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Role of cyclooxygenase-2, but not cyclooxygenase-1, on type II collagen-induced arthritis in DBA/1J mice

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Abstract

The purpose of this paper is to explore the contribution of isoforms of cyclooxygenase (COX) to chronic inflammation in DBA/1J mice with type II collagen-induced arthritis (CIA). To address this question pharmacologically, we tested the effects of selective inhibitors of COX-1 and COX-2 on paw edema and the formation of arachidonic acid metabolites in the inflamed paws immunized with type II collagen (CII). Oral administration of FR140423 (3-(difluoromethyl)-1-(4-methoxyphenyl)-5-[4-(methylsulfinyl)phenyl]pyrazole), a selective inhibitor of COX-2, showed a dose-dependent anti-inflammatory effect in mouse CIA with ED_{50} value of 0.20 mg/kg. Indomethacin, a non-selective inhibitor of COX, also inhibited paw edema in this arthritic model. In contrast, the selective COX-1 inhibitors, FR122047 (1-[(4,5-bis(4-methoxyphenyl)-2-thiazoyl)carbonyl]-4-methylpiperazine hydrochloride) and SC-560 (5-(4-chlorophenyl)-1-(4-methoxyphenyl)-3-trifluoromethylpyrazole), had no effect in mouse CIA model. The increase of prostaglandin (PG) E_2 and thromboxane (TX) B_2 in the mouse inflamed paws was associated with the development of paw edema induced by CII. FR140423 dose dependently inhibited the levels of PGE_2 and TXB_2 in the CIA mouse paws with ED_{50} values of 0.20 and 0.12 mg/kg, respectively, similar to indomethacin. In contrast, FR122047 and SC-560 had no effect. These results suggest that COX-2, but not COX-1, contributes to the edema and the formation of PGE_2 and TXB_2 in mouse CIA model.

Keywords: FR140423; Cyclooxygenase-2; Anti-inflammation; Type II collagen arthritis; Prostaglandin formation

1. Introduction

Metabolites of the arachidonic acid cascade have been shown to play an important role in inflammation and the pathophysiology of autoimmune diseases such as rheumatoid arthritis [1]. The rate-limiting pathway in the metabolism of arachidonic acid is controlled by the COX, which catalyzes a key step in the synthesis of PGs and TXB_2 [2]. COX exists at least in two isoforms: constitutive COX-1 and inducible COX-2 [3,4]. COX-1 is constitutively expressed in different cell types and is involved in the homeostatic function of PGs. In contrast, COX-2 is induced by a number of mitogenic and inflammatory stimuli and plays a key role in the onset of the inflammatory response [3,5,6].

CIA in mice has been proposed as a useful animal model of chronic inflammatory diseases that has a number of characteristics in common with human rheumatoid arthritis [7]. In this model, a chronic inflammatory arthropathy is induced in susceptible strains of rodents and primates by intradermal injection of native CII [8]. This animal model for the study of rheumatoid arthritis has been widely used for evaluation of traditional NSAIDs, which inhibit the activity of both COX isozymes [9]. NSAIDs, which include indomethacin and etodolac, significantly inhibit the development of CIA in mice, indicating a potential role for COX in this arthritic model [10,11].

Another approach to understanding the role of COX isoforms in inflammation was to use gene deletion technology in mice. Using this approach, Myers *et al.* [12] reported that mice deficient in COX-2 has a low incidence and severity of CIA compared with findings in wild-type

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Abbreviations: CII, type II collagen; CIA, type II collagen-induced arthritis; CFA, complete Freund's adjuvant; COX, cyclooxygenase; ICFA, incomplete Freund's adjuvant; LT, leukotriene; NSAID, non-steroidal anti-inflammatory drug; PG, prostaglandin; TX, thromboxane.

