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 PGE_2 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-μm 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 μg/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 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 μg 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′-cagctggacctctttccaac-3′; muc5ac, 5′-aactctgcccaccacaagc-3′ and 5′-tgccatctatccaatcagtccaat-3′; *muc6*, 5′-tgctgtctccagcacaaaac-3′ and 5′-tcagaagtctgcgtcactgc-3′; *gapdh*, 5′-atgtatccgttgtggatctgac-3′ and 5′-cctgcttcaccaccttcttg-3′; *actin*, 5′-gtcgtaccactggcattgtg-3′ and 5′-gctgatcttgcccttgagac-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 μg/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 manufacture'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 μg/ml SBA-HRP (lectin) in phosphate-buffered saline) for 1 h at 37 °C. After further washing, each well was finally incubated with 100 μl 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 μg protein) was diluted with 40 mM Tris-HCl (pH 6.8) buffer containing 3 mM $MgSO_4$, 15 mM KCl, 5 mM NaN_3 and 2 mM ouabain and pre-incubated with each tested chemical in the presence or absence of 50 μM 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 μg protein, 75 μl) was pre-incubated with each tested chemical in 96 well plates for 5 min at room temperature. Then 25 μl adenylate cyclase assay buffer (final concentrations; 50 mM Tris-HCl (pH 7.4), 100 mM KCl, 10 mM $MgCl_2$, 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 μl 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 ± 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 PGE_2 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 PGE_2 level were similar between the two treatment groups (Fig. 3A and B), suggesting that mucosal cell death rather than gastric PGE_2 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 PGE_2 level had been lowered by pre-administration of aspirin (10 mg/kg). This regimen reduced PGE_2 to a negligible level, with subsequent administration of F-LOX or LOX having no effect (Fig. 3 C). 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 PGE_2 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

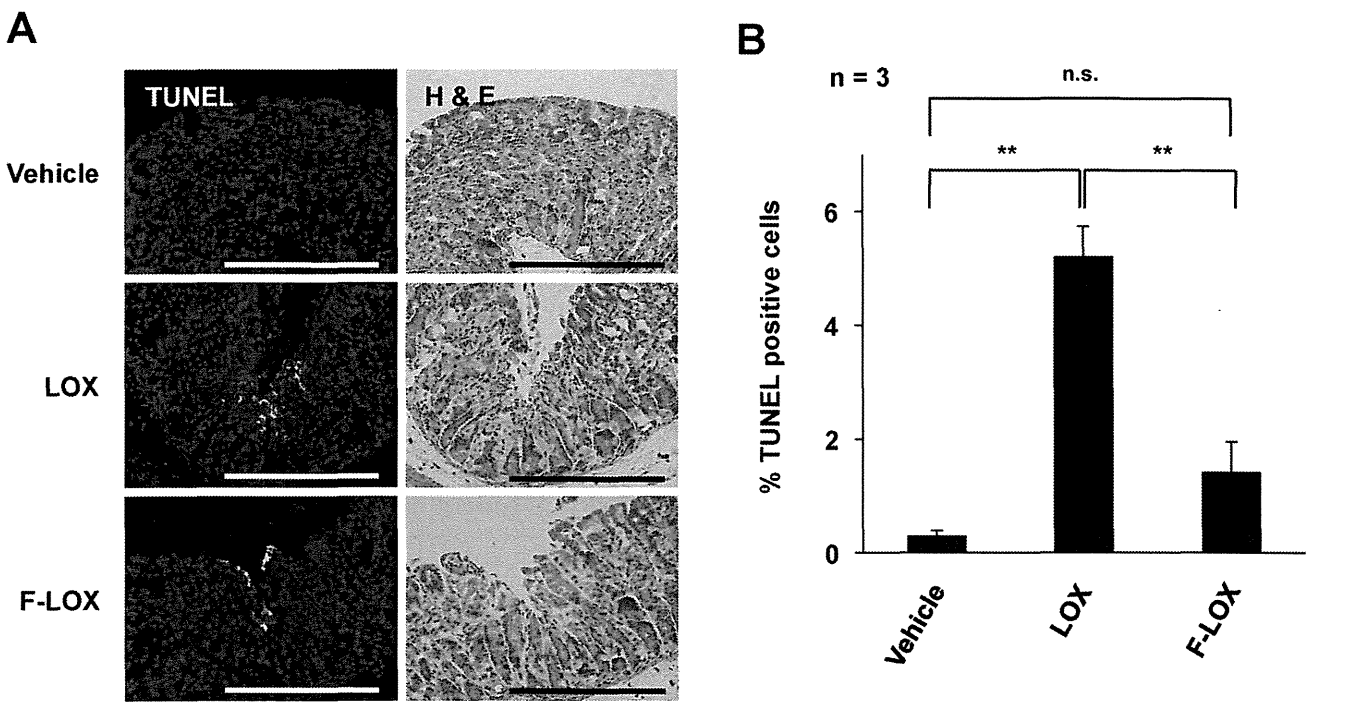

Fig. 2. Gastric mucosal cell death following oral administration of F-LOX or LOX. Wistar rats were orally administered 100 or 106.7 mg/kg of LOX or F-LOX, respectively, and their stomachs were removed 8 h later. A, sections of gastric tissues were prepared and subjected to TUNEL assay, DAPI staining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

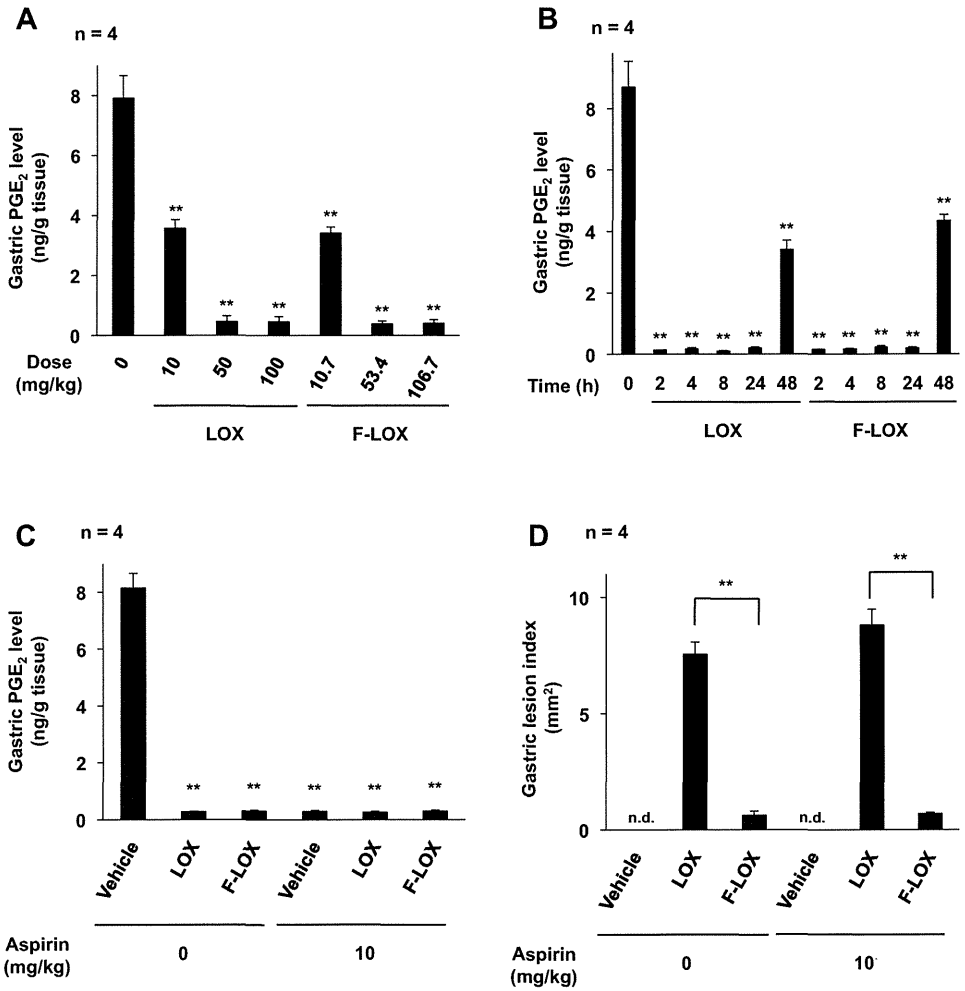

Fig. 3. Decrease in gastric PGE2 level in response to oral administration of F-LOX or LOX. Wistar rats were orally administered either the indicated doses (A), 200 or 213.4 mg/kg (B), or 100 or 106.7 mg/kg (C, D) of LOX or F-LOX, respectively. Rats were orally pre-administered the indicated dose of aspirin, 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 PGE_2 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 PGE_2 level in both cases was similar to that obtained following oral administration of the drugs (Fig. 4 C). 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

