A COX-2 inhibitor prevents the esophageal inflammation--metaplasia--adenocarcinoma sequence in rats

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Barrett’s esophagus (BE) and esophageal adenocarcinoma (ADC) is associated with reflux of duodenal contents. Cyclooxygenase (COX)-2 is over-expressed in BE and ADC, and supposedly contributes to esophageal carcinogenesis. The aim of this study is to investigate what effect a COX-2 inhibitor has on esophageal adenocarcinogenesis in rats. A series of 90 rats underwent a duodenoesophageal reflux procedure and were divided into 2 groups. One group was given commercial chow (control group), and the other was given experimental chow containing celecoxib (celecoxib group). The animals were sacrificed sequentially, at the 10th, 20th, 30th and, finally, 40th week after surgery, and their esophagi were examined. In the control group, esophagitis, columnar-lined epithelium (CLE) and ADC were first observed at the 10th week, 20th week and 30th week, respectively. Their incidences sequentially increased and at the 40th week reached 100, 89 and 47%, respectively. In the celecoxib group, the esophagitis was mild and the incidence of CLE was significantly lower at each week ($P < 0.001$), compared with the control group, and ADC was not observed throughout the experiment ($P < 0.05$). COX-2 expression was observed predominantly in the stroma of inflamed esophageal epithelia, and up-regulated at the 10th and 20th week ($P < 0.05$, respectively). PGE2 level and proliferative activity were also up-regulated in both groups, but they were lower in the celecoxib group than in the control group ($P < 0.05$). Apoptosis was observed to increase with celecoxib treatment ($P < 0.05$). Celecoxib is effective in preventing CLE and ADC by suppressing esophagitis in rats.

Introduction

Recently the incidence of esophageal adenocarcinoma (ADC) has risen rapidly (1,2). Because the prognosis of esophageal ADC is extremely poor, prevention of this disease is of great importance (3–5). Barrett’s esophagus (BE) is assumed to be the result of chronic esophageal reflux and a precursor lesion of ADC (6–9). Reflux of duodenal contents in addition to gastric juice presumably contributes to the development of these diseases (10–12). Several groups of investigators have reported the influence of duodenal refluxate in humans (13–15) and animal models (16–21). The concentration of bile acids in esophageal refluxate correlate with the degree of esophageal mucosal injury (15). In the case of patients with chronic gastro-esophageal reflux disease some acid suppressors such as proton pump inhibitors control symptoms of reflux esophagitis, but they fail to heal mucosal injury (22,23). We observed that duodeno-esophageal reflux can sequentially induce columnar-lined epithelium (CLE) and ADC in rat models (16,17,21).

Several chemopreventive targets for esophageal carcinogenesis have been registered (24–26), but the precise development-mental mechanisms of these diseases are still unclear. One of the most important epidemiological findings is that intake of aspirin is associated with a decrease in the risk of developing esophageal cancer (27), suggesting that non-steroidal anti-inflammatory drugs may reduce esophageal carcinogenesis by inhibiting cyclooxygenase (COX). COX is a rate-limiting enzyme in arachidonic acid metabolism and two isozymes have been identified, COX-1 and COX-2 (28). COX-1 is considered to be a constitutively expressed housekeeping gene, involved in the homeostasis of various physiological functions (29). COX-2 is responsible for many mitogenic and inflammatory stimuli (30–32) and is also related to neoplastic conditions in several cancers (33–37). Human esophagitis, BE and ADC contain up-regulated COX-2 (38–40). COX-2 plays an important role in some aspects of tumorigenesis, such as the increase in cell proliferation, the reduction in apoptosis and the promotion of angiogenesis (28,41,42). Furthermore, recent reports suggest an antitumor effect of selective inhibitors of COX-2 in esophageal carcinogenesis (43–46).

The aim of this study was to determine the association of COX-2 expression and duodeno-esophageal reflux and the effect of a specific COX-2 inhibitor in the genesis of CLE and esophageal ADC.

Materials and methods

Animals and treatment procedures

Eight-week-old male Fisher 344 rats were purchased from Charles River Japan (Kanagawa, Japan). Each cage contained three animals, which were kept in our animal center for 2 weeks before surgical intervention. Rats were given standard solid chow (CRF-1; Charles River Japan) and water and maintained on a 12 h light/dark cycle. A total of 120 rats underwent operative procedures and an additional 10 rats, which did not undergo operative procedures, were monitored in order to obtain normal esophageal tissue.

