treatment of RGM1 cells with F-LOX increased the cellular level of cAMP to the extent similar to that induced by PGE₂ (Fig. 8C). Since neither LOX nor F-LOX activated adenylate cyclase activity in membrane fraction prepared from guinea pig gastric mucosa (Fig. 8D), F-LOX seems to activate adenylate cyclase indirectly. Furthermore, pre-treatment of cells with SQ22536 suppressed not only PGE₂- but also F-LOX-induced expression levels of mRNAs corresponding to mucin proteins (Fig. 8E). Results in Fig. 8 suggest that direct interaction of F-LOX with the gastric mucosa increases the level of mucin through increase in the cellular level of cAMP.

4. Discussion

F-LOX is a derivative of LOX, which is a leading NSAID on the Japanese market due to its relatively lower incidence of

gastrointestinal complications than other traditional NSAIDs [31,32]. The results of a previous study, in which we demonstrated that, compared to LOX, F-LOX has lower ulcerogenic activity but similar anti-inflammatory activity, led us to propose that F-LOX is likely to represent a safer NSAID. However, before this compound can be developed for clinical use, it is first necessary to understand the molecular mechanism governing its low ulcerogenic activity.

In order to address this, we initially determined the dose-response profile of F-LOX, which revealed that its ulcerogenic activity is less than one-twentieth that of LOX when orally administered. Furthermore, the fact that the time-course for the production of gastric lesions observed with F-LOX was similar to that obtained with LOX demonstrated that the former NSAID has lower rather than slower ulcerogenic activity.

We have previously suggested that both gastric mucosal cell death due to the membrane permeabilization activity of NSAIDs and a decrease in the gastric level of PGE₂ due to COX inhibition are

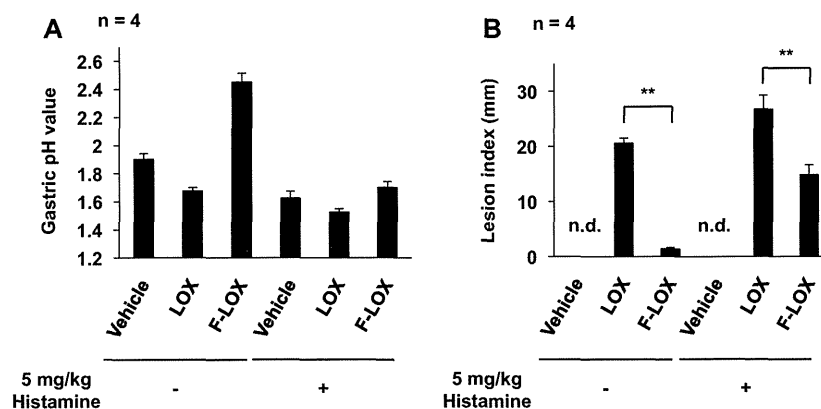


Fig. 6. Effects of pre-administration of histamine on the production of gastric lesions by F-LOX and LOX. SD rats were orally pre-administered the indicated dose of histamine, 1 h after which they were orally administered 100 or 106.7 mg/kg of LOX or F-LOX, respectively. The stomach was then removed 4 h later. A, gastric pH value was determined as described in the legend of Fig. 5. B, the stomach was scored for damage. Values are mean \pm S.E.M. ** $P < 0.01$; n.d., not detected.

key factors in the production of gastric lesions *in vivo* [16,20]. Based on this hypothesis, NSAIDs without membrane permeabilization activity or those without the ability to decrease the gastric level of PGE₂ (such as COX-2 selective NSAIDs) would represent a therapeutically beneficial option. Among the clinically used NSAIDs that we tested, LOX had the weakest membrane permeabilization activity [30] and, among its derivatives, F-LOX had the weakest such activity [34]. Therefore, it is not surprising that in the current study less gastric mucosal cell death was observed with F-LOX than LOX. Furthermore, as neither LOX nor F-LOX display COX-2 selectivity [34], it is not unexpected that F-LOX decreased the gastric level of PGE₂ to a similar extent to LOX. We also showed that the lower ulcerogenic activity of F-LOX occurred even in the presence of an inhibitor of prostaglandin synthesis (aspirin). Taken together these results led us to conclude that the lower ulcerogenic activity of F-LOX compared with LOX involved gastric mucosal cell death rather than the gastric level of PGE₂.

In contrast to the above findings following oral NSAID administration, the ulcerogenic activities of F-LOX and LOX were indistinguishable when the drugs were administered subcutaneously. It is known that most NSAIDs produce more gastric lesions when administered orally rather than subcutaneously, which is in accordance with what we observed in the case of LOX in the present study. However, F-LOX produced more gastric lesions following subcutaneous administration than following oral administration of the drug. By way of explanation for the opposite effect of F-LOX, we consider a possibility that direct interaction of this NSAID with the gastric mucosa somehow confers a protective effect, but that this protection requires relatively higher concentrations of F-LOX, which can only be achieved by its oral administration. In support of this idea, we found that oral but not subcutaneous pre-administration of F-LOX protected against the formation of gastric lesions induced by subsequent administration of LOX or indomethacin.

A possible explanation for the lower ulcerogenic activity of F-LOX with oral administration than that with subcutaneous administration is that F-LOX but not its active metabolite (F-LOX-OH) confers a protective effect, because we recently found that the conversion of F-LOX to F-LOX-OH occurred very rapidly and the serum level of F-LOX-OH peaked within 1 h after either oral or intravenous administration of F-LOX [49]. However, this idea was ruled out by following observations in this study; the ulcerogenic activity of F-LOX-OH was much lower than that of LOX with their oral administration; oral pre-administration of F-LOX-OH also suppressed the production of gastric lesions induced by subsequent oral administration of LOX; as well as F-LOX (see

below), F-LOX-OH increased the level of mucin in culture medium *in vitro*.