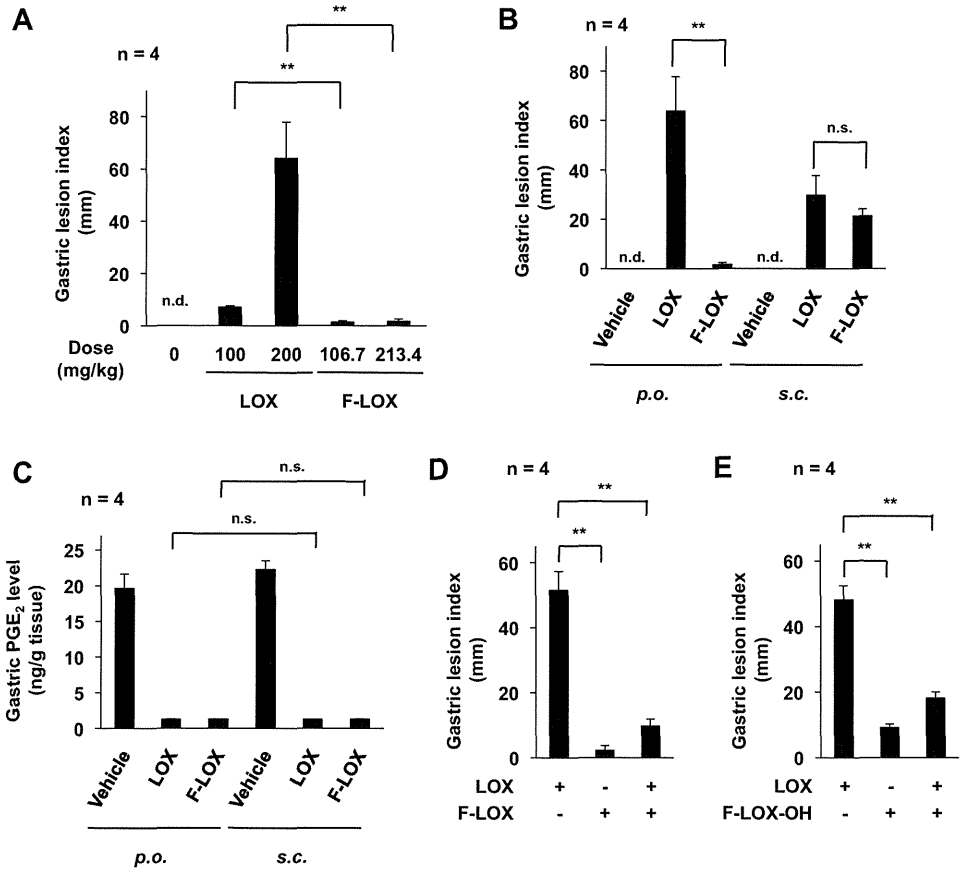

Fig. 4. Protective effect of orally administered F-LOX on the gastric mucosa. (A) SD rats were orally administered the indicated doses of LOX or F-LOX. (B and C) SD rats were orally (*p.o.*) or subcutaneously (*s.c.*) administered 200 or 213.4 mg/kg of LOX or F-LOX, respectively. (D and E) SD rats were 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 PGE_2 level was determined by EIA 4 h after the administration of LOX. Values are mean ± 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. PGE_2 stimulates the production of mucus through both EP_1 and EP_4 receptors [47]. As shown in Fig. 8A, pre-treatment of RGM1 cells with antagonists for EP_1 and EP_4 receptors suppressed PGE_2-induced production of mucin but not F-LOX-induced one (Fig. 8A). EP receptor subtypes are coupled to different intracellular signaling pathways. The EP_1 receptor is coupled to Ca^{2+} mobilization and activation of EP_4 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 Ca^{2+} 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

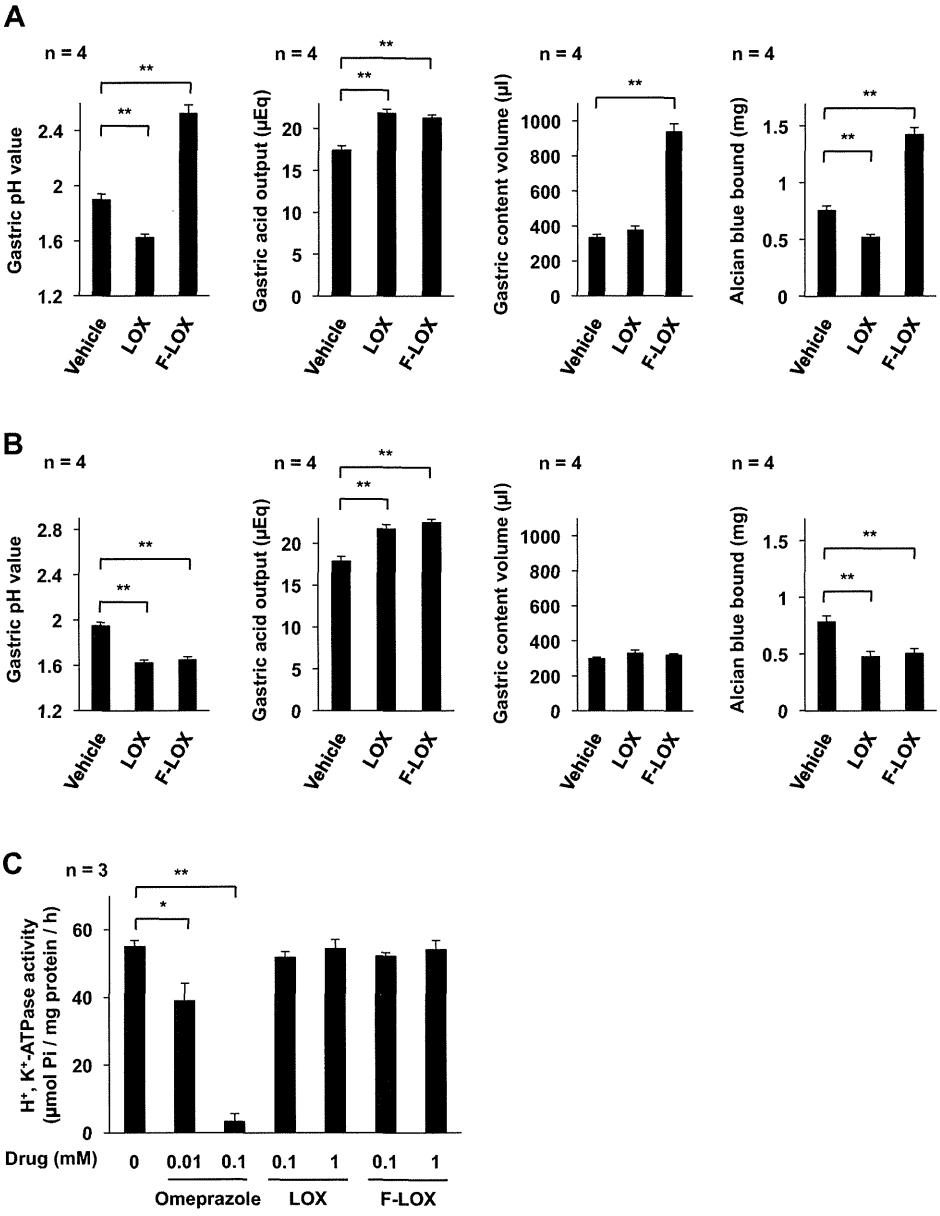

Fig. 5. Effect of F-LOX and LOX on gastric pH and mucus content. SD rats were orally (A) or subcutaneously (B) administered 100 or 106.7 mg/kg of LOX or F-LOX, respectively, 1 h after which the pylorus was ligated and the rats were maintained for a further 3 h. The gastric pH value, gastric acid output, 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 PGE_2 (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 PGE_2- 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 PGE_2 due to COX inhibition are

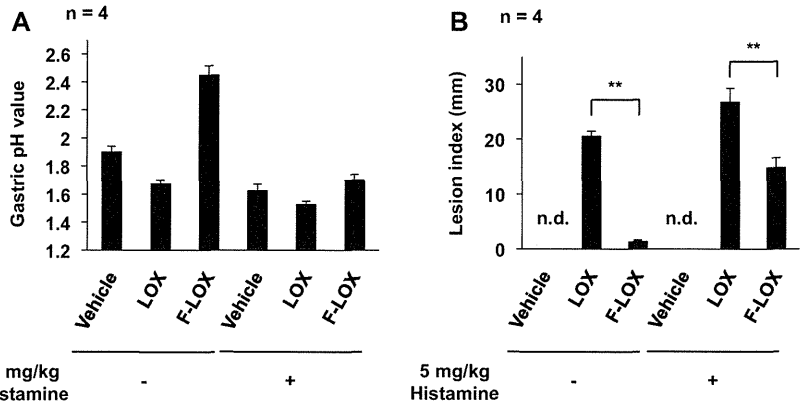

Fig. 6. Effects of pre-administration of histamine on the production of gastric lesions by F-LOX and LOX. SD rats were orally pre-administered the indicated dose of histamine, 1 h after which they were orally administered 100 or 106.7 mg/kg of LOX or F-LOX, respectively. The stomach was then removed 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 ± 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 PGE_2 (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 PGE_2 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 PGE_2.

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 PGE_2 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 PGE_2 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