After 24 h fasting the rats underwent diethyl ether inhalation anesthesia. A rat surgical model for the esophageal metaplasia–adenocarcinoma sequence induced by duodeno-esophageal reflux, established by Miwa et al. in 1996 (17), was employed. Briefly, total gastrectomy was performed through an upper middle incision. The duodenal stump was closed with sutures and then the esophageal stump was anastomosed to the jejunum ~4 cm distal to the Treitz ligament in an end-to-side fashion. Intestinal anastomosis was carried out with interrupted full-thickness stitches using 7-0 atrumatic braided silk sutures. This procedure allowed the duodenal contents to flow back directly into the esophagus. The rats were given water and commercial chow 24 h after...
surgery. The Institutional Animal Care and Use Committee of the Graduate School of Medical Science, Kanazawa University approved this study.

Study design
Ninety of 120 animals operated on survived longer than 1 week after surgery. These were randomly divided into two groups: the control group (n = 60) were given commercial rat chow (CRF-1); the celecoxib group (n = 30) were given experimental rat chow premixed with celecoxib (500 p.p.m., 100 mg/kg body wt/day). Celecoxib was obtained from Searle Co. (Skokie, IL). An additional 10 rats were monitored without any intervention and given commercial rat chow to obtain normal esophageal tissue. All rats were weighed every 10 weeks throughout the experiment. Randomly selected rats were killed by diethyl ether inhalation in the 10th, 20th, 30th and 40th weeks after surgery. The timing of death was designed according to a previous study (21). Ten animals in the control group and five animals in the celecoxib group were killed every 10 weeks until the 30th week after surgery. All remaining animals were killed in the 40th week.

The dosage of celecoxib was based on a previous tumor suppression study with exogenous carcinogens of rats (47,48). Rats without surgical intervention that were given experimental chow containing celecoxib (1500 or 500 p.p.m.) exhibited no toxic side-effects. However, all rats with duodeno-esophageal reflux given experimental chow containing celecoxib (1500 p.p.m., 300 mg/kg body wt/day) died.

Tissue preparation
The entire esophagus and jejunum of each rat (including the anastomosis) were resected. Finding the threads identified the site of anastomosis. After the specimen was opened longitudinally, two 1 mm wide longitudinal slices of the esophageal mucosa were immediately frozen and stored at −70 °C for analysis. COX-2 mRNA expression and prostaglandin E2 (PGE₂) levels. The remaining samples were fixed in 10% formalin for 24 h and then cut at 2 mm intervals along the longitudinal section. The samples were embedded in paraffin for hematoxylin and eosin staining and immunohistochemistry.

Pathological assessment
Pathological assessment was carried out on 5 μm sections of each block stained with hematoxylin and eosin. The pathological changes due to duodeno-esophageal reflux were defined as: regenerative thickening (RT), epithelial thickening to more than twice that of normal epithelium, with anacanthosis and elongation of the papillae and parakeratosis; basal cell hyperplasia (BCH), basal cell layer thickening of >15% of the epithelium, stratified structure of the epithelium kept; erosion, lack of epithelium with cellular infiltration; CLE, the presence of columnar metaplasia with incomplete intestinal metaplasia in esophageal squamous epithelium; ADC, epithelial growth with atypical cells and structure and invasion of the submucosal layer.