In order to understand the molecular mechanism governing the protective activity of F-LOX, we examined the effect of its oral administration on gastric levels of aggressive (acid) and defensive (mucus) factors, demonstrating that the gastric pH value and mucus content were higher following oral administration of the drug. This result is surprising because it is known that PGE₂ inhibits the secretion of acid and stimulates the production of mucus, and therefore that NSAIDs affect these responses in the opposite direction through their inhibitory effects on COX and prostaglandin synthesis [8,9]. This was reflected by the finding that both oral and subcutaneous administration of LOX caused a decrease in gastric pH and mucus. Similarly, in contrast to its oral administration, subcutaneous administration of F-LOX lowered the gastric pH and mucus content. However, the F-LOX-dependent decrease in the gastric level of PGE₂ was indistinguishable following oral and subcutaneous administration, suggesting that orally administered F-LOX, in other words, direct interaction of relatively higher concentrations of F-LOX with the gastric mucosa exerts its protective effects through a COX-independent mechanism.

Oral administration of F-LOX but not that of LOX increased the gastric content volume, however, both of these drugs increased the gastric acid output. On the other hand, subcutaneous administration of both LOX and F-LOX increased the gastric acid output but did not affect the gastric content volume. We also found that neither LOX nor F-LOX affects the H⁺,K⁺-ATPase activity *in vitro*. These results suggest that direct interaction of relatively higher concentrations of F-LOX with the gastric mucosa increases the gastric pH value through increasing the gastric content volume. However, the mechanism for this increase is unclear at present.

In order to test the contribution of gastric pH value to the production of lesions after oral administration of F-LOX, we examined the effect of a stimulator for gastric acid secretion, histamine. Following oral pre-administration of 5 mg/kg histamine, the difference in gastric pH value in response to oral F-LOX and LOX treatment became less apparent. Similarly, the difference in the production of gastric lesions was reduced, suggesting that the higher gastric pH value contributes to the lower gastric lesion index after oral administration of F-LOX.

We also found that the expression of mRNAs corresponding to mucin proteins was up-regulated not only at the gastric mucosa after oral administration of F-LOX but also in cultured RGM1 cells treated with this NSAID. Furthermore, a F-LOX-dependent increase in the level of mucin was observed in the culture medium, these