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and COX-1-deficient mice, suggesting an important role for COX-2 in mouse CIA model. To better understand the role of COX-2 in mouse CIA, we tested the anti-inflammatory effect of a novel selective COX-2 inhibitor such as FR140423, 3-(difluoromethyl)-1-(4-methoxyphenyl)-5-[4-(methylsulfinyl)phenyl]pyrazole [13]. Additionally, SC-560, 5-(4-chlorophenyl)-1-(4-methoxyphenyl)-3-trifluoromethylpyrazole [14,15], and FR122047, 1-[(4,5-bis(4-methoxyphenyl)-2-thiazoyl)carbonyl]-4-methylpiperazine hydrochloride [16,17], provided a pharmacological tool to analyze the role of COX-1-derived PGs in inflammation. Herein the anti-inflammatory profile of COX inhibitors described above has been characterized in a model of chronic arthritis induced by CII in DBA/1J mice. Moreover, the contribution of two COX isoforms in mouse arthritic model is discussed.

2. Materials and methods

2.1. Animals

These experiments were conducted in accordance with the Ethical guidelines of the International Association for the Study of Pain [18]. In addition, the experimental work was reviewed by the Animal Ethical Committee of Fujisawa Pharmaceutical for Animal Experimentation.

Male DBA/1J mice (15–20 g, Charles River Japan) were used at the age of 6 weeks. The animals were housed five per cage for at least 5 days on a 12-hr light–dark cycle (light on from 07:00 to 19:00 hr) in a controlled temperature ($23 \pm 1^{\circ}$) and humidity ($55 \pm 5\%$) environment. The animals were given standard laboratory food and tap water *ad libitum* before the experiment.

2.2. Induction of type II collagen-induced arthritis

CII, isolated and purified from bovine articular cartilage, was purchased from Collagen Research Centre and dissolved overnight at 4° in 0.1 M acetic acid at a concentration of 10 mg/mL. The solution was emulsified in an equal volume of CFA containing *Mycobacterium tuberculosis* strain H37 Rv (Wako Pure Chemical Industries). Each mouse was immunized subcutaneously at the base of the tail with 125 μg CII in 25 μL cold emulsion [19]. Booster injections of 125 μg of CII emulsified in CFA were given on day 21. The drugs were given orally once a day from day 21 to day 32 after the first CII immunization. For the time course study, volume of all four paws below the joint for each mouse was measured before and 10, 21, 25, 29 and 32 days after the first immunization by using the Volume Meter TK-105 (Neuroscience) as an indication of arthritis. The results were expressed as edema as follows:

$$\text{Edema}\,(\Delta\,\mu\text{L}) = V_t - V_0$$

V_t: total volume of all four paws after CII immunization; V_0: total volume of all four paws before CII immunization.

The anti-inflammatory effect was expressed as the difference in paw edema compared with that of vehicle-treated CIA-control mice.

2.3. Measurement of eicosanoids production in the inflamed mouse paws

The technique of Opas *et al.* was used [20]. At selected times after immunization, mice were euthanized by CO_2 inhalation and all four paws were amputated. The paws were then placed immediately into *n*-hexane cooled by dry ice–acetone for 30 s. Frozen paws were then stored at -70° until needed for extraction of arachidonic acid metabolites.

Frozen paw tissue was pulverized in a glass homogenizer fitted with a Teflon pestle under cooling in 5 mL extraction buffer (75% methanol, 25% 0.1 M sodium acetate, adjusted to pH 3 with HCl). The extracted tissue was centrifuged at 1500 *g* for 10 min at 4°. The resulting supernatant fluid was filtered through gauze and diluted with distilled water to a final concentration of 15% methanol. This solution was applied to a C_{18} Sep-Pak cartridge (Waters) that was prewashed with 10 mL of methanol, distilled water and 15% methanol. After loading Sep-Pak, the columns were sequentially washed with 5 mL of 15% methanol, distilled water and petroleum ether. The samples were eluted with 2 mL of methyl formate [21,22], evaporated under nitrogen gas, dissolved in 1 mL phosphate-buffered saline and assayed for the arachidonic acid metabolites PGE_2, TXB_2, LTB_4 and LTC_4 by radioimmunoassay (Amersham).