COX-2 mRNA expression and PGE₂ production
COX-2 mRNA expression and PGE₂ production were measured in esophageal mucosa from five randomly selected rats from each group (the control group and the celecoxib group), killed in the 10th, 20th, 30th and 40th weeks after undergoing the operative procedure, and normal esophageal tissue obtained from rats which had not undergone the operative procedure and were killed in the 40th week. Total RNA was extracted from the tissue using Isogen Solution (Nippon Gene Co. Ltd, Tokyo, Japan). After heat denaturation at 68 °C for 15 min with 500 pmol oligo(dT) primer, 10 μg RNA was reverse transcribed at 37 °C for 60 min into first-strand cDNA in reverse transcription solution (400 U Moloney murine leukemia virus reverse transcriptase (Invitrogen Japan, Tokyo, Japan), 50 mM Tris–HCl, pH 8.3, 7.5 mM KCl, 3 mM MgCl₂, 0.01 M DTT, 0.5 mM each dNTP and 16 U RNasin (Promega, Madison, WI) with a total volume of 100 μl. The reverse transcribed cDNA solution corresponding to 100 ng total RNA was quantitatively amplified with primers and a probe specific for the targeted cDNA using an ABI Prism 7700 Sequence Detector (TaqMan). PCR was carried out using the following thermocycler conditions: 50 °C for 2 min and 95 °C for 5 min, followed by 45 cycles of 95 °C for 15 s and 60 °C for 1 min. Data were analyzed using Sequence Detection Software (PE Biosystems Japan). The specific COX-2 primer and probe sequences were TGGATGGAAGACTACTGTCACAACA (forward primer), 6-FAM-5'-CCATGATCGAAGCCGATTGCTTGAAT-3'-TAMRA (probe) and GCCGATGCGGTAGTATAGTG (reverse primer).

Each sample was weighed and then homogenized in 0.5 ml of buffer (0.1 M phosphate plus 1 mM EDTA plus 10 μg indomethacin). Then 0.5 ml of ethanol was added and vortexed. After leaving at room temperature for 5 min, we removed the precipitate by centrifugation at 1500 r.p.m. for 10 min at 4 °C. The supernatant was diluted with 2 ml of ace buffer and purified with an SPE cartridge (Cayman Chemicals, Ann Arbor, MI). The samples were assayed with an EIA kit (Cayman Chemicals) according to the manufacturer’s instructions. PGE₂ levels were expressed as ng PGE₂/g tissue.

Immunohistochemistry and the TUNEL method
Localization of COX-2 protein was determined by immunohistochemical staining using specific antibodies. The Envision System (Dako, Glostrup, Denmark) was used with autoclaw acceleration. Deparaffinized 5 μm sections of a formalin fixed, paraffin embedded block were immersed in absolute methanol containing 0.3% hydrogen peroxide, then covered with normal goat serum (1:30). Sections were incubated overnight at 4 °C with primary antibody to rat COX-2 protein (diluted 1:100) (Transduction Laboratory, Lexington, KY). The sections were treated with labeled polymer (Dako) for 2 h. Immersing the sections in 3,3′-diaminobenzil tetrahydrochloride developed the reaction products. The slides were counterstained lightly with hematoxylin.

To determine the proliferative activity of esophageal epithelia, immunohistochemical staining for Ki-67 protein was performed. Monoclonal mouse anti-rat Ki-67 antigen (Dako) was used as the primary antibody with the immunohistochemical staining procedure as above. Blocks from five randomly selected rats from each group were stained. The specimens were qualitatively evaluated as proliferative activity under 400 × microscopic magnification. Ki-67-labeled cells were counted per 1000 epithelial basal cells (Ki-67 labeling index) in the esophageal epithelium ~5 mm oral of the anastomosis.

Further, apoptosis was evaluated in the same blocks using the TUNEL method (Apoptosis In Situ Detection Kit; Wako, Osaka, Japan) according to the manufacturer’s instructions. Apoptotic cells were expressed as the number of apoptotic cells per 1000 epithelial cells in the esophageal epithelium ~5 mm oral of the anastomosis. These results are used as an apoptotic index.

Statistical analysis
Fisher’s exact test was used for statistical analysis of the incidence of pathological findings. COX-2 expression, PGE₂ production, proliferative activity and apoptotic index were expressed as the mean value ± 1 SD. Comparisons between groups were made using the Mann-Whitney U-test. Data management and statistical analysis were performed using StatView software (SAS Co., Berkeley, CA). Differences were considered as significant when the P value was <0.05.

Results
General observations
Seventy-two of 90 rats were used for this study. The effective numbers of rats examined in the control and celecoxib groups were as follows: 10 and 5 in weeks 10, 20 and 30; 19 and 8 in week 40 after surgery (Table II). A total of 18 rats (11 in the control group and 7 in the celecoxib group) died of complications, such as malnutrition, pneumonia and unknown causes. No significant differences in mortality were noted between the two groups. The 18 rats which were lost were excluded from the assessment because specimens were taken too late for investigation. Body weight did not significantly differ between the groups at 0, 10, 20, 30 and 40 weeks, as shown in Table I.