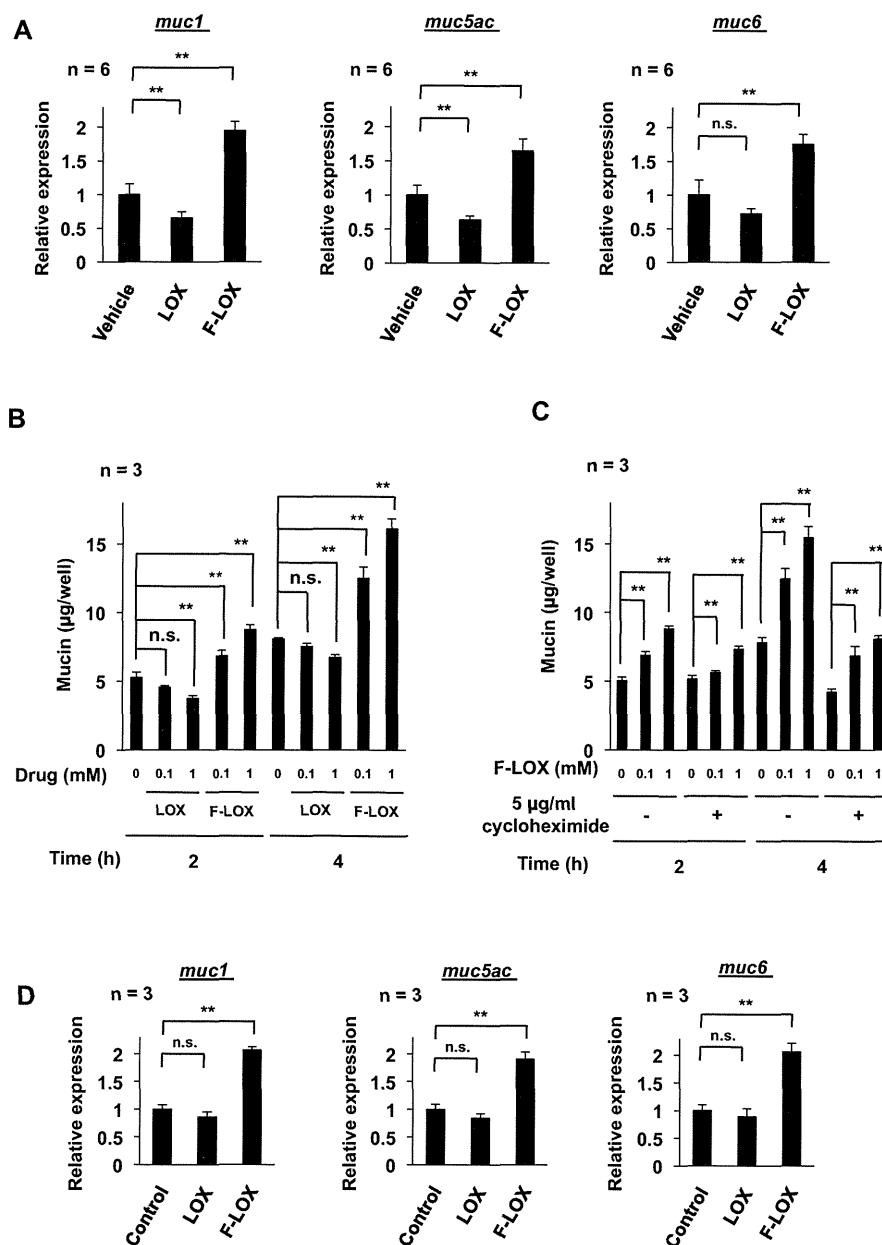


Fig. 7. Effect of F-LOX and LOX on the production and secretion of mucin. (A) SD rats were orally administered 100 or 106.7 mg/kg of LOX or F-LOX, respectively, and the gastric mucosa was removed 4 h later. Total RNA was extracted and subjected to real-time RT-PCR using a specific primer for each gene. Values normalized to the *gapdh* gene are expressed relative to the control sample. (B) RGM1 cells were incubated with the indicated concentrations of LOX or F-LOX for the indicated periods. (C) RGM1 cells were pre-incubated with 5 µg/ml of cycloheximide for 1 h, after which the medium was exchanged to one containing indicated concentrations of F-LOX and cultured the indicated periods. (B and C) The amount of mucin in the culture medium was determined by ELLA. (D) RGM1 cells were incubated with 1 mM of LOX or F-LOX for 4 h. Expression of each mRNA was examined as described above. Values normalized to the *actin* gene are expressed relative to the control sample. Values are mean ± S.E.M. ***P* < 0.01; n.s., not significant.

changes occurring even when the cells were pre-treated with an inhibitor of protein synthesis. These results suggest that F-LOX affects both production and secretion of mucin.

Since PGE₂ stimulates production of mucin through both EP₁ and EP₄ receptors [47], we consider the possibility that F-LOX is an agonist for these receptors. However, this idea was ruled out by the observation that pre-treatment of RGM1 cells with antagonists for EP₁ and EP₄ suppressed PGE₂-induced production of mucin but not F-LOX-induced one. We then tested whether F-LOX directly affects the intracellular signalling pathway coupled with EP₁ or EP₄ receptor (Ca²⁺ mobilization or activation of adenylylase and resulting increase in the cellular level of cAMP, respectively) and found that pre-treatment of RGM1 cells with an inhibitor of

adenylylase suppresses F-LOX-dependent increase in the level of mucin and that treatment of cells with F-LOX increases the cellular level of cAMP. Furthermore, we found that pre-treatment of cells with SQ22536 also suppressed F-LOX-induced expression levels of mRNAs corresponding to mucin proteins. These results suggested that F-LOX increases the level of mucin through increase in the cellular level of cAMP.

In line with improvements in diagnostic procedures, it has become clear that NSAIDs induce lesions not only in the stomach but also in the small intestine. However, clinical protocols for the treatment of NSAID-induced lesions of the small intestine have not been established. This is because acid secretion is not as important in the development of these lesions compared with gastric lesions,

