Ulcerogenic Influence of Selective Cyclooxygenase-2 Inhibitors in the Rat Stomach with Adjuvant-Induced Arthritis

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ABSTRACT

Cyclooxygenase (COX)-2 inhibitors have been developed as new gastric sparing anti-inflammatory drugs. We previously reported that the ulcerogenic response to conventional nonselective COX inhibitors, such as indomethacin and aspirin, was markedly increased in arthritic rats. The ulcerogenic effect of selective COX-2 inhibitors in arthritic animals, however, remains unknown. The present study was designed to examine the influence of selective COX-2 inhibitors, such as rofecoxib and celecoxib, on gastric mucosal integrity in rats with adjuvant-induced arthritis. Arthritis was induced in male dark Agouti rats by injection of Freund’s complete adjuvant into the right hind paw. Two weeks after the injection, the animals were fasted for 18 h, various COX inhibitors were administered orally, and the mucosa was examined for lesions 4 h later. Oral administration of indomethacin caused hemorrhagic gastric lesions in both normal and arthritic rats, although the severity of lesions was significantly greater in the latter group. In contrast, neither rofecoxib nor celecoxib caused any gastric damage in normal rats, but both drugs provoked hemorrhagic gastric lesions in arthritic rats. The expression of COX-2 mRNA and immuno-positive cells was observed in the gastric mucosa of arthritic but not normal rats. The gastric mucosal prostaglandin (PG) E2 content was significantly elevated in arthritic rats in a rofecoxib-sensitive manner. In conclusion, COX-2 inhibitors produce gastric lesions in arthritic rats, similar to the nonselective COX-inhibitors. COX-2 is up-regulated in the stomach of arthritic rats, and PGs produced by COX-2 play a role in maintaining the integrity of the gastric mucosa.

Gastroenteropathy is the most common side effect among patients taking nonsteroidal anti-inflammatory drugs (NSAIDs) for inflammatory disorders, especially rheumatoid arthritis. The pathogenesis of NSAID-induced gastric lesions is generally considered to involve the depletion of endogenous prostaglandins (PGs) caused by inhibition of cyclooxygenase (COX) activity (Vane, 1971; Whittle, 1981). There are two isoforms of COX; COX-1 is a constitutive enzyme and is expressed in various tissues, including the stomach, whereas COX-2 is an inducible enzyme, expressed in few tissues under normal conditions, but rapidly up-regulated in response to various cytokines and growth factors (Feng et al., 1993; Kargman et al., 1993; O’Neill and Fold-Hutchinson, 1993). It is, therefore, assumed that COX-1 is a housekeeping enzyme that maintains gastric mucosal integrity under physiological conditions, whereas COX-2 is responsible for inflammation (Xie et al., 1992; Seibert et al., 1994; Langenbach et al., 1995). Recently, several selective COX-2 inhibitors have been developed as new gastric sparing anti-inflammatory drugs.

We previously reported that the gastric lesions caused by conventional NSAIDs such as indomethacin and aspirin were markedly aggravated in rats with experimentally induced arthritis and suggested an increase in the susceptibility of the gastric mucosa to NSAIDs in arthritic animals (Kato et al., 1999, 2001a,b). The gastric ulcerogenic effect of selective COX-2 inhibitors in arthritic animals remains unknown, however.

In this study, therefore, we investigated the influence of selective COX-2 inhibitors, such as rofecoxib and celecoxib, on the integrity of gastric mucosa in rats with adjuvant-induced arthritis and compared the effects with those of indomethacin, a conventional NSAID.

Materials and Methods

Animals. Male dark Agouti rats (140–160 g; SLC, Shizuoka, Japan) were used. The animals were fed standard rat chow and tap water ad libitum. All experimental procedures described were ap-
proved by the Experimental Animal Research Committee of Kyoto Pharmaceutical University.

**Induction of Arthritis.** Arthritis was induced by injection of 50 μl of Freund’s complete adjuvant (FCA; 10 mg/ml of heat-killed *Mycobacterium tuberculosis* H37Ra suspended in paraffin oil) into the plantar region of the right hindfoot. Normal rats were housed in the same manner for the same period of time so that aged and batch-matched normal and arthritic rats were used in all of the experiments. The severity of arthritis was assessed by measuring the paw volume (edema) by plethysmometry. Since the paw edema in the left (uninjected) hindfoot was observed from 10 days and reached a maximum 14 days after the injection of FCA, we used the animals of 14 days after the injection in all ulcerogenic experiments as arthritic rats. The animals with or without arthritis were deprived of food but allowed free access to tap water for 18 h before the experiments. In some case, we examined the anti-inflammatory effects of rofecoxib and indomethacin in adjuvant-induced arthritic rats. Chronic and systemic inflammation was evaluated by the development of left (uninjected) paw edema 10 days after FCA injection. Rofecoxib (3–30 mg/kg) and indomethacin (3 mg/kg) were administered orally once daily for 3 days, starting from day 7 following FCA injection.