Histopathological findings
In the control group the distal portions of the esophagi were macroscopically thickened and irregular. In some parts of the rough epithelia small nodular elevations were observed (Figure 1A). Severe squamous esophagitis, such as erosion, Table I. Change in body weight

<table>
<thead>
<tr>
<th>Group</th>
<th>Weight (g)</th>
<th>Initial</th>
<th>Final</th>
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<tbody>
<tr>
<td>Control</td>
<td>190 ± 12</td>
<td>197 ± 32</td>
<td></td>
</tr>
<tr>
<td>Celecoxib</td>
<td>191 ± 5</td>
<td>206 ± 31</td>
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Body weights at the initial and final points of the experiment were not significantly different between the two groups.
RT and BCH of the esophageal epithelia were observed in distal portions of the esophagi from an early phase after surgery (Figure 2A). CLE developed in distal portions of the esophagi, i.e. the squamous epithelium was replaced by columnar cell lined epithelium comprising absorptive cells with brush borders, along with incomplete intestinal metaplasia (specialized type). CLE was observed from the 10th week (1/10) and sequentially increased to 89% (17/19) in the 40th week (Table II). Dysplastic changes developed in CLE and ADC was observed from the 30th week (10%) and sequentially increased to 47% in the 40th week (Table II). In the celecoxib group the distal portions of the esophagi in all rats were smooth and without thickening (Figure 1B). Squamous esophagitis was very mild compared with the control group (Figure 2B). Pathological changes indicating accelerated cell proliferation, such as RT and BCH, were significantly suppressed through the experiment \((P < 0.05)\). The incidence of CLE in the celecoxib group was lower than in the control group in the 10th, 20th and 30th weeks and statistically lower in the 40th week \((P < 0.005)\). Moreover, no ADC was observed in the celecoxib group throughout this experiment \((P < 0.05)\), as shown in Table II.

**Immunohistochemistry of COX-2**

Every animal that suffered from reflux demonstrated COX-2 protein expression in the lower esophagus. COX-2 immunoreactivity was mainly observed in infiltrating cells and fibroblasts in the stroma. There were few squamous epithelial cells positive for COX-2 protein, but some epithelial cells of CLE and ADC strongly expressed COX-2 protein (Figure 3). COX-2 protein immunoreactivity was observed in both groups at all times.

**COX-2 mRNA expression and PGE₂ induction**

COX-2 mRNA expression was greater in the esophagi of rats with duodeno-esophageal reflux as compared with the results in normal esophagi of those rats without surgical intervention \((P < 0.005)\). COX-2 mRNA expression in the control group markedly increased in the 10th and 20th weeks and sequentially decreased in the 30th and 40th weeks. COX-2 mRNA expression in the celecoxib group increased as in the control group. COX-2 mRNA expression was not affected by celecoxib (Figure 4).

\[\text{PGE}_2\] level increased in the esophagi of rats with duodeno-esophageal reflux compared with normal esophageal tissue.
PGE2 level peaked in the 10th week and sequentially decreased in the 20th, 30th and 40th weeks. In the celecoxib group PGE2 induction was significantly suppressed at all times compared with the control group \((P < 0.05)\) (Figure 5). COX-2 and PGE2 levels did not differ in rats that had and did not have cancer.

**Proliferative activity and apoptosis**

Ki-67 immunoreactivity was observed in basal cells of the esophageal epithelia. Ki-67 labeling index was higher in esophageal epithelia with duodeno-esophageal reflux than in normal epithelia \((P < 0.01)\). The proliferative activity increased in the 10th and 20th weeks and decreased sequentially in the 30th and 40th weeks, similarly to COX-2 mRNA expression and PGE2 induction. Celecoxib significantly suppressed the Ki-67 labeling index in the 10th, 20th, 30th and 40th weeks \((P < 0.05)\) (Figure 6).

Apoptosis calculated by the TUNEL method increased in the esophageal epithelia of rats with duodeno-esophageal reflux compared with normal esophageal epithelia \((P < 0.05)\). Celecoxib significantly increased the apoptotic index in the 10th, 20th, 30th and 40th weeks \((P < 0.05)\), as shown in Figure 7.