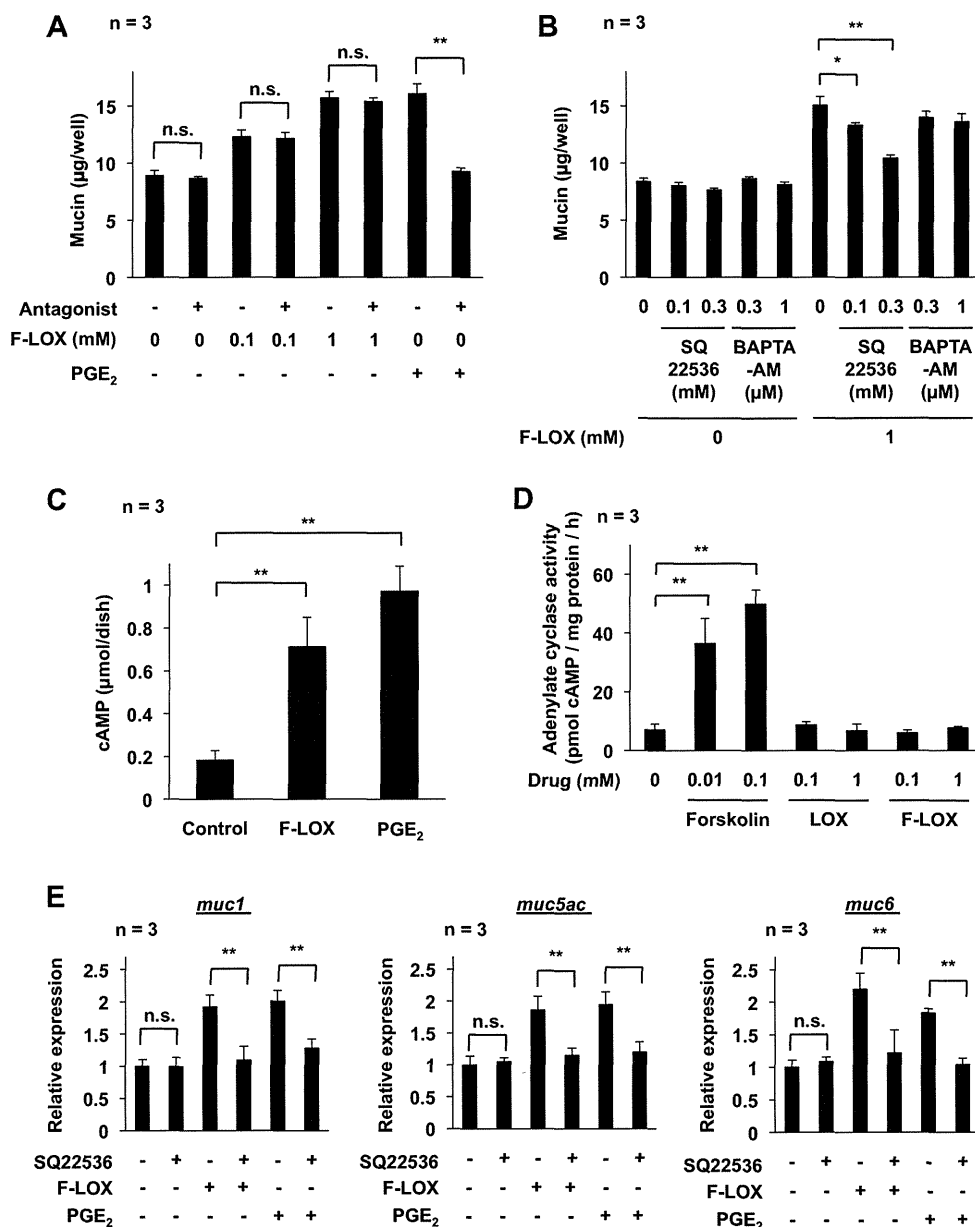


Fig. 8. Molecular mechanism for F-LOX-mediated alteration of production of mucin. RGM1 cells were pre-incubated with 1 μg/ml ONO-8711 (EP₁ antagonist) and 0.1 μg/ml ONO-AE2-227 (EP₄ antagonist) for 0.5 h, after which the medium was exchanged to one containing indicated concentrations of F-LOX and cultured for 4 h (A). RGM1 cells were pre-incubated with the indicated concentrations of SQ22536 (an inhibitor of adenylate cyclase) or BAPTA-AM for 1 h, after which the medium was exchanged to one containing indicated concentrations of F-LOX and cultured for 4 h (B). (A and B) The amount of mucin in the culture medium was determined by ELLA. (C) RGM1 cells were incubated with the indicated concentrations of F-LOX for 0.5 h and cellular cAMP levels were determined by EIA. (D) The activity of adenylate cyclase in membrane fraction prepared from guinea pig gastric mucosa was measured in the presence of indicated concentrations of forskolin (an activator of adenylate cyclase), LOX or F-LOX as described in the experimental procedures. (E) RGM1 cells were pre-incubated with the indicated concentrations of SQ22536 for 1 h, after which the medium was exchanged to one containing indicated concentrations of F-LOX and cultured for 4 h. Expression of each mRNA was examined as described in the legend of Fig. 7. Values are mean ± S.E.M. ***P* < 0.01; **P* < 0.05; n.s., not significant.

as a result of which acid-controlling drugs are not efficacious [50,51]. In this study, we found that orally administered F-LOX produced fewer small intestine lesions than LOX. Given that the direct cytotoxicity of NSAIDs seems to be involved in, and mucus is protective against, NSAID-induced damage of the small intestine [52,53], this reduced ulcerogenic activity of F-LOX may be the result of its lower cytotoxicity and ability to stimulate mucus synthesis; the development of F-LOX for clinical use is therefore worth considering. However, the extent of the difference between F-LOX and LOX was not as apparent in the small intestine as in the stomach. This may be due to the fact that the pH-increasing activity by F-LOX does not contribute to the suppression of lesion production in the small intestine.

In conclusion, this study has revealed that F-LOX is an unique NSAID, because this NSAID has protective effect against the gastric mucosa and this unique activity contribute to the ulcerogenic activity of this NSAID.

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Minimally invasive surgery for esophageal epiphrenic diverticulum: the results of 133 patients in 25 published series and our experience

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Abstract Esophageal epiphrenic diverticula are uncommon. Traditionally, thoracotomy has been the preferred surgical approach. Recently, minimally invasive approaches have been reported in a few series. However, the best surgical approach remains uncertain. In this study, we review the results of 25 articles discussing laparoscopic or thoracoscopic surgery. From January 1995 to December 2008, there were a total of 133 patients reported in English-language journals in PubMed. Nineteen patients (14 %) underwent thoracoscopic surgery, 112 (84 %) laparoscopic surgery and two patients (2 %) were treated using a combination approach. The diverticulectomy was performed using an endostapler device in all patients. A myotomy was added in 103 patients (83 %). A fundoplication was added in 106 patients (85 %). There were two deaths during surgery (2 %). The post-operative morbidity rate was 21 %. The most severe complication was suture-line leakage, which occurred in 20 patients (15 %). Recently, we successfully treated a patient with an epiphrenic esophageal diverticulum by performing a minimally invasive laparoscopic transhiatal resection and Heller myotomy with Dor fundoplication after observing its enlargement on radiological and endoscopic examinations over 2 years. We believe laparoscopic transhiatal resection and Heller myotomy with Dor fundoplication may therefore become

the standard treatment modality for minimally invasive surgery for esophageal epiphrenic diverticulum.

Keywords Achalasia · Epiphrenic diverticulum · Laparoscopy · Thoracoscopy · Transhiatal approach

Introduction

Epiphrenic diverticula occur in the lower one-third of the esophagus and are pulsion diverticula of the acquired and false type. They are rare entities, with an estimated prevalence of approximately 0.015 % in the general population based on radiological data [1]. In large series reporting clinical findings, 50–80 % of patients had minimal or no symptoms [2, 3]. These diverticula were often derived from esophageal motor abnormalities, such as a lack of coordination between the distal esophagus and lower esophageal sphincter (LES) [4]. Patients with no symptoms or mildly symptomatic epiphrenic diverticula can be managed conservatively [4]. However, if a diverticulum associated with dysmotility is enlarging and making symptoms worse, surgical intervention is indicated [4].

Minimally invasive treatments of epiphrenic diverticula have been reported in a few series since 1995; however, the best surgical approach remains uncertain. We reviewed the literature to provide an overview of the different approaches that have been used and to determine which technique(s) provide the best outcome. A search for English-language articles in PubMed from 1995 to 2008 was performed using the term “epiphrenic diverticulum AND thoracoscopy OR laparoscopy.” We reviewed the results of 25 articles containing 133 cases who were treated via either laparoscopic or thoracoscopic surgery.

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In addition, we recently successfully treated an epiphrenic esophageal diverticulum by performing minimally invasive laparoscopic transhiatal resection and Heller myotomy with Dor fundoplication after observing the enlargement of the diverticulum on radiological and endoscopic examinations over a 2-year period. We herein describe our surgical approach.

Review of previously published series

In this study, we reviewed the results of a series of 25 articles involving either laparoscopic or thoracoscopic surgery [4–28]. From January 1995 to December 2008, there were a total of 133 patients.