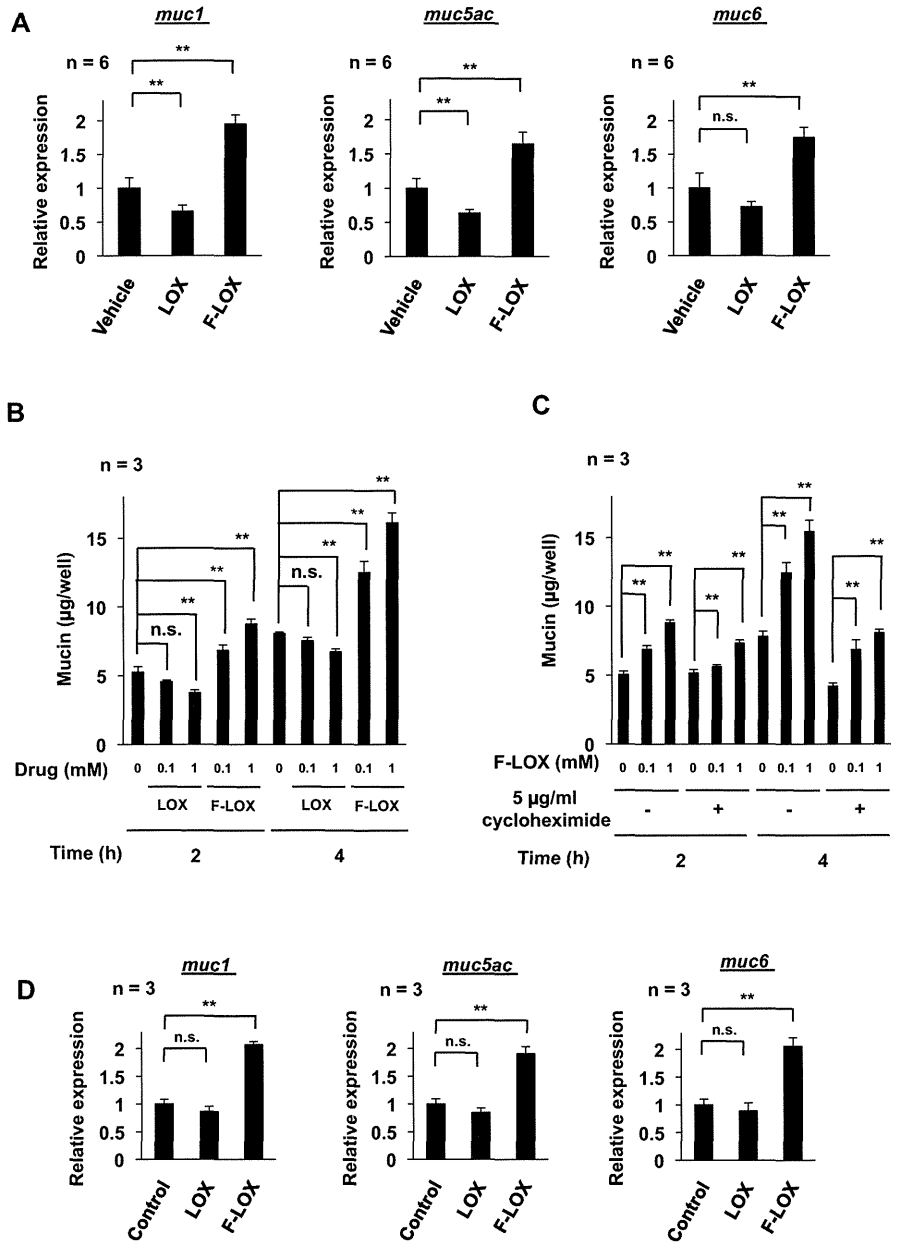

Fig. 7. Effect of F-LOX and LOX on the production and secretion of mucin. (A) SD rats were orally administered 100 or 106.7 mg/kg of LOX or F-LOX, respectively, and the gastric mucosa was removed 4 h later. Total RNA was extracted and subjected to real-time RT-PCR using a specific primer for each gene. 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 PGE_2 stimulates production of mucin through both EP_1 and EP_4 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 EP_1 and EP_4 suppressed PGE_2-induced production of mucin but not F-LOX-induced one. We then tested whether F-LOX directly affects the intracellular signalling pathway coupled with EP_1 or EP_4 receptor (Ca^{2+} mobilization or activation of adenylate cyclase and resulting increase in the cellular level of cAMP, respectively) and found that pre-treatment of RGM1 cells with an inhibitor of adenylate cyclase 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,

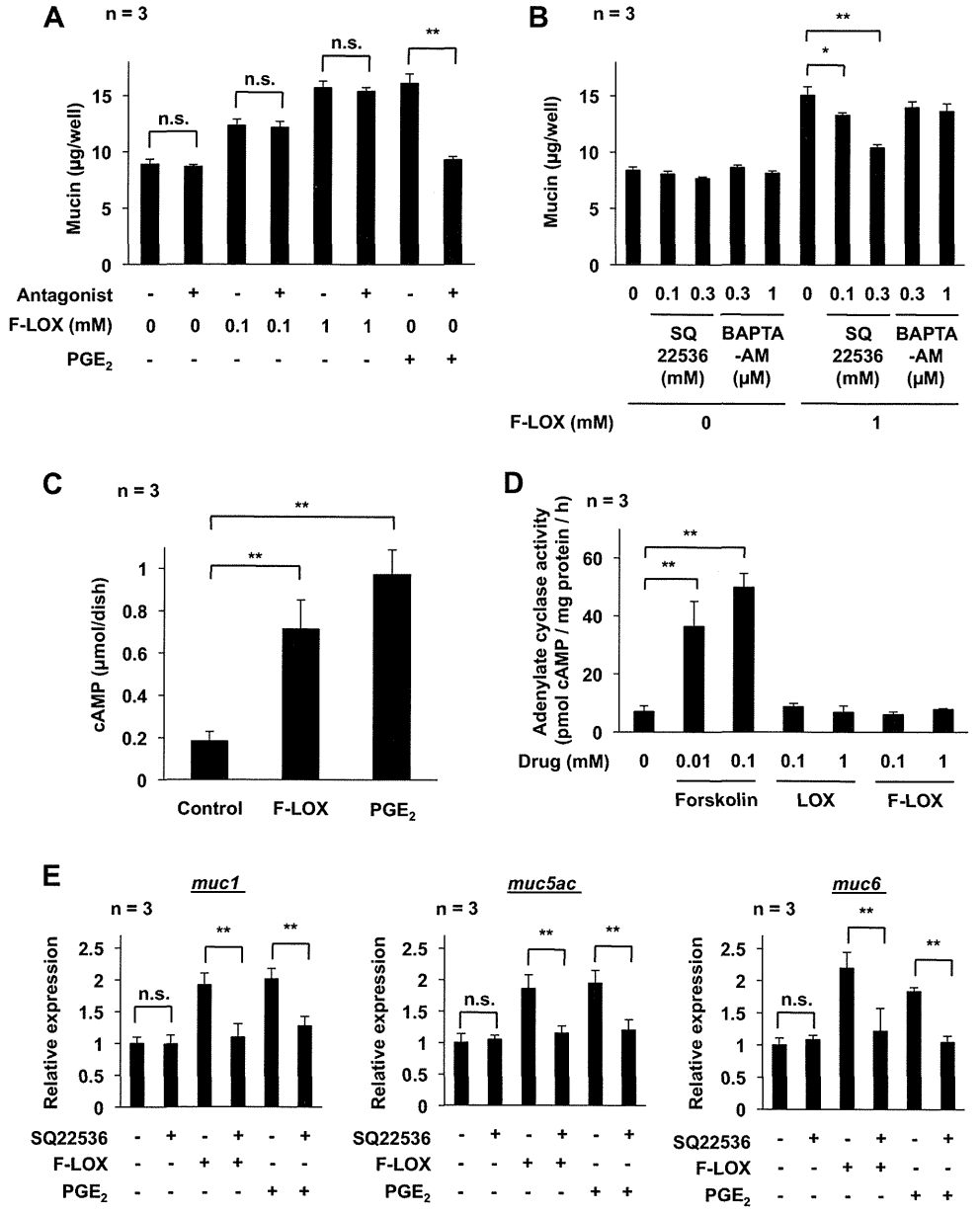

Fig. 8. Molecular mechanism for F-LOX-mediated alteration of production of mucin. RGM1 cells were pre-incubated with 1 μg/ml ONO-8711 (EP_1 antagonist) and 0.1 μg/ml ONO-AE2-227 (EP_4 antagonist) for 0.5 h, after which the medium was exchanged to one containing indicated concentrations of F-LOX and cultured 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.

References

[1] Holzer P. Neural emergency system in the stomach. Gastroenterology 1998;114:823–39.
[2] Miller TA. Protective effects of prostaglandins against gastric mucosal damage: current knowledge and proposed mechanisms. Am J Physiol 1983;245: G601–23.
[3] Smalley WE, Ray WA, Daugherty JR, Griffin MR. Nonsteroidal anti-inflammatory drugs and the incidence of hospitalizations for peptic ulcer disease in elderly persons. Am J Epidemiol 1995;141:539–45.