The Ki-67 labeling index and apoptotic index of the columnar epithelium could not be estimated, because parts of columnar epithelium were fragile and easily dispersed through repeated dipping during the immunostaining process.

**Discussion**

The present study demonstrated that reflux of duodenal contents induces esophagitis and subsequently causes CLE and ADC in rats. In this sequence COX-2 was upregulated, accompanied by PGE2 production, and cell proliferation and apoptosis were accelerated in the esophageal...
epithelium. A specific COX-2 inhibitor, celecoxib, suppressed the inflammatory process and prevented the development of CLE and ADC.

The role of COX-2 in carcinogenesis has been investigated in various carcinomas (33–38). With regard to esophageal carcinogenesis, increased COX-2 expression in BE and ADC has been reported (37,38). Zimmermann et al. observed that COX-2 expression was revealed immunohistochemically in 91% of 172 squamous cell carcinomas and in 78% of 27 adenocarcinomas and suggested that COX-2-derived prostaglandins might play an important role in the regulation of proliferation and apoptosis of esophageal tumor cells (37). Zhang et al. observed that reflux of duodenal contents into the esophagus led to increased COX-2 expression in cultured esophageal cells and tissue (38). COX-2 and eicosanoids, particularly PGE2, are known to promote tumorigenesis through mechanisms including immune suppression, cell proliferation, anti-apoptosis and proangiogenesis (42,43). In the present experimental study COX-2 expression was significantly increased and inhibition of COX-2 suppressed the degree of reflux esophagitis and the development of CLE and ADC. This fact suggests that the increased expression of COX-2 plays a pivotal role in promoting esophageal adenocarcinogenesis in rats.

There have been three papers regarding a preventing effect of selective COX-2 inhibitors on the development of experimental esophageal carcinoma (44–46). Li et al. reported that esophageal tumorigenesis induced by N-nitrosomethylbenzylamine was prevented by administration of a selective COX-2 inhibitor, JTE-522, to rats (44). Chen et al. described how a COX-2 inhibitor, sulindac, had a chemopreventive effect on esophageal adenocarcinogenesis induced by esophagogastrroduodenal anastomosis in rats (46). Buttar et al. examined a preventive effect against esophageal ADC of MF-tricyclic and sulindac in rats with esophageal reflux (45). In that report, MF-tricyclic and sulindac reduced the relative risk of development of ADC by 55 and by 79%, respectively, compared with the control. Moderate to severe esophagitis was more common in the control, compared with the MF-tricyclic and sulindac groups, and the prevalence of BE was not different among the three groups.

The question as to whether a COX-2 inhibitor may inhibit the growth of columnar metaplasia has been raised. Buttar et al.

did not observe any suppressive action of COX-2 inhibitors on the development of CLE. On the other hand, our sequential observations make it clear that CLE was observed even 10 weeks after surgery and its incidence increased with the passage of time in the control group while it was unchanged in the celecoxib group. This could be interpreted as a suppressive action of PGE2 induced by celecoxib.

Our recent work has demonstrated that anti-reflux surgery does not lead to regression of CLE but prevents the development of esophageal adenocarcinoma in the rat model (51). This implies that CLE is an early stage in carcinogenesis and is an irreversible pathological change, and that persistent inflammation may promote the process from CLE to ADC. Earlier administration of a COX-2 inhibitor would possibly be more effective in preventing this carcinogenesis.

In conclusion, duodeno-esophageal reflux induces an inflammation-metaplasia-adenocarcinoma sequence in rats. Progression was associated with expression of COX-2, overproduction of PGE2 and increased cell proliferation. A specific COX-2 inhibitor, celecoxib, reduced PGE2 production and inhibited carcinogenesis throughout the entire course.

References


Fig. 7. Apoptosis in the reflux model and the effect of celecoxib. The apoptotic index increased in esophageal epithelium with duodeno-esophageal reflux compared with normal esophageal epithelia (\( P < 0.05 \)). Celecoxib significantly increased apoptotic index (\( P < 0.05 \)) (Mann–Whitney U-test).

A COX-2 inhibitor prevents adenocarcinoma

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Received March 13, 2004; revised November 11, 2004; accepted November 14, 2004