Clinical data

The clinical data are summarized in Table 1. There were 47 male and 54 female patients, with a mean age of 60 years (range 32–84). The average maximum size of the diverticula was 6 cm (range 3.3–10) in diameter. A hiatal hernia was present in 12 patients (9 %).

Motility findings

The motility findings are also summarized in Table 1. Motility disorders were identified in 108 patients (68 %). In 31 patients (29 %), the esophageal motor disorder was characterized as achalasia, 32 patients (30 %) had diffuse esophageal spasm (DES) or non-specific esophageal motility disorder (NEMD), and 10 patients (9 %) had a

hypertensive lower esophageal sphincter (HLES). One patient had pseudoachalasia in association with a gastrointestinal stromal tumor (GIST) [8].

Surgical treatments

Table 2 summarizes the data about the surgical treatments used in the 133 patients. Nineteen patients (14 %) were treated with a thoracoscopic approach, 112 patients (84 %) with a laparoscopic approach, and two patients (2 %) underwent a combination approach. The diverticulectomy was performed using an endostapler in all patients. A myotomy was added in 103 patients (83 %). A fundoplication was added in 106 patients (85 %). The techniques used for fundoplication were the Dor in 53 patients (50 %), Toupet in 35 (33 %), Nissen in 16 (15 %), Belsey in two (2 %).

Mortality and morbidity

The mortality and morbidity of patients in these 25 articles are summarized in Table 3. There were two surgical deaths (2 %). One patient died on post-operative day 4 of an acute myocardial infarction [16]. Another patient died on post-operative day 61 from renal failure after re-operation for a suture-line leak [17]. The post-operative morbidity rate was 21 %. The most severe complication was suture leakage, which occurred in 20 patients (15 %). No differences in complication rates between laparoscopic or thoracoscopic approaches were identified.

Table 2 Surgical treatments used in the 25 published series

| Surgical treatments | |
|---|----------------|
| Surgical procedures <i>n</i> (%) | <i>n</i> = 133 |
| Thoracoscopic | 19 (14) |
| Diverticulectomy | 8 (6) |
| Diverticulectomy + myotomy | 2 (2) |
| Diverticulectomy + myotomy + fundoplication | 2 (2) |
| Unknown | 7 (5) |
| Laparoscopic | 112 (84) |
| Diverticulectomy | 7 (5) |
| Diverticulectomy + myotomy | 1 (1) |
| Diverticulectomy + fundoplication | 6 (5) |
| Diverticulectomy + myotomy + fundoplication | 98 (74) |
| Thoracoscopic + laparoscopic | 2 (2) |
| Unknown | 2 (2) |
| Techniques used for fundoplication <i>n</i> (%) | <i>n</i> = 106 |
| Dor | 53 (50) |
| Toupet | 35 (33) |
| Nissen | 16 (15) |
| Belsey | 2 (2) |

Table 1 Patient demographics in the 25 published series

| Patient demographics | |
|--|-------------------------|
| Demographics and preoperative data | |
| Gender (M/F/unknown) | 47/54/32 |
| Patient's age (years) | 60 (32–84) ^a |
| Maximum diverticulum size (cm) | 6 (3.3–10) ^a |
| Location of diverticulum (L/R/unknown) | 4/13/116 |
| Motility findings <i>n</i> (%) | <i>n</i> = 108 |
| Achalasia | 31 (29) |
| DES or NEMD | 32 (30) |
| HLES | 10 (9) |
| Undetected abnormality | 35 (32) |
| Unknown | 25 |

M male, *F* female, *L* left side of the esophagus, *R* right side of the esophagus, *DES* diffuse esophageal spasm, *NEMD* non-specific esophageal motility disorder, *HLES* hypertensive lower esophageal sphincter

^a Data are expressed as the means (range)

Table 3 Mortality and morbidity in the 25 published series

| Mortality and morbidity <i>n</i> (%) | <i>n</i> = 133 |
|--------------------------------------|----------------|
| Mortality | 2 (2) |
| Morbidity | 28 (21) |
| Staple-line leak | 20 (15) |
| Dysphagia | 4 (3) |
| Pneumonitis | 3 (2) |
| GERD | 3 (2) |
| Diverticulum recurrence | 1 (1) |

GERD gastro-esophageal reflux disease

Case presentation

A 41-year-old male presented to our department for treatment of an epiphrenic esophageal diverticulum causing dysphagia. He had been diagnosed with achalasia 2 years prior, and the diverticulum had not existed at that point. Barium swallow, computed tomography (CT) and gastrointestinal endoscopy undertaken by the Department of Internal Medicine over a 2-year period showed the appearance and gradual enlargement of the epiphrenic esophageal diverticulum, resulting in worsened dysphagia and vomiting (Fig. 1). Barium swallow and CT examinations showed an 8 cm in diameter epiphrenic diverticulum into which barium accumulated before influx into the stomach (Fig. 2). Gastrointestinal endoscopy showed considerable food residue in the diverticulum, but there was no ulceration or evidence of malignancy (Fig. 3). Manometric examination showed a hypertensive (42 mmHg) non-relaxing LES and non-peristaltic contractions of the esophageal body. Surgery was indicated due to the patient's symptoms, the size of the diverticulum, and the associated motor abnormality. Thus, we performed laparoscopic transhiatal diverticulectomy with Heller myotomy and Dor fundoplication.