[4] Hawkey CJ. Nonsteroidal anti-inflammatory drug gastropathy. Gastroenterology 2000;119:521–35.
[5] Lichtenberger LM. Where is the evidence that cyclooxygenase inhibition is the primary cause of nonsteroidal anti-inflammatory drug (NSAID)-induced gastrointestinal injury. Topical injury revisited. Biochem Pharmacol 2001;61:631–7.
[6] Whittle BJ. New dogmas or old. Gut 2003;52:1379–81.
[7] Gupta M, Eisen GM. NSAIDs and the gastrointestinal tract. Curr Gastroenterol Rep 2009;11:345–53.
[8] Levine RA, Schwartzel EH. Effect of indomethacin on basal and histamine stimulated human gastric acid secretion. Gut 1984;25:718–22.
[9] Phillipson M, Johansson ME, Henriksnas J, Petersson J, Gendler SJ, Sandler S, et al. The gastric mucus layers: constituents and regulation of accumulation. Am J Physiol Gastrointest Liver Physiol 2008;295:G806–12.
[10] Ligumsky M, Golanska EM, Hansen DG, Kauffman GJ. Aspirin can inhibit gastric mucosal cyclo-oxygenase without causing lesions in rat. Gastroenterology 1983;84:756–61.
[11] Ligumsky M, Sestieri M, Karmeli F, Zimmerman J, Okon E, Rachmilewitz D. Rectal administration of nonsteroidal antiinflammatory drugs Effect on rat gastric ulcerogenicity and prostaglandin E2 synthesis. Gastroenterology 1990;1245–9.
[12] Tanaka K, Tomisato W, Hoshino T, Ishihara T, Namba T, Aburaya M, et al. Involvement of Intracellular Ca2+ Levels in Nonsteroidal Anti-inflammatory Drug-induced Apoptosis. J Biol Chem 2005;280:31059–67.
[13] Tsutsumi S, Gotoh T, Tomisato W, Mima S, Hoshino T, Hwang HJ, et al. Endoplasmic reticulum stress response is involved in nonsteroidal anti-inflammatory drug-induced apoptosis. Cell Death Differ 2004;11:1009–16.
[14] Tomisato W, Tanaka K, Katsu T, Kakuta H, Sasaki K, Tsutsumi S, et al. Membrane permeabilization by non-steroidal anti-inflammatory drugs. Biochem Biophys Res Commun 2004;323:1032–9.
[15] Tomisato W, Tsutsumi S, Rokutan K, Tsuchiya T, Mizushima T. NSAIDs induce both necrosis and apoptosis in guinea pig gastric mucosal cells in primary culture. Am J Physiol Gastrointest Liver Physiol 2001;281:G1092–100.
[16] Aburaya M, Tanaka K, Hoshino T, Tsutsumi S, Suzuki K, Makise M, et al. Heme oxygenase-1 protects gastric mucosal cells against non-steroidal anti-inflammatory drugs. J Biol Chem 2006;281:33422–32.
[17] Tsutsumi S, Namba T, Tanaka KI, Arai Y, Ishihara T, Aburaya M, et al. Celecoxib upregulates endoplasmic reticulum chaperones that inhibit celecoxib-induced apoptosis in human gastric cells. Oncogene 2006;25:1018–29.
[18] Namba T, Hoshino T, Tanaka K, Tsutsumi S, Ishihara T, Mima S, et al. Up-regulation of 150-kDa oxygen-regulated protein by celecoxib in human gastric carcinoma cells. Mol Pharmacol 2007;71:860–70.
[19] Ishihara T, Hoshino T, Namba T, Tanaka K, Mizushima T. Involvement of up-regulation of PUMA in non-steroidal anti-inflammatory drug-induced apoptosis. Biochem Biophys Res Commun 2007;356:711–7.
[20] Tomisato W, Tsutsumi S, Hoshino T, Hwang HJ, Mio M, Tsuchiya T, et al. Role of direct cytotoxic effects of NSAIDs in the induction of gastric lesions. Biochem Pharmacol 2004;67:575–85.
[21] Vane J. Towards a better aspirin. Nature 1994;367:215–6.
[22] Silverstein FE, Faich G, Goldstein JL, Simon LS, Pincus T, Whelton A, et al. Gastrointestinal toxicity with celecoxib vs nonsteroidal anti-inflammatory drugs for osteoarthritis and rheumatoid arthritis: the CLASS study: A randomized controlled trial. Celecoxib Long-term Arthritis Safety Study. JAMA 2000;284:1247–55.
[23] Bombardier C, Laine L, Reicin A, Shapiro D, Burgos VR, Davis B, et al. Comparison of upper gastrointestinal toxicity of rofecoxib and naproxen in patients with rheumatoid arthritis. VIGOR Study Group. N Engl J Med 2000;343:1520–8. 2 p following 8.
[24] FitzGerald GA, Patrono C. The coxibs, selective inhibitors of cyclooxygenase-2. N Engl J Med 2001;345:433–42.
[25] Mukherjee D, Nissen SE, Topol EJ. Risk of cardiovascular events associated with selective COX-2 inhibitors. JAMA 2001;286:954–9.
[26] Mukherjee D. Selective cyclooxygenase-2 (COX-2) inhibitors and potential risk of cardiovascular events. Biochem Pharmacol 2002;63:817–21.
[27] McAdam BF, Catella LF, Mardini IA, Kapoor S, Lawson JA, FitzGerald GA. Systemic biosynthesis of prostacyclin by cyclooxygenase (COX)-2: the human pharmacology of a selective inhibitor of COX-2. Proc Natl Acad Sci USA 1999;96:272–7.
[28] Catella LF, McAdam B, Morrison BW, Kapoor S, Kujubu D, Antes L, et al. Effects of specific inhibition of cyclooxygenase-2 on sodium balance, hemodynamics, and vasoactive eicosanoids. J Pharmacol Exp Ther 1999;289:735–41.
[29] Belton O, Byrne D, Kearney D, Leahy A, Fitzgerald DJ. Cyclooxygenase-1 and -2-dependent prostacyclin formation in patients with atherosclerosis. Circulation 2000;102:840–5.
[30] Yamakawa N, Suemasu S, Kimoto A, Arai Y, Ishihara T, Yokomizo K, et al. Low direct cytotoxicity of loxoprofen on gastric mucosal cells. Biol Pharm Bull 2010;33:398–403.
[31] Misaka E, Yamaguchi T, Iizuka Y, Kamoshida K, Kojima T, Kobayashi K, et al. Anti-inflammatory, Analgesic and Antipyretic Activities of Sodium 2-[4-(2-oxocyclopentan-1-ylmethyl)phenyl] Propionate Dihydrate (CS-600)*. Pharmacometrics 1981;21:753–71.
[32] Kawano S, Tsuji S, Hayashi N, Takei Y, Nagano K, Fusamoto H, et al. Effects of loxoprofen sodium, a newly synthesized non-steroidal anti-inflammatory drug, and indomethacin on gastric mucosal haemodynamics in the human. J Gastroenterol Hepatol 1995;10:81–5.
[33] Sugimoto M, Kojima T, Asami M, Iizuka Y, Matsuda K. Inhibition of prostaglandin production in the inflammatory tissue by loxoprofen-Na, an anti-inflammatory prodrug. Biochem Pharmacol 1991;42:2363–8.
[34] Yamakawa N, Suemasu S, Matoyama M, Kimoto A, Takeda M, Tanaka K, et al. Properties and synthesis of 2-{2-fluoro (or bromo)-4-[(2-oxocyclopentyl)-methyl]phenyl}propanoic acid: nonsteroidal anti-inflammatory drugs with low membrane permeabilizing and gastric lesion-producing activities. J Med Chem 2010;53:7879–82.
[35] Takeuchi K, Ueki S, Okabe S. Importance of gastric motility in the pathogenesis of indomethacin-induced gastric lesions in rats. Dig Dis Sci 1986;31:1114–22.
[36] Tanaka A, Matsumoto M, Hayashi Y, Takeuchi K. Functional mechanism underlying cyclooxygenase-2 expression in rat small intestine following administration of indomethacin: relation to intestinal hypermotility. J Gastroenterol Hepatol 2005;20:38–45.
[37] Asano T, Tanaka K, Yamakawa N, Adachi H, Sobue G, Goto H, et al. HSP70 confers protection against indomethacin-induced lesions of the small intestine. J Pharmacol Exp Ther 2009;330:458–67.
[38] Filaretova L, Tanaka A, Miyazawa T, Kato S, Takeuchi K. Mechanisms by which endogenous glucocorticoid protects against indomethacin-induced gastric injury in rats. Am J Physiol Gastrointest Liver Physiol 2002;283:G1082–89.
[39] Anson ML. The estimation of pepsin, trypsin, papain, and cathepsin with hemoglobin. J Gen Physiol 1938;22:79–89.
[40] Bolton JP, Palmer D, Cohen MM. Stimulation of mucus and nonparietal cell secretion by the E2 prostaglandins. Am J Dig Dis 1978;23:359–64.
[41] Kobayashi I, Kawano S, Tsuji S, Matsui H, Nakama A, Sawaoka H, et al. RGM1, a cell line derived from normal gastric mucosa of rat. In Vitro Cell Dev Biol Anim 1996;32:259–61.
[42] Ohara S, Watanabe T, Hotta K. Comparative study of carbohydrate portion of gastrointestinal mucins using enzyme-linked lectin-binding assay (ELLA). Comp Biochem Physiol B Biochem Mol Biol 1997;116:167–72.
[43] Fujii T, Takahashi Y, Ikari A, Morii M, Tabuchi Y, Tsukada K, et al. Functional association between K + -Cl- cotransporter-4 and H+,K + -ATPase in the apical canalicular membrane of gastric parietal cells. J Biol Chem 2009;284:619–29.
[44] Fujii T, Ohira Y, Itomi Y, Takahashi Y, Asano S, Morii M, et al. Inhibition of P-type ATPases by [(dihydroindenyl)oxy]acetic acid (DIOA), a K+ -Cl- cotransporter inhibitor. Eur J Pharmacol 2007;560:123–6.
[45] Yoda A, Hokin LE. On the reversibility of binding of cardiotonic steroids to a partially purified (Na + K)-activated adenosinetriphosphatase from beef brain. Biochem Biophys Res Commun 1970;40:880–6.
[46] Oner SS, Kaya AI, Onaran HO, Ozcan G, Ugur O. Beta2-Adrenoceptor, Gs and adenylate cyclase coupling in purified detergent-resistant, low density membrane fractions. Eur J Pharmacol 2010;630:42–52.
[47] Ohnishi A, Shimamoto C, Katsu K, Ito S, Imai Y, Nakahari T. EP1 and EP4 receptors mediate exocytosis evoked by prostaglandin E(2) in guinea-pig antral mucous cells. Exp Physiol 2001;86:451–60.
[48] Coleman RA, Smith WL, Narumiya S. International Union of Pharmacology classification of prostanoid receptors: properties, distribution, and structure of the receptors and their subtypes. Pharmacol Rev 1994;46:205–29.
[49] Yamakawa N, Suemasu S, Watanabe H, Tahara K, Tanaka KI, Okamoto Y, et al. Comparison of pharmacokinetics between loxoprofen and its derivative with lower ulcerogenic activity, fluoro-loxoprofen. Drug Metab Pharmacokinet 2012.
[50] Goldstein JL, Eisen GM, Lewis B, Gralnek IM, Aisenberg J, Bhadra P, et al. Small bowel mucosal injury is reduced in healthy subjects treated with celecoxib compared with ibuprofen plus omeprazole, as assessed by video capsule endoscopy. Aliment Pharmacol Ther 2007;25:1211–22.
[51] Aabakken L, Bjornbeth BA, Weberg R, Viksmoen L, Larsen S, Osnes M. NSAID-associated gastroduodenal damage: does famotidine protection extend into the mid- and distal duodenum. Aliment Pharmacol Ther 1990;4:295–303.
[52] Somasundaram S, Sigthorsson G, Simpson RJ, Watts J, Jacob M, Tavares IA, et al. Uncoupling of intestinal mitochondrial oxidative phosphorylation and inhibition of cyclooxygenase are required for the development of NSAID-enteropathy in the rat. Aliment Pharmacol Ther 2000;14:639–50.
[53] Basivireddy J, Vasudevan A, Jacob M, Balasubramanian KA. Indomethacin-induced mitochondrial dysfunction and oxidative stress in villus enterocytes. Biochem Pharmacol 2002;64:339–49.