**Evaluation of Gastric Mucosal Lesions.** The animals were orally administered indomethacin (3, 10, and 30 mg/kg), rofecoxib (3, 10, 30, and 100 mg/kg), or celecoxib (100 mg/kg) and were killed under deep ether anesthesia 4 h later. Then, the stomachs were removed, inflated by injecting 7 ml of 2% formalin, immersed in 2% formalin for 10 min to fix the gastric wall, and opened along the greater curvature. The area (square millimeters) of each lesion that developed in the granular mucosa was measured under a dissecting microscope with a square grid (10×), summed per stomach, and used as a lesion score. The person measuring the lesion did not know the treatment given to the animal.

**Measurement of PGE2 in the Gastric Mucosa.** The animals were killed by deep ether anesthesia 2 h after oral administration of indomethacin (10 mg/kg) or rofecoxib (3 and 10 mg/kg) on day 14 following FCA injection. The corpus mucosa was isolated, weighed, and put in a tube containing 100% ethanol plus 100 μM indomethacin to prevent further synthesis of PGE2. This concentration of indomethacin did not interfere with the measurement of PGE2 by enzyme immunoassay (Fukutake et al., 1994). Then, samples were homogenized with a polytron homogenizer (IKA, Tokyo, Japan) and centrifuged at 12,000 rpm for 10 min at 4°C. After the supernatant of each sample had been evaporated with nitrogen gas, the residue was resolved in assay buffer solution and used for determination of PGE2. The concentration of PGE2 was measured using an enzyme immunoassay (Amersham Biosciences, Little Chalfont, Buckinghamshire, UK).

**Analyses of COX-1 and COX-2 mRNA by Reverse Transcription-PCR.** The animals were killed by deep ether anesthesia on day 14 following FCA injection, and the gastric mucosa was removed, frozen in liquid nitrogen, and stored at −80°C until use. The total RNA of each sample was extracted using Sepharose RNA-I (Nacalai Tesque, Kyoto, Japan). Total RNA primed by random hexadeoxyribonucleotide was reverse-transcribed with RevertAse Ace-alpha (TOYOBO, Osaka, Japan). The sequences of sense and antisense primers for the rat COX-1 were 5′-AACCGTTGTTGTAGTCTTGGT-CAA-3′ and 5′-AGAGAAGGCCCCCTCAG-AGCTCGTG-3′, respectively, giving an 887-bp PCR product (Feng et al., 1993). For the rat COX-2, the sequences of sense and antisense primers were 5′-TGATGA-CTGCCCAACTCCCTAG-3′ and 5′-AAATGTGAAAGTGTCCGGACGC-3′, respectively, giving a 702-bp PCR product (Feng et al., 1993). For the rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH), a constitutively expressed gene, the sequences were 5′-GAACGGGAAGCTCACTGGCATGGC-3′ and 5′-AATGTTGAGCTCAGGGATGCCAGG-3′, respectively, giving a 310-bp PCR product (Iso et al., 1995). An aliquot of the reverse transcription reaction product served as a template for PCR. The reaction profile for COX-1 was 35 cycles of denaturation for 30 s at 96°C and annealing for 2 s at 65°C; for COX-2, 35 cycles of denaturation for 30 s at 94°C and annealing for 1 s at 62.5°C; for GAPDH, 25 cycles of denaturation for 30 s at 94°C, annealing for 1 s at 65°C, and extension for 30 s at 74°C on a thermal cycler (TP-240; TAKARA, Shiga, Japan) using KOD dash polymerase (TOYOBO). A portion of the PCR mixture was electrophoresed in 2% agarose gel in Tris-acetic acid-EDTA buffer (40 mM Tris buffer, 2 mM EDTA, and 20 mM acetic acid; pH 8.1), and the gel was stained with ethidium bromide and photographed.