The patient was positioned in a reverse-Trendelenburg (30°) supine position with legs spread; the surgeon stood between the patient's legs (Fig. 4a). Two 12-mm trocars and three 5-mm trocars were placed (Fig. 4b). Initially, a pneumoperitoneum was established, enabling us to maintain a constant abdominal pressure of 10 mmHg. We used a flexible laparoscope to obtain a clear surgical field via a transhiatal approach. The abdominal esophagus was isolated and encircled with a silicon tube for safe traction. The esophagus was then retracted inferiorly, and the diverticular pouch was identified and completely cleaned of all adherent tissue. We resected the diverticulum at its base using an endostapler (Endo-GIA Universal, Tyco Healthcare, Tokyo, Japan) four times (Fig. 5a). We took care to extend the staple line in a straight line (Fig. 5b). Intraoperative endoscopy was used to insure complete identification of the

diverticulum using insufflation and deflation, and to identify the esophageal lumen during the resection of the diverticulum to prevent esophageal stricture (Fig. 6). The site of the operation was submerged in saline, and air was introduced into the esophagus via the endoscope to check for leaks. The myotomy subsequently involved 5 cm of the anterior wall of the lower esophagus and 2 cm of the stomach, from 3 cm above the distal limit of the neck of the diverticulum (Fig. 5c). The gastric fundus adjoined the right side of the myotomy, including the distal 1/3 of the staple line after diverticulectomy (Fig. 5d). The fundus was also anchored at the right crural pillar. Drains were placed below the liver, the left diaphragm, in both sides of the mediastinum, and into the left thoracic cavity. The total duration of the operation was 320 min. Histopathology of the resected specimen showed false diverticula without any malignant findings.

On post-operative day 4, oral intake was started after the esophagogram demonstrated no leaks (Fig. 7). The patient was swallowing well without any further episodes of dysphagia 18 months after the operation.

Discussion

There is increasing consensus for the laparoscopic treatment of esophageal diverticula. Technical factors support this choice, including better visualization of the esophago-gastric junction, easier myotomy and performance of antireflux wrap, and better alignment of the stapler cartridge to the longitudinal axis of the esophagus. Potential disadvantages could be a difficult dissection of the upper part of the diverticular neck and the major risks of pleural lesions [16]. According to previous studies in the literature, the indications for thoracoscopic surgery are (1) suspected mediastinal adhesions, (2) a fairly large size of diverticulum, (3) after failed laparoscopic resection of epiphrenic diverticula. [10, 17, 24].

There have been controversies about whether an esophagomyotomy or an antireflux procedure should be performed in addition to diverticulectomy. Some authors recommend a routine esophagomyotomy in every patient, irrespective of manometric results [3, 9]. The exact association between esophageal diverticula and esophageal motility disorders is unclear. In some patients, stationary manometry may fail to demonstrate intermittent esophageal dysmotility. However, many articles suggest that epiphrenic diverticula occur in association with motility dysfunction of the esophagus. Nehra et al. [29] have suggested the use of 24-h motility recording in this category of dysfunction. In their cohort of 21 patients, motility abnormalities were found in 100 % of cases. Removing the diverticulum without addressing the abnormal motility has been associated with a higher incidence of diverticulum

Fig. 1 A barium swallow examination showed the appearance and gradual enlargement of the epiphrenic esophageal diverticulum. **a** July 2006; **b** February 2007; **c** July 2008

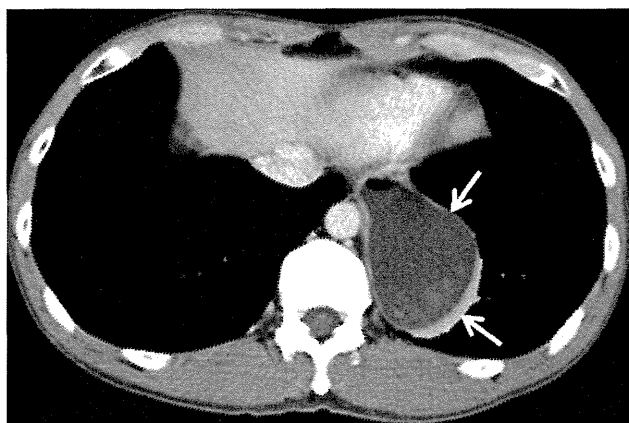
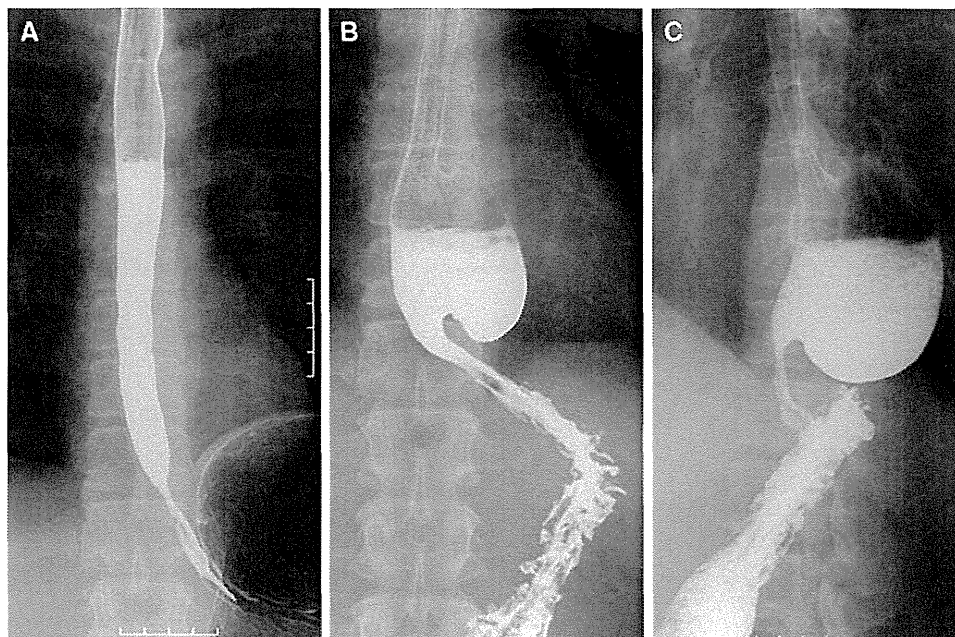


Fig. 2 CT showed an 8 cm in diameter epiphrenic diverticulum (arrows)