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REVIEW ARTICLE

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

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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.

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].

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 *n* (%)	*n* = 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)

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 *n* (%)	*n* = 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 *n* (%)	*n* = 106
Dor	53 (50)
Toupet	35 (33)
Nissen	16 (15)
Belsey	2 (2)

Table 3 Mortality and morbidity in the 25 published series

Mortality and morbidity *n* (%)	*n* = 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 esophagogastric 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

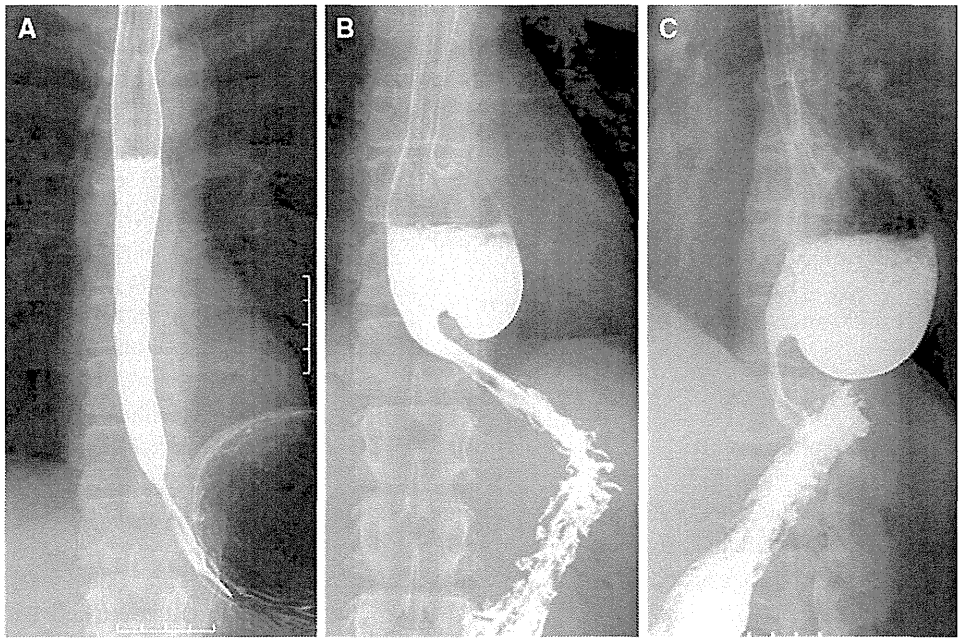

Fig. 1 A barium swallow examination showed the appearance and gradual enlargement of the epiphrenic esophageal diverticulum. **a** July 2006; **b** February 2007; **c** July 2008

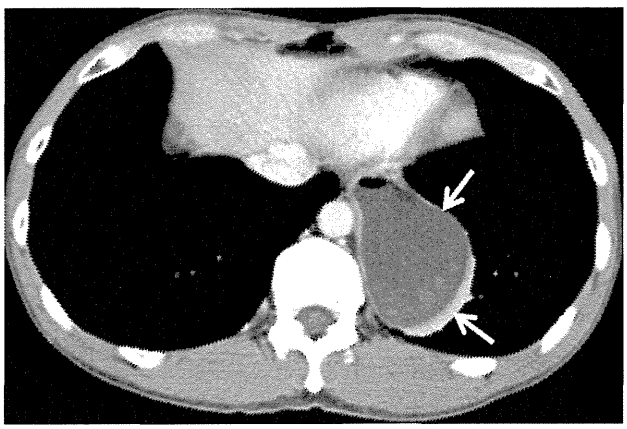

Fig. 2 CT showed an 8 cm in diameter epiphrenic diverticulum (*arrows*)

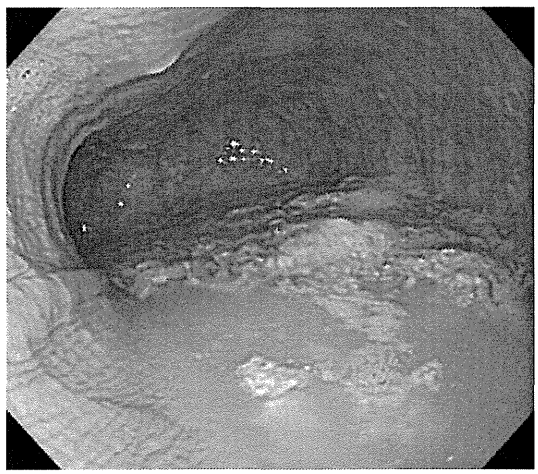

Fig. 3 Gastrointestinal endoscopy showed considerable food residue in the diverticulum

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

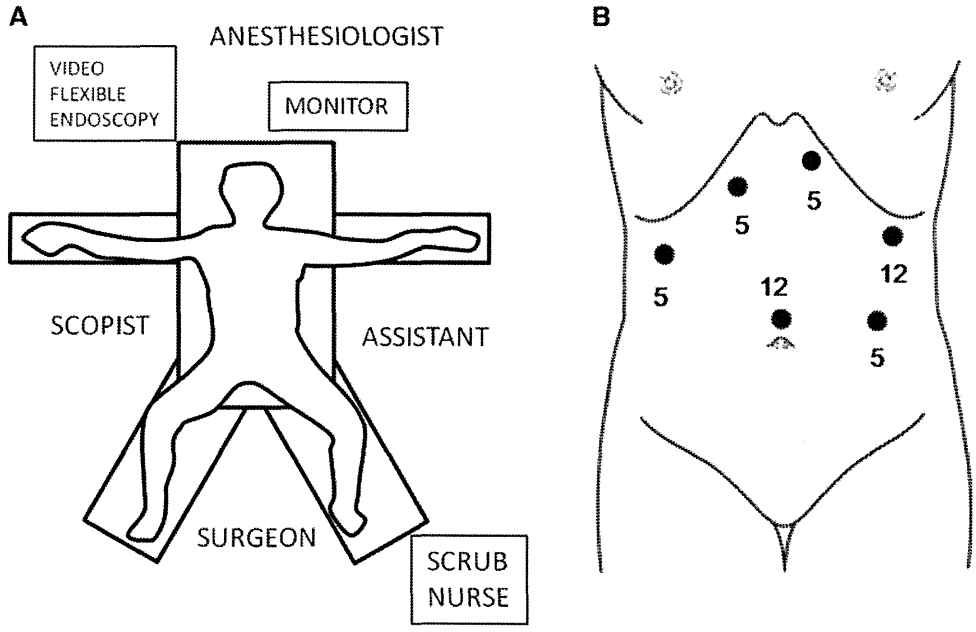

Fig. 4 **a** Surgical team positioning **b** trocar placement (two 12-mm trocars and three 5-mm trocars)

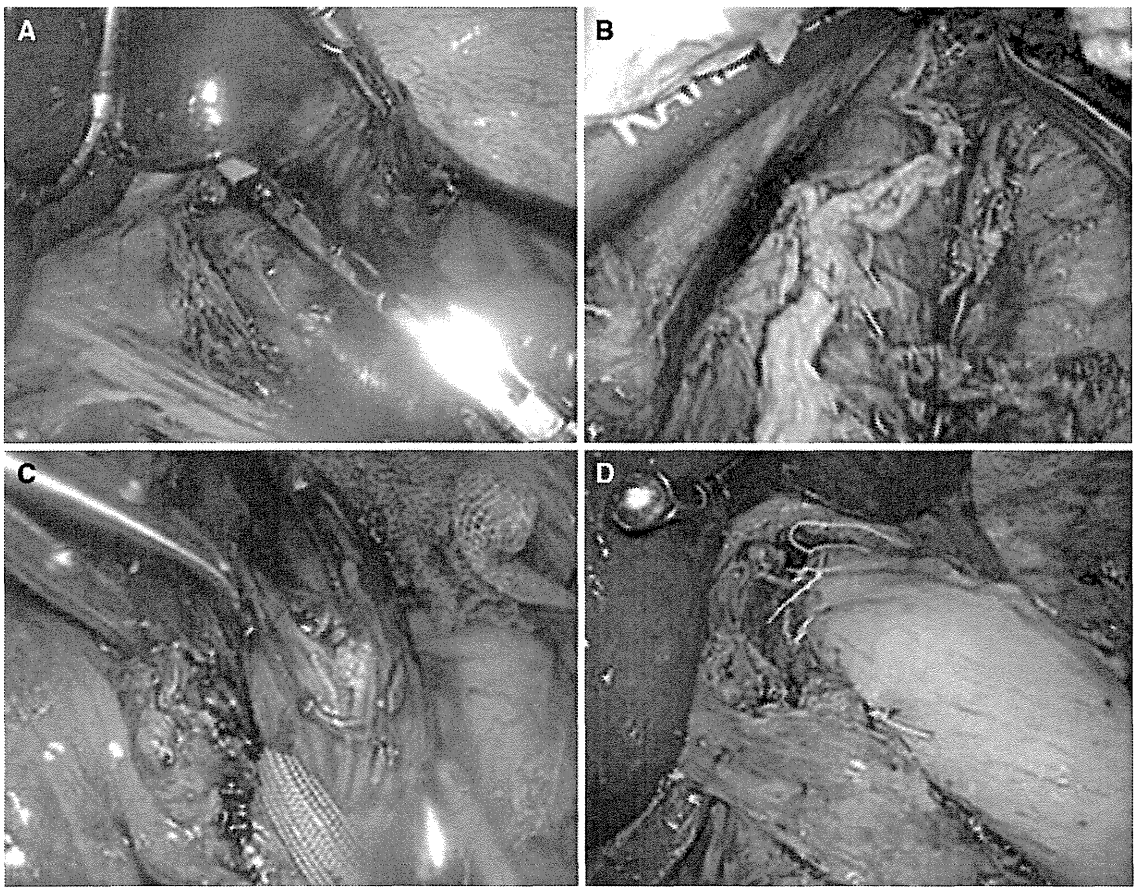

Fig. 5 Intraoperative procedures. **a** Resection of the diverticulum using an endostapler four times; **b** taking care to extend the staple line in a straight line; **c** myotomy involved 5 cm of the anterior wall of the lower esophagus and 2 cm of stomach; **d** the gastric fundus adjoined the right side of the myotomy, including a distal 1/3 of the staple line

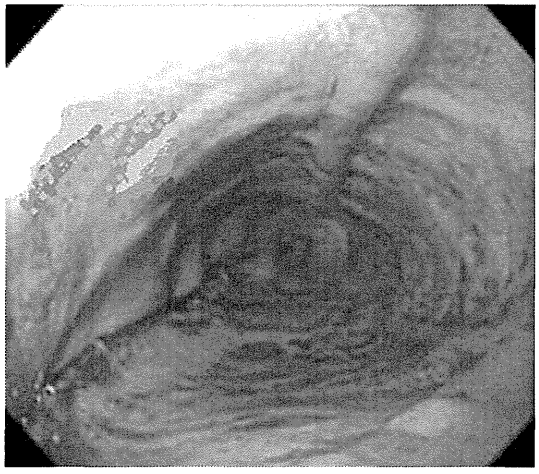

Fig. 6 An intraoperative flexible endoscope view of the esophageal lumen after diverticulectomy

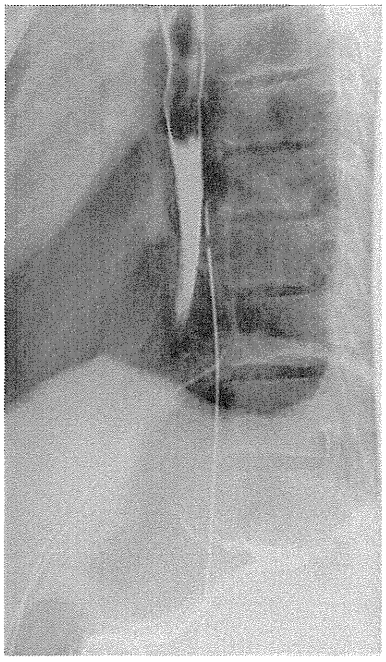

Fig. 7 A post-operative esophagogram demonstrated no leaks

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.

References

1. Wheeler D. Diverticula of the foregut. Radiology. 1947;49:476–81.
2. Bruggeman LL, Seaman WB. Epiphrenic diverticula. An analysis of 80 cases. Am J Roentgenol Radium Ther Nucl Med. 1973;119:266–76.
3. Benacci JC, Deschamps C, Trastek VF, Allen MS, Daly RC, Pairolero PC. Epiphrenic diverticulum: results of surgical treatment. Ann Thorac Surg. 1993;55:1109–13.
4. Zaninotto G, Portale G, Costantini M, Merigliano S, Guirroli E, Rizzetto C, et al. Long-term outcome of operated and unoperated epiphrenic diverticula. J Gastrointest Surg. 2008;12:1485–90.
5. Peracchia A, Rosati R, Bona S, Fumagalli U, Bonavina L, Chella B. Laparoscopic treatment of functional diseases of the esophagus. Int Surg. 1995;80:336–40.
6. Saw EC, McDonald TP, Kam NT. Video-assisted thoracoscopic resection of an epiphrenic diverticulum with esophagomyotomy and partial fundoplication. Surg Laparosc Endosc. 1998;8:145–8.
7. Chami Z, Fabre JM, Navarro F, Domergue J. Abdominal laparoscopic approach for thoracic epiphrenic diverticulum. Surg Endosc. 1999;13:164–5.
8. Mainprize KS, Dehn TC. Laparoscopic management of pseudoachalasia, esophageal diverticulum, and benign esophageal stromal tumor. Dis Esophagus. 2001;14:73–5.
9. Rosati R, Fumagalli U, Bona S, Zago M, Celotti S, Bisagni P, et al. Laparoscopic treatment of epiphrenic diverticula. J Laparoendosc Adv Surg Tech A. 2001;11:371–5.

10. van der Peet DL, Klinkenberg-Knol EC, Berends FJ, Cuesta MA. Epiphrenic diverticula: minimal invasive approach and repair in five patients. Dis Esophagus. 2001;14:60–2.
11. Neoral C, Aujesky R, Bohanes T, Klein J, Kral V. Laparoscopic transhiatal resection of epiphrenic diverticulum. Dis Esophagus. 2002;15:323–5.
12. Champion JK. Thoracoscopic Belsey fundoplication with 5-year outcomes. Surg Endosc. 2003;17:1212–5.
13. Fraiji E Jr, Bloomston M, Carey L, Zervos E, Goldin S, Banasiak M, et al. Laparoscopic management of symptomatic achalasia associated with epiphrenic diverticulum. Surg Endosc. 2003;17:1600–3.
14. Pitchford TJ, Price PD. Laparoscopic Heller myotomy with epiphrenic diverticulectomy. JSLS. 2003;7:165–9.
15. Costantini M, Zaninotto G, Rizzetto C, Narne S, Ancona E. Oesophageal diverticula. Best Pract Res Clin Gastroenterol. 2004;18:3–17.
16. Del Genio A, Rossetti G, Maffetton V, Renzi A, Brusciano L, Limongelli P, et al. Laparoscopic approach in the treatment of epiphrenic diverticula: long-term results. Surg Endosc. 2004;18:741–5.
17. Fernando HC, Luketich JD, Samphire J, Alvelo-Rivera M, Christie NA, Buenaventura PO, et al. Minimally invasive operations for esophageal diverticula. Ann Thorac Surg. 2005;80:2076–80.
18. Tedesco P, Fisichella PM, Way LW, Patti MG. Cause and treatment of epiphrenic diverticula. Am J Surg. 2005;190:891–4.
19. Valentini M, Pera M, Vidal O, Lacima G, Belda J, de Lacy AM. Incomplete esophageal myotomy and early recurrence of an epiphrenic diverticulum. Dis Esophagus. 2005;18:64–6.
20. Feo CV, Sortini D, Liboni A. Oesophageal achalasia with epiphrenic diverticulum with laparoscopic approach: a 6-year follow-up. Dig Surg. 2006;23:27.
21. Motoyama S, Maruyama K, Okuyama M, Sasaki K, Sato Y, Ogawa J. Laparoscopic long esophagomyotomy with Dor fundoplication using a transhiatal approach for an epiphrenic esophageal diverticulum. Surg Today. 2006;36:758–60.
22. Granderath FA, Pointner R. Laparoscopic transhiatal resection of giant epiphrenic esophageal diverticulum. Dis Esophagus. 2007;20:353–7.
23. Kinoshita Y, Udagawa H, Tsutsumi K, Ueno M, Mine S, Ehara K, et al. Modified Dor fundoplication technique following diverticulectomy and myotomy for epiphrenic diverticulum combined with esophageal motility disorders: prevention of esophageal leak at the diverticulectomy staple line. Esophagus. 2008;5:57–60.
24. Miyashita M, Ohkawa K, Akagi I, Nomura T, Makino H, Tajiri T. Esophagoscopy-guided thoracoscopic resection of large epiphrenic esophageal diverticulum. Esophagus. 2008;5:225–8.
25. Palanivelu C, Rangarajan M, John SJ, Parthasarathi R, Senthilkumar R. Laparoscopic transhiatal approach for benign supradiaphragmatic lesions of the esophagus: a replacement for thoracoscopy? Dis Esophagus. 2008;21:176–80.
26. Palanivelu C, Rangarajan M, Maheshkumaar GS, Senthilkumar R. Minimally invasive surgery combined with perioperative endoscopy for symptomatic middle and lower esophageal diverticula: a single institute's experience. Surg Laparosc Endosc Percutan Tech. 2008;18:133–8.
27. Silecchia G, Casella G, Recchia CL, Bianchi E, Lomartire N. Laparoscopic transhiatal treatment of large epiphrenic esophageal diverticulum. JSLS. 2008;12:104–8.
28. Melman L, Quinlan J, Robertson B, Brunt LM, Halpin VJ, Eagon JC, et al. Esophageal manometric characteristics and outcomes for laparoscopic esophageal diverticulectomy, myotomy, and partial fundoplication for epiphrenic diverticula. Surg Endosc. 2009;23:1337–41.
29. Nehra D, Lord RV, DeMeester TR, Theisen J, Peters JH, Crookes PF, et al. Physiologic basis for the treatment of epiphrenic diverticulum. Ann Surg. 2002;235:346–54.
30. Allen TH, Clagett OT. Changing concepts in the surgical treatment of pulsion diverticula of the lower esophagus. J Thorac Cardiovasc Surg. 1965;50:455–62.
31. Fekete F, Vonns C. Surgical management of esophageal thoracic diverticula. Hepatogastroenterology. 1992;39:97–9.
32. Evander A, Little AG, Ferguson MK, Skinner DB. Diverticula of the mid- and lower esophagus: pathogenesis and surgical management. World J Surg. 1986;10:820–8.
33. Rosati R, Fumagalli U, Bona S, Bonavina L, Peracchia A. Diverticulectomy, myotomy, and fundoplication through laparoscopy: a new option to treat epiphrenic esophageal diverticula? Ann Surg. 1998;227:174–8.
34. Belsey R. Functional disease of the esophagus. J Thorac Cardiovasc Surg. 1966;52:164–88.
35. Effler DB, Barr D, Groves LK. Epiphrenic diverticulum of the esophagus: surgical treatment. Arch Surg. 1959;79:459–67.
36. Kjellin AP, Granqvist S, Ramel S, Thor KB. Laparoscopic myotomy without fundoplication in patients with achalasia. Eur J Surg. 1999;165:1162–6.
37. Wang PC, Sharp KW, Holzman MD, Clements RH, Holcomb GW, Richards WO. The outcome of laparoscopic Heller myotomy without antireflux procedure in patients with achalasia. Am Surg. 1998;64:515–20.
38. Richards WO, Torquati A, Holzman MD, Khaitan L, Byrne D, Lutfi R, et al. Heller myotomy versus Heller myotomy with Dor fundoplication for achalasia: a prospective randomized double-blind clinical trial. Ann Surg. 2004;240:405–12.
39. Gawande AS, Batiuchok W, Barman AA, Mule JE. Carcinoma within lower esophageal (epiphrenic) diverticulum. N Y State J Med. 1972;72:1749–51.
40. Fujita H, Kakegawa T, Shima S, Kumagaya Y. Carcinoma within a middle esophageal (parabronchial) diverticulum: a case report and the review of the literature. Jpn J Surg. 1980;10:142–8.

REVIEW

Molecular Approaches and Modern Clinical Strategies for the Management of *Helicobacter pylori* Infection in Japan

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Thirty years have passed since Warren and Marshall's discovery of *Helicobacter pylori* (*H.pylori*). Since then, not only peptic ulcer diseases and chronic gastritis but also non-cardia gastric cancers have been recognized as diseases originating from *H. pylori* infection. Several combination therapies consisting of multiple antibiotics have been developed as first- or second-line regimens to eradicate *H. pylori* infection. Our extensive experience in the field of anti-*H. pylori* medicine suggests that clinicians should consider a possible role for unidentified, invisible pathogens to elucidate the pathogenesis and improve the treatment of refractory diseases of unknown etiology. (doi: 10.2302/kjm.2012-0001-RE; Keio J Med 61 (4) : 109–119, December 2012)

***Keywords*: *Helicobacter pylori*, CagA, indication, diagnosis, eradication**

Introduction

Classical bacteriology techniques have demonstrated that *Helicobacter pylori* (*H.pylori*) is a gram-negative, flagellated bacterium that expresses catalase and urease, enzymes which help neutralize host responses and enable intragastric colonization. One of the early benefits of this basic research was the realization that virtually all strains of *H. pylori* produce urease. This finding led to the development of accurate diagnostic tests, including the rapid urease test and the urea breath test. A stool antigen test has also emerged as an informative, noninvasive means by which to diagnose infection via detection of bacterial antigens.

Before the discovery of intragastric bacteria 30 years ago, there were several reports of spiral gastric bacteria dating back to 1890 (**Table 1**).[1] The modern discovery of *H. pylori* may have been delayed by Palmer's declaration in 1954 that there were no microorganisms in the human stomach. At that time, microorganisms were believed to be unable to survive in the acidic gastric environment, and intragastric spiral bacteria had been observed only post-mortem;[2] however, the presence of organisms in the gastric mucosa had been described since the 1890s. In Japan, Kasai and Kobayashi of the Kitasato Institute reported propagation of a spirochete-like organism, probably modern *Helicobacter felis*, from the stomachs of dogs and cats but not from laboratory animals.[3] They showed that when rabbits infected with these spirochetes were inoculated with *virus fixe*, marked hemorrhagic lesions were produced in the gastric mucosa. In addition, spirochetes inoculated into the mouse gastric mucosa could be eradicated by arsaminol (a classic equivalent of bismuth).[4] Sixty five years later, scientists and doctors again attempted to treat gastric diseases associated with "spirochetes," i.e., *Helicobacter pylori* infection. To commemorate the achievements of Kobayashi and coworkers in the Taisho era, the Rokuzo Kobayashi Memorial Symposium on *Helicobacter pylori* was held on May 11th, 2002, in Kita-kan Hall at Keio University Mita Campus, Tokyo, Japan.[4,5]

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Table 1 History of *Helicobacter* research

1892	Bizzozero	Discovery of snake-like bodies in a dog stomach
1906	Krienitz	Discovery of spirochete-like bodies in human stomach
1919	Kasai and Kobayashi	Establishment of an animal model for *H. felis* infection and first *Helicobacter* eradication experiment
1938	Doenges	Isolation of spirochete-like organism from a human stomach
1954	Palmer	Denied the existence of microorganisms in the stomach
1976	Lieber	Reduction of gastric NH_3 by ampicillin
1982	Warren and Marshall	Detection and isolation of *Campylobacter*-like organism from biopsied gastric specimen (*Campylobacter pyloridis*)
1994	NIH Consensus Development Conference	Recommendation of *H. pylori* eradication therapy for all patients with peptic ulcer disease
2002	Marshall	Keio Medical Science Prize
2005	Warren and Marshall	Nobel Prize in Physiology and Medicine

Pathogenesis

The most-investigated bacterial toxins of *H. pylori* are associated with a segment of bacterial DNA referred to as the *cag* pathogenicity island (*cag* PAI). Genes within the *cag* PAI encode proteins such as CagE, an ATPase that drives a type IV secretory apparatus enabling bacterial macromolecules, especially the toxin CagA, to translocate into the host cell. The intact *cag* PAI of *H. pylori* plays a significant role in the pathogenesis of chronic gastritis in humans because the *cag* PAI is associated with increased chemokine expression and greater inflammation in gastric mucosal specimens.[6] After the CagA protein is injected into the host cell cytoplasm through the type IV secretory system, the EPIYA motif of CagA is tyrosine-phosphorylated by host Src kinases, and the phosphorylated protein subsequently alters the gastric epithelial morphology. Src homology-2 domain-containing phosphatase 2 (SHP2) is able to bind the EPIYA-B, EPIYA-C, and EPIYA-D motifs.[7] Importantly, however, CagA with the EPIYA-D motif has a higher binding affinity for SHP2 than does CagA with the EPIYA-C motif.[8] The sequence flanking the tyrosine phosphorylation site of the EPIYA-D motif (EPIYATIDF), but not that flanking of the EPIYA-C motif (EPIYATIDD), perfectly matches the consensus high-affinity binding sequence for the SH2 domains of SHP2. The CagA proteins of strains from distinct geographic populations appear to be phosphorylated to different degrees, resulting in graded effects on intracellular signaling.[9]

The biological half-life of intracellular CagA in gastric epithelial cells was recently reported to be approximately 200 min,[10] and the activity of CagA as an epigenetic oncoprotein thus does not persist for a long period in any single cell. Therefore, the risk of gastric cancer may be determined by various factors that influence the stability of intracellular CagA. We recently reported that intracellular CagA is degraded by p53 degradation-induced autophagy;[11] however, a specific mutation in p53 increases the intracellular stability of CagA by inhibiting its autophagic degradation. These findings suggest that disrupting the autophagic degradation of CagA increases the risk of developing *H. pylori*-associated gastric cancer.

VacA is the second-most extensively studied *H. pylori* virulence factor. In addition to inducing vacuolation, VacA also promotes several cellular activities, including membrane channel formation and the release of cytochrome *c* from mitochondria and consequent apoptosis. VacA can also specifically inhibit T-cell activation and proliferation.[12] Further studies have confirmed that the risks for both cancer and peptic ulcers are associated strongly with the *cagA/vacA s1m1* genotype but rarely with the *vacA s2m2* polymorphism.[13]

Oxygen-derived free radicals released from activated neutrophils extravasated from microvascular beds are considered to be potential toxic factors involved in *H. pylori*-induced gastric mucosal injury because *H. pylori* exhibits chemotactic activity for neutrophils.[1,14] Neutrophil infiltration of the gastric mucosa leads to the development of the initial lesions of *H. pylori*-associated gastritis. Neutrophils express the enzyme myeloperoxidase, which, in the presence of Cl^-, produces the potent oxidant hypochlorous anion (OCl^-) from H_2O_2. This hypochlorous anion reacts with ammonia, produced from urea by *H. pylori*-associated urease, to yield the lipophilic cytotoxic oxidant monochloramine (NH_2Cl), which freely penetrates biological membranes to oxidize intracellular components (**Fig. 1**, left).[1] 8-Hydroxy-2-deoxyguanosine (8-OHdG), which results from the attack of a singlet hydroxyl or oxygen radical on guanine, is one of the forms of DNA damage most commonly induced by reactive oxygen species. Patients with *cagA*-positive strains of *H. pylori* have higher 8-OHdG levels than do *cagA*-negative or *H. pylori*-negative patients.[15] We previously reported greater enhancement of neutrophil-derived gastric mucosal luminol-dependent chemiluminescence levels in

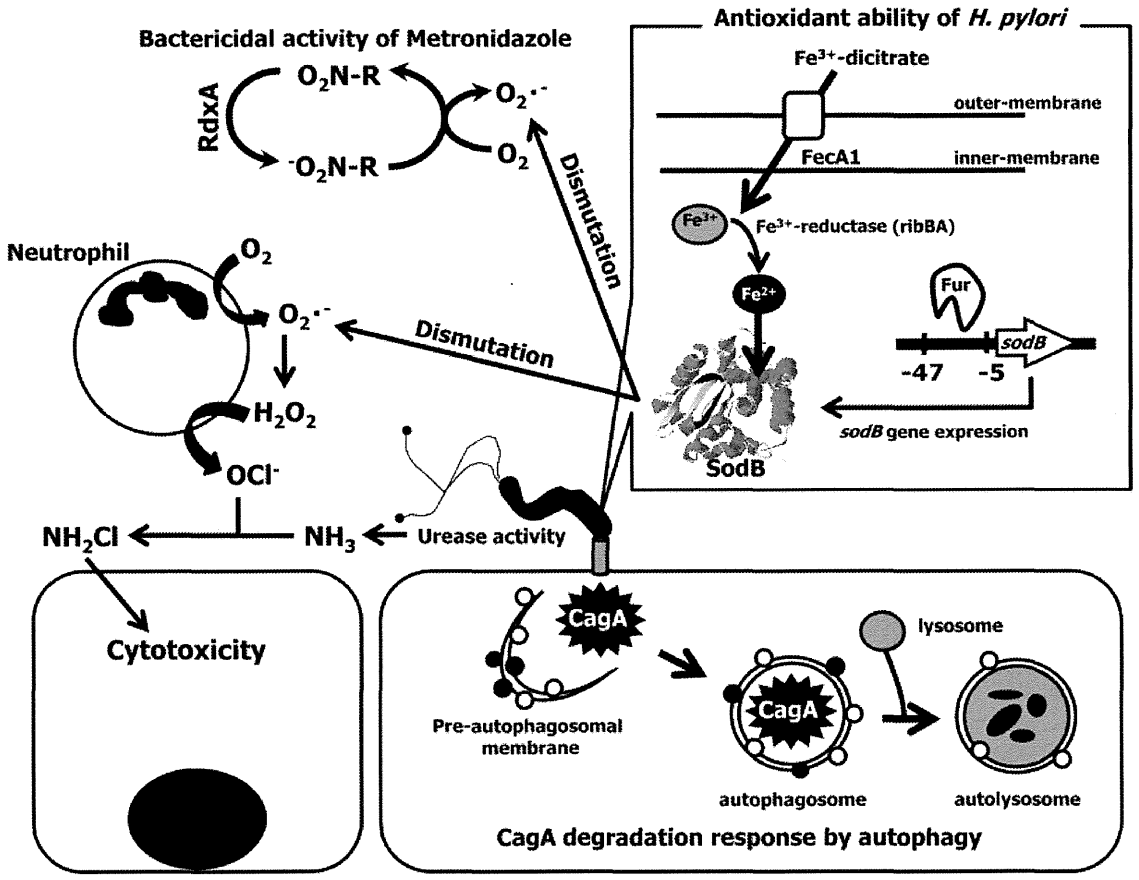

Fig. 1 Molecular approaches to *Helicobacter pylori* infection.
RdxA, NADPH nitroreductase; Fur, ferric uptake regulator; FecA1, a Fe^{3+}-dicitrate transporter homolog.

cagA-positive patients than in *cagA*-negative patients.[16] *cagA*-positive patients are characterized by greater oxidative DNA damage, both overall and at younger ages, in the presence of multifocal atrophy.

Infection of gastric epithelial cells with *H. pylori* increases the accumulation of intracellular reactive oxygen species (ROS). Increased intracellular oxidative stress may play a role in the induction of programmed cell death.[17] We recently detected increased mitochondrial ROS production in *H. pylori*-infected gastric epithelial cells. This finding suggests that *H. pylori* infection alters the mitochondria. In addition, the antioxidant *N*-acetyl cysteine inhibited induction of autophagy and induced the accumulation of intracellular CagA.[11] These results suggest that intracellular oxidative stress is involved in the induction of autophagy required for CagA degradation (**Fig. 1**, lower right).[11]

Metronidazole, a major component of the second-line *H. pylori* eradication regimen used in Japan, enters cells by diffusion, and its antimicrobial toxicity depends on the reduction of its nitro group to nitro anion radicals and the subsequent generation of superoxide radicals ($O_2^{\cdot-}$).[18,19] In detail, the NADPH nitroreductase of *H. pylori* reduces the nitro group of metronidazole to anion radicals that induce oxidative stress and produce DNA strand breaks, causing rapid cell death.[18] *H. pylori* expresses only a single superoxide dismutase, the iron-cofactored enzyme SodB, which has 53.5% identity with the *Escherichia coli* protein FeSod.[20] SodB prevents the interaction between iron and superoxide and also catalyzes the dismutation of superoxide ($O_2^{\cdot-}$) into oxygen (O_2) and hydroperoxide (H_2O_2). The mRNA expression of *sodB* in *H. pylori* is directly regulated by the ferric uptake regulator (Fur) protein.[21] We recently found metronidazole-resistant strains of *H. pylori* with amino acid mutations in Fur that significantly reduced its binding affinity for its operator sequence in the *sodB* promoter (Fur-Box), resulting in derepression of *sodB* mRNA expression.[22] In other words, metronidazole resistance is due to enhanced scavenging activity of cytotoxic superoxide ($O_2^{\cdot-}$), a major bactericidal component of metronidazole, because of the derepression of *H. pylori* SodB expression by mutant Fur (**Fig. 1**, left).[22]

Ferrous iron (Fe^{2+}), an essential cofactor for many enzymes and metalloproteins, is necessary for the basal

functions of all cells as well as for SodB activation.[23] We recently demonstrated that the FecA1 protein, a Fe^{3+}-dicitrate transporter homolog, is essential for SodB activation but not for the bioactivity of *H. pylori*.[24] *fecA1* mRNA expression is derepressed in metronidazole-resistant *H. pylori* strains with mutations in Fur.[24] Deletion of *fecA1* dramatically decreased the minimal inhibitory concentrations (MICs) of metronidazole for *H. pylori* strains with Fur mutations,[24] suggesting that the activation of SodB by mutant Fur is supported by the FecA1-dependent Fe^{2+} supply system (**Fig. 1**, upper right).

Notably, *in vitro* study has shown that metronidazole-susceptible *H. pylori* became metronidazole resistant after several passages on agar plates containing sub-inhibitory concentrations of metronidazole.[25] From these findings, it can be readily assumed that metronidazole-susceptible *H. pylori* may become metronidazole resistant through repeated exposure to sub-inhibitory concentrations of metronidazole. Therefore, it is important to understand the initial anti-metronidazole response of metronidazole-susceptible *H. pylori* to prevent the acquisition of resistance and consequent increased incidence of metronidazole-resistant *H. pylori*.

Five families of multidrug efflux transporters have been described in bacteria.[26] One of these five efflux systems, the RND family, has three components, namely, the inner membrane efflux proteins, a periplasmic efflux protein, and an outer membrane efflux protein (the TolC, or TolC homolog protein).[27] Four RND families have been identified in *H. pylori* (HP0605 to HP0607; HefABC and HP0971 to HP0969; HefDEF and HP1327 to HP1329; HefGHI and HP1489 to HP1487), and these proteins are reportedly involved in the development of multidrug resistance.[28] We recently explored the variations in transcription of the RND efflux pump systems in the initial phases of the development of metronidazole resistance *in vitro*. In this study, the MICs of metronidazole for 9 out of 10 metronidazole-susceptible strains cultured on plates containing sub-inhibitory concentrations of metronidazole increased to levels similar to those for metronidazole-resistant strains. In the newly metronidazole-resistant strains, exposure to metronidazole significantly increased the expression of the TolC efflux pump (*hefA*) with no decrease in the metronidazole-reduction activity, suggesting that overexpression of the TolC efflux pump *hefA* may be the first step in the acquisition of metronidazole resistance in *H. pylori*.[29]

H. pylori and gastric cancer – molecular approach

Gastric epithelium changes during the progression from inflammation to cancer, with evidence of disruption of normal epithelial cell differentiation and recruitment of inflammatory cells.[30] Coincident with the development of atrophic gastritis and intestinal metaplasia is the loss of expression of the gastric morphogen sonic hedgehog (Shh).[31,32] Loss of Shh is reportedly an early change in carcinogenesis of the gastric mucosa, prior to neoplastic transformation. We have demonstrated in humans that the suppression of Shh expression in the gastric mucosa by *H. pylori* infection was restored after eradication of the infection and that earlier eradication more fully restored Shh expression in the gastric mucosa.[33,34]

MicroRNAs (miRNAs) are post-transcriptional regulators of gene expression that are involved in development, cell proliferation, and immune responses. Recent studies have shown that some miRNAs act as tumor suppressors or oncogenes in gastric cancer.[35] Some miRNAs, including *miR-146*, *miR-155*, *miR-21*, *miR-27a*, *miR-106–93-25*, the *miR-221–222* clusters, and the *miR-200* family, are possibly involved in *H. pylori* infection and associated gastric cancers.[36] miRNA expression profiling may be a powerful tool for clinical cancer diagnosis, and regulation of miRNA expression could be a novel strategy for the chemoprevention of human gastrointestinal cancers.[37]

H. pylori and gastric mucosa-associated lymphoid tissue lymphoma – molecular approach

H. pylori eradication has become widely accepted as an initial treatment strategy for stage I gastric marginal zone B cell lymphoma of the mucosa-associated lymphoid tissue (MALT) type. *H. pylori*-positive low-grade gastric MALT lymphoma regresses both endoscopically and histopathologically after *H. pylori* eradication in 60%–80% of such cases.[38] The presence of a t(11;18)(q21;q21) translocation appears to be a major predictor of failure to respond. This translocation is associated with an *API2-MALT1* fusion; the former is involved in the regulation of apoptosis, and the latter is a caspase-like protein with an as yet unknown biological function. The fusion causes suppression of apoptosis. Several studies have reported that MALT lymphomas with this translocation respond only rarely or not at all to *H. pylori* eradication.

Methylation of the *p16/INK4a* gene was observed in 60% of MALT lymphomas; however, *p16* gene methylation status did not correlate with the presence of *API2-MALT1* fusion or any other clinicopathological factor, suggesting that aberrant methylation of the *p16* gene might be an early event in MALT lymphomagenesis.[39] Examination of the methylation profiles of eight CpG islands, namely *p15, p16, p73, hMLH1, DAPK, MINT1, MINT2,* and *MINT31,* revealed that more than four genes were methylated in *H. pylori*-dependent MALT lymphomas while fewer than two genes were methylated in non-*H. pylori*-dependent cases, indicating that the pathogenesis of gastric MALT lymphomas, including the aberrant DNA methylation pattern, may differ between *H. pylori*-dependent and non-*H. pylori*-dependent cases.[40] Aberrant DNA methylation thus plays critical roles in the