The efficiencies of recovery as determined by injection of radiolabeled arachidonic acid metabolites into amputated paws were as follows (mean percent ± SEM, N = 3): PGE_2, $46.7 \pm 4.3\%$; TXB_2, $41.2 \pm 4.5\%$; LTB_4, $41.7 \pm 3.6\%$ and LTC_4, $64.7 \pm 8.1\%$.

2.4. Drugs

The following drugs were used: indomethacin was obtained from Sigma. SC-560, FR122047 and FR140423 were chemically synthesized at Fujisawa Pharmaceutical. These drugs were suspended and diluted in 0.5% methylcellulose.

2.5. Statistical analysis

The results were expressed as mean ± SEM. Statistical significance was analyzed using the ANOVA followed by Dunnett's multiple comparison test. The difference between groups was considered statistically significant when $P < 0.05$. ED_{50} values and 95% confidence limits (95% CL) were calculated from the dose-percent inhibition relations by computer log-linear analysis [23].

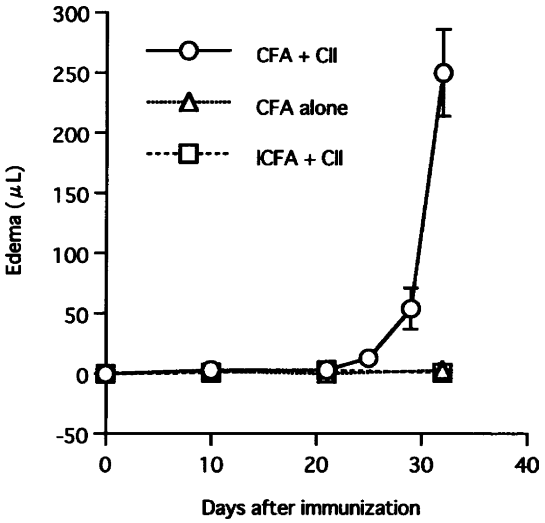

Fig. 1. Time course of type II collagen-induced arthritic mouse paw edema. Male DBA/1J mice were immunized with CII in CFA, as described in Section 2. Paw volume was measured before and various times after the first injection of CII in CFA (open circles), CFA alone (open triangles) or CII in ICFA (open squares), and the pre-injection volume was subtracted from these values. Values are mean ± SEM, N = 5.

3. Results

3.1. Induction of paw edema in mouse CIA model

Figure 1 shows the time course of CIA mouse paw edema. The injection of CII emulsified in CFA, followed by a booster injection on day 21, resulted in an increase in the total volume of all four paws. The mean edema in the inflamed paws on day 32 was 250 ± 36 μL. In the CFA alone group and ICFA plus CII group, no edema was found in either paw.

3.2. Anti-inflammatory effect of drugs in mouse CIA model

We evaluated the anti-inflammatory effect of different COX inhibitors on CIA in mice. As shown in Fig. 2, oral administration of FR140423 (0.0032–3.2 mg/kg) and indomethacin (0.0032–3.2 mg/kg) to CIA mice resulted in a significant suppression of the paw edema, and showed a therapeutic effect in a dose-dependent manner with ED_{50} values (95% CL) of 0.20 (0.013–13) and 0.15 (0.015–2.2) mg/kg, respectively. On the other hand, FR122047 and SC-560 at doses between 0.032 and 3.2 mg/kg (p.o.) had no significant effect on the edema formation in mouse CIA (Fig. 3).

3.3. Formation of arachidonic acid metabolites in the CIA mouse paws

The formation of PGE_2 and TXB_2 was examined in the CIA mouse paws (Fig. 4). Increases in the levels of PGE_2 and TXB_2 were evident after the booster immunization and