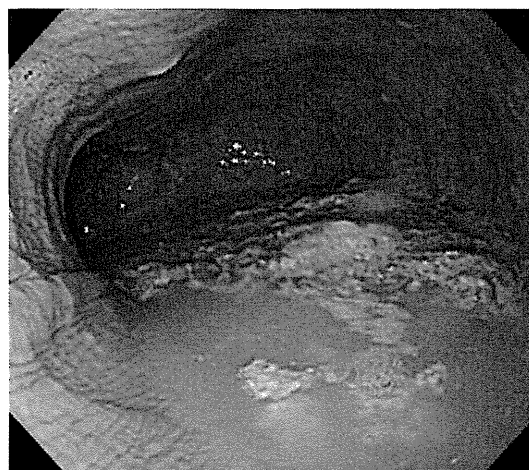


Fig. 3 Gastrointestinal endoscopy showed considerable food residue in the diverticulum

recurrence and a suture-line leak rate of 10–20 % [3, 30–33]. There is general agreement that it is necessary to perform a myotomy in association with diverticulectomy to correct the underlying motor dysfunction [34, 35].

The reflux rate, defined by symptoms or with the assistance of the pH probe for patients with achalasia who are status post-Heller myotomy without an antireflux procedure, range from 14 to 57 % [36–38]. Some authors recommend routine fundoplication in every patient with myotomy for esophageal diverticulum [9, 24]. However, controversy exists with regard to the choice of operation between Nissen, Toupet or Dor fundoplication. We recommend Dor fundoplication, which has three advantages for the Heller myotomy. First, it is a much simpler operation than Toupet or Nissen fundoplication. Second, it prevents a pseudodiverticulum secondary to an uncovered

myotomy. Finally, it prevents a major leak if intraoperative perforations of the esophageal mucosa occur [9]. In fact, Dor fundoplication was chosen in most patients in the literature. We also think an antireflux wrap should be used to cover over the staple line as widely as possible to prevent esophageal leak, in addition to the myotomy site. However, we were unable to cover the whole staple line in our experience, because the wrapped stomach was not large enough. However, the small wrapped size might be sufficient to cover just the distal 1/3 of the staple line.

Almost all surgeons used intraoperative endoscopy. Intraoperative flexible endoscopy was helpful in (1) correct identification of the cranial and distal limits of the neck of the diverticulum by transillumination; (2) aspiration of

Fig. 4 **a** Surgical team positioning **b** trocar placement (two 12-mm trocars and three 5-mm trocars)

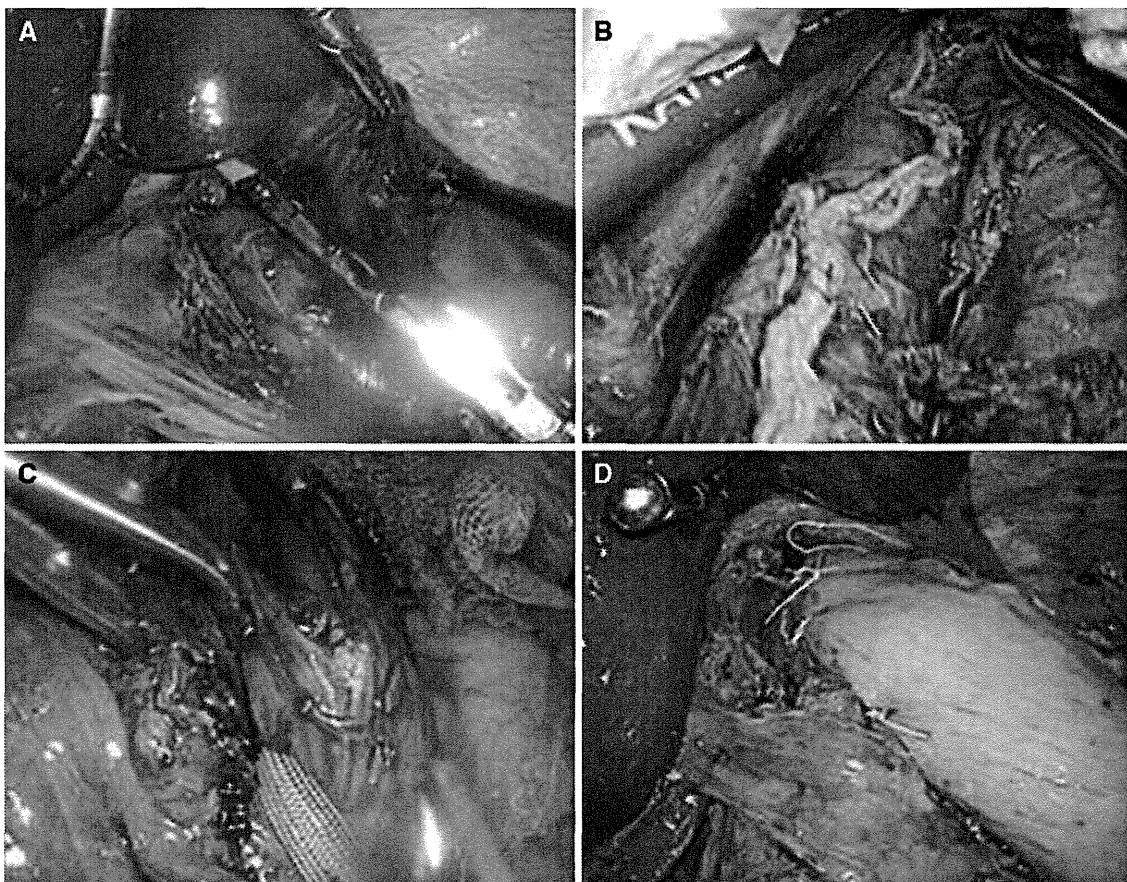
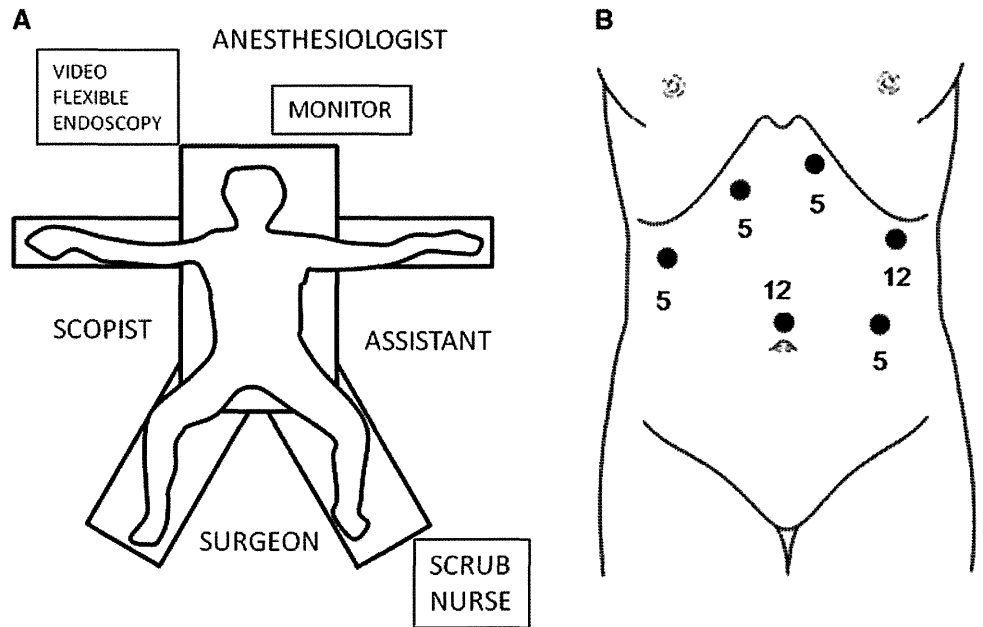