**Immunostaining of COX-1 and COX-2.** Immunostaining of COX-1 isoforms in the gastric mucosa was performed 14 days after FCA injection. The stomach was removed and washed in phosphate-buffered saline (PBS). Stomachs were fixed in 4% paraformaldehyde for 6 h and washed in PBS containing 10, 15, and then 20% sucrose, in that order. The specimens were embedded in octamethyl transcrip- 

**Preparation of Drugs.** Drugs used were heat-killed *Mycobacterium tuberculosis* (H37Ra: Difco, Detroit, MI), paraffin oil (Wako, Osaka, Japan), indomethacin, Evans blue (Sigma-Aldrich, St. Louis, MO), rofecoxib, and celecoxib (synthesized in our department). All COX inhibitors were suspended in hydroxy-propyl-cellulose (NIPPON SODA, Tokyo, Japan) solution. Evans blue was dissolved in saline. All drugs were prepared immediately before use and administered p.o. in a volume of 0.5 ml/100 g b.wt. or i.v. in a volume of 0.1 ml/100 g b.wt.

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Statistical Analysis. Data are presented as the mean ± S.E. for four to six rats per group. Statistical analyses were performed using a two-tailed unpaired t test and Dunnett’s multiple comparison test, and values of P < 0.05 were considered to be significant.

Results

Gastric Ulcerogenic Response to Indomethacin, Rofecoxib, and Celecoxib. Oral administration of indomethacin (3, 10, and 30 mg/kg) caused hemorrhagic lesions in the gastric mucosa of normal rats in a dose-dependent manner, the lesion score at the doses of 10 and 30 mg/kg being 2.2 ± 0.9 and 9.8 ± 1.6 mm², respectively (Fig. 1). The gastric ulcerogenic response to indomethacin was markedly aggravated in arthritic rats, and severe lesions were observed even at the dose of 3 mg/kg (Fig. 2). On the other hand, neither rofecoxib (3, 10, 30, and 100 mg/kg) nor celecoxib (100 mg/kg) induced any damage in the stomach of normal rats even at the highest dose (100 mg/kg). In arthritic rats, however, these drugs also provoked gastric lesions at the dose of 10 mg/kg or greater, similar to indomethacin, and for rofecoxib the severity of the lesions increased dose dependently, the lesion score being 1.0 ± 0.71, 13.0 ± 4.0, 25.0 ± 5.1, and 51.8 ± 9.4 mm², respectively.

Effects of Rofecoxib and Indomethacin on Gastric Mucosal PGE2 Content. The mucosal PGE2 content in normal rat stomachs was 57.9 ± 7.0 pg/g tissue. In arthritic rats, the PGE2 content was 128.6 ± 11.1 pg/g tissue, significantly higher than in normal rats (Fig. 3). Oral administration of indomethacin (10 mg/kg) almost totally reduced the mucosal PGE2 content in both normal and arthritic rats, the inhibition being 99.9 and 99.7%, respectively. In contrast, rofecoxib (3 and 10 mg/kg) did not affect the PGE2 content in normal rats (49.2 ± 5.5 and 63.6 ± 12.7 pg/g tissue, respectively) but significantly decreased the PGE2 content in arthritic rats toward the level observed in normal rats (80.2 ± 12.2 and 58.5 ± 9.3 pg/g tissue, respectively).

Expression of COX-1 and COX-2 in the Stomach of Normal and Arthritic Rats. The expression of COX-2 mRNA was evident in the stomach of arthritic rats on day 14 following the FCA injection, although the gene expression of COX-2 was not detected in the gastric mucosa of normal rats; the relative density of COX-2/GAPDH in normal and arthritic rats were 0.02 ± 0.01 and 0.53 ± 0.08, respectively, and there were significant differences between these rats.

Changes in Microvascular Permeability in the Stomach. In normal rats, the amount of extravasated dye in the gastric mucosa after 30 min was 48.3 ± 4.3 μg/g tissue (Fig. 6). This value increased significantly to 70.3 ± 8.9 μg/g tissue
The injection of FCA into the plantar region of the hindfoot 10 days later, the increase in paw volume being 0.86 ± 0.07 ml (Table 1). Daily oral administration of rofecoxib (3, 10, and 30 mg/kg) for 3 days, starting from day 7 following FCA injection, significantly prevented the development of paw edema in a dose-dependent manner, the inhibition being 52.0, 72.1, and 87.4%, respectively. Likewise, indomethacin (3 mg/kg) almost totally prevented the paw edema observed after FCA injection (89.8%). On the other hand, the gain of body weight was markedly attenuated by induction of arthritis, the body weight in normal and arthritic rats on day 10 following FCA injection being 160.0 ± 3.8 and 128.2 ± 4.5 g, respectively. Daily administration of rofecoxib (3–30 mg/kg) for 3 days significantly prevented the loss of body weight, the value being 137.0 ± 2.4, 147.0 ± 1.7, and 152.0 ± 2.9 g, respectively. Likewise, indomethacin also prevented the decrease in body weight, the value being 149.8 ± 4.7 g.

**Discussion**

Selective COX-2 inhibitors have been used safely in patients without causing gastric damage (Hawkey, 1999; Langman et al., 1999; Goldstein et al., 2000). In the present study, we observed that COX-2 inhibitors, such as rofecoxib and celecoxib, induced gastric mucosal lesions in rats with adjuvant-induced arthritis, similar to conventional NSAIDs such as indomethacin, although they did not cause any damage in normal rat stomachs, unlike indomethacin. We also found that in arthritic rat stomachs, the generation of PGs was significantly enhanced with a marked expression of COX-2. These findings suggest that PGs produced by COX-2 play a part in maintaining the integrity of the gastric mucosa in arthritic rats.

Conventional NSAIDs that inhibit nonselectively both COX-1 and COX-2 activities cause gastrointestinal damage in experimental animals and humans (Vane, 1971; Katayama et al., 1978; Whittle, 1981). COX-1 is constitutively expressed in the gastrointestinal tract and maintains mucosal integrity through the continuous generation of PGs, whereas COX-2 is induced predominantly in certain inflammatory cells by various cytokines, endotoxins, tumor promoters, and growth factors, as well as in response to tissue injury (Feng et al., 1993; Kargman et al., 1993; O’Neill and Fold-Hutchinson, 1993). It is believed that inhibition of COX-1 activity is critical in the ulcerogenic response to NSAIDs, whereas COX-2 mediates the synthesis of PGs responsible for the inflammatory response (Xie et al., 1992; Seibert et al., 1994; Langenbach et al., 1995). It has been demonstrated that new anti-inflammatory drugs with a several hundred-fold higher selectivity for COX-2 have minimal gastric toxicity in animals and humans (Futaki et al., 1993; Boyce et al., 1994; Hawkey, 1999; Langman et al., 1999; Warner et al., 1999; Goldstein et al., 2000; Silverstein et al., 2000). Thus, selective COX-2 inhibitors are believed to be clinically useful as safe new anti-inflammatory drugs.

We recently reported that gastric mucosal lesions induced by NSAIDs, such as aspirin and indomethacin, were significantly aggravated in experimentally induced arthritic animals when compared with normal animals (Kato et al., 1999, 2001a,b). The increased ulcerogenic response to indomethacin depended on the degree of arthritic change, with maximal aggravation observed 14 days after the injection of FCA. In addition, we also reported that healing of chronic gastric ulcers was significantly impaired in arthritic animals (Kato et al., 2001a,b). These findings together suggest that systemic chronic inflammation during adjuvant-induced arthritis has certain deleterious influences on the ulcerogenic and healing responses in the stomach. Although mere speculation, arthritic conditions may modify the gastric ulcerogenic response to selective COX-2 inhibitors. In the present study, we thus investigated whether COX-2 inhibitors, such as rofecoxib and celecoxib, have any ulcerogenic effect in the stomachs of arthritic rats. Consistent with the findings of others, these COX-2 inhibitors did not induce any gastric damage in normal rats. We found, however, that they produced severe hemorrhagic lesions in the stomach of arthritic rats, similar to indomethacin, a conventional NSAID. These results suggest that PGs produced by COX-2 plays a role in maintaining the integrity of the gastric mucosa in arthritic rats.

In the present study, we also observed that the 
PGE$_2$ content of the stomach was significantly higher in arthritic rats than normal rats. Certainly, indomethacin reduced the mucosal 
PGE$_2$ content in both normal and arthritic rats. In contrast, rofecoxib, a COX-2 inhibitor, did not affect gastric 
PGE$_2$ content in normal rats but significantly decreased the 
PGE$_2$ content observed in arthritic rats to the level of normal rats. Furthermore, we confirmed the apparent expression of
COX-2 in the stomach of arthritic rats but not in normal rats. These results strongly suggest that the increase in PG production in arthritic rat stomachs is brought about by COX-2 activity. Indeed, immunohistochemical analysis showed that COX-1 was expressed in all gastric mucosa of both normal and arthritic rats, with especially strong staining being observed in mucous neck cells and epithelial cells, whereas COX-2 immuno-positive cells were seen in the gastric mucosa of arthritic rats but not normal rats. Interestingly, we also observed that the number of monocytes/macrophages was increased in arthritic rats. When performing double immunostaining for COX-2 and monocytes/macrophages, we found that COX-2-positive cells mostly consisted of monocytes/macrophages. Therefore, it is assumed that COX-2 is expressed mainly in the macrophages in the gastric mucosa of arthritic rats.

It remains unclear, however, how the macrophages accumulate in the gastric mucosa of arthritic rats and how COX-2 is up-regulated in these cells. In the present study, we observed that the microvascular permeability in the rat stomach was increased in arthritic rats compared with normal rats when determined as the amount of extravasated dye. Because it is known that the amount of extravasated dye increases in tissue with inflammation or damage including vascular injury (Menkin and Menkin, 1930; Szabo et al., 1985), it is possible to speculate that the stomach of arthritic rats is in such a condition. If so, it would be understandable in arthritic rats for the macrophages to migrate into the gastric mucosa and COX-2 to be expressed in these cells. Indeed, we observed in the immunohistochemical study that the surface of the gastric mucosal epithelium was rough in arthritic rats compared with normal rats. It is likely, therefore, that some injury might be existed in the stomach of the arthritic rats. Adjuvant arthritis is often used for animal models of rheumatoid arthritis, and these arthritic animals are known to suffer from chronic systemic inflammation and severe pain. It is possible that the increases in COX-2 expression and macrophage number in the stomach occur in association with inflammation or stress caused by pain and that the higher amount of PGE2 produced by COX-2 plays a role.

Fig. 5. Immunohistochemical study for COX-1, COX-2, and monocytes/macrophages in the stomach of normal and arthritic rats. A, COX-1 in a normal rat; B, COX-1 in an arthritic rat; C, COX-2 in a normal rat; D, COX-2 in an arthritic rat; E, monocytes/macrophages in a normal rat; F, monocytes/macrophages in an arthritic rat; G, double staining for COX-2 and monocytes/macrophages in an arthritic rat (400×).
in maintaining the mucosal integrity or inhibiting the progression of mild injury in arthritic rat stomachs. Certainly, further study is required to verify such a concept.

The most interesting finding of this study is that selective COX-2 inhibitors produced gastric mucosal lesions in rats with arthritis. Takahashi et al. (2000) reported that COX-2 inhibitors caused gastric lesions in Helicobacter pylori-infected animals in the stomachs, of which COX-2 was expressed. We also reported that NS-398, a selective COX-2 selective inhibitor, attenuated the adaptive gastric protection induced by a mild irritant, especially when COX-2 expression was observed in the stomach (Yamamoto et al., 1999). Based on these findings, it can be speculated that selective inhibitors of COX-2 have deleterious influences on the stomach when a significant overexpression of COX-2 occurs under various conditions, including H. pylori infection, severe arthritis and stressful stimulation.

The mechanism by which NSAIDs cause gastric damage still remains unresolved. In general, there is some discrepancy between PGE₂ levels and ulcerogenicity induced by NSAIDs. Indeed, we have previously reported that indomethacin at the dose of 5 mg/kg almost totally inhibited PGE₂ production in the stomach but induced very few gastric damage in rats (Takeuchi et al., 1986). It is assumed that other mechanisms, in addition to PG deficiency, are involved in the gastric ulcerogenicity of NSAIDs. In the present study, rofecoxib at 10 mg/kg totally prevented the increase in PGE₂ contents and produced gross lesions in arthritic rat stomachs while this agent at 3 mg/kg induced only slight damage in the stomach, despite significantly attenuating the increase in PGE₂ contents. Thus, it is likely that the gastric PGE₂ level is not necessarily correlated with the ulcerogenicity of COX-2 inhibitors in arthritic rats, suggesting the involvement of factors other than PG deficiency in this phenomenon.

Certainly, we confirmed in this study the anti-ulcer effects of rofecoxib and indomethacin on adjuvant-induced arthritis in rats. Daily administration of rofecoxib for 3 days dose dependently prevented the development of paw edema at 10 days after FCA injection, and a significant effect was observed even at 3 mg/kg. These results suggest that the doses of rofecoxib used for evaluation of the ulcerogenic effect are within the effective dose range for inflammation in this animal model. It is still unclear at present, however, whether or not COX-2 inhibitors really cause gastric damage in patients with rheumatoid arthritis. Further study should certainly be done on this point in future.

In summary, COX-2 inhibitors, such as rofecoxib and celecoxib, cause gastric mucosal lesions in arthritic rats, similar to conventional NSAIDs such as indomethacin that inhibit nonselectively both COX-1 and COX-2. Thus, it is assumed that PGs derived from COX-2 play an important role in maintaining the integrity of the gastric mucosa in arthritic rats. Finally, when using selective COX-2 inhibitors to treat the patients with rheumatoid arthritis, one should pay attention to the severity and pathogenesis of the disease to prevent side effects.

Reference


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