Fig. 5 Intraoperative procedures. **a** Resection of the diverticulum using an endostapler four times; **b** taking care to extend the staple line in a straight line; **c** myotomy involved 5 cm of the anterior wall of the

lower esophagus and 2 cm of stomach; **d** the gastric fundus adjoined the right side of the myotomy, including a distal 1/3 of the staple line

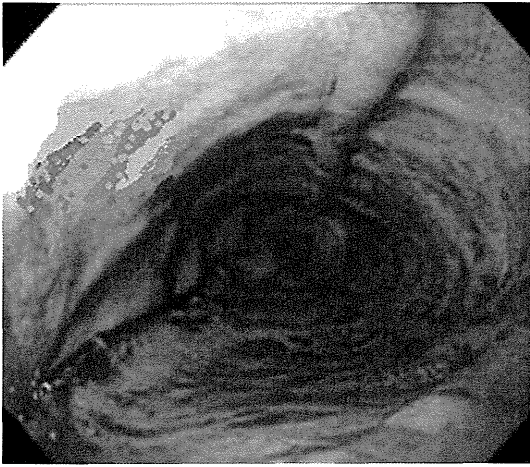


Fig. 6 An intraoperative flexible endoscope view of the esophageal lumen after diverticulectomy

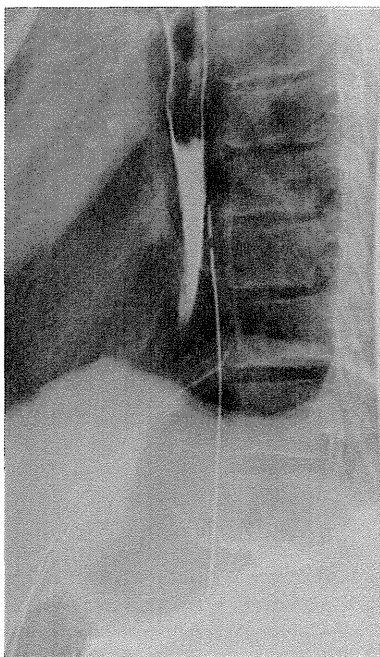


Fig. 7 A post-operative esophagogram demonstrated no leaks

residual food from the diverticulum; (3) helping the dissection by insufflation/desufflation of the pouch; (4) identification of the esophageal lumen during the resection of the diverticulum to prevent esophageal stricture; (5) checking for staple-line leakage after the diverticulum resection and the myotomy [27]. It is important to take care not to leave the muscular layer defect, and intraoperative endoscopy was also useful to confirm the correction of the muscular defect by insufflation/desufflation of the pouch.

According to the pertinent literature, the incidence of cancer development within esophageal diverticula is 0.3–3 % [39, 40]. There was no cancer in any of the 133

patients who underwent minimally invasive surgery for epiphrenic diverticula in the current report, however, it is important to rule out malignancy within a diverticulum preoperatively.

We successfully treated an epiphrenic esophageal diverticulum by performing minimally invasive laparoscopic transhiatal resection and a Heller myotomy with Dor fundoplication. For gastroenterological surgeons who have the experience and skills of laparoscopic Heller and Dor operations for esophageal achalasia, this surgical approach will be the easiest, safest and most effective procedure for esophageal epiphrenic diverticulum. The procedure still carries a significant morbidity related mainly to suture leakage, therefore, intraoperative prevention by adequate surgical technique, and an appropriate treatment strategy in the event of suture leakage, must be provided. We took care to extend the staple line in a straight line and to avoid leaving any muscular defect during resection of the diverticulum in order to prevent suture leakage.

Unfortunately, because of the rarity of the condition, a randomized trial of surgical treatment will be difficult to conduct. Therefore, we reviewed the results of 133 patients in a series of 25 published articles and our personal experience. We believe that a laparoscopic transhiatal resection of an esophageal diverticulum and Heller myotomy with Dor fundoplication may therefore become the standard modality using minimally invasive surgery for the treatment of an esophageal epiphrenic diverticulum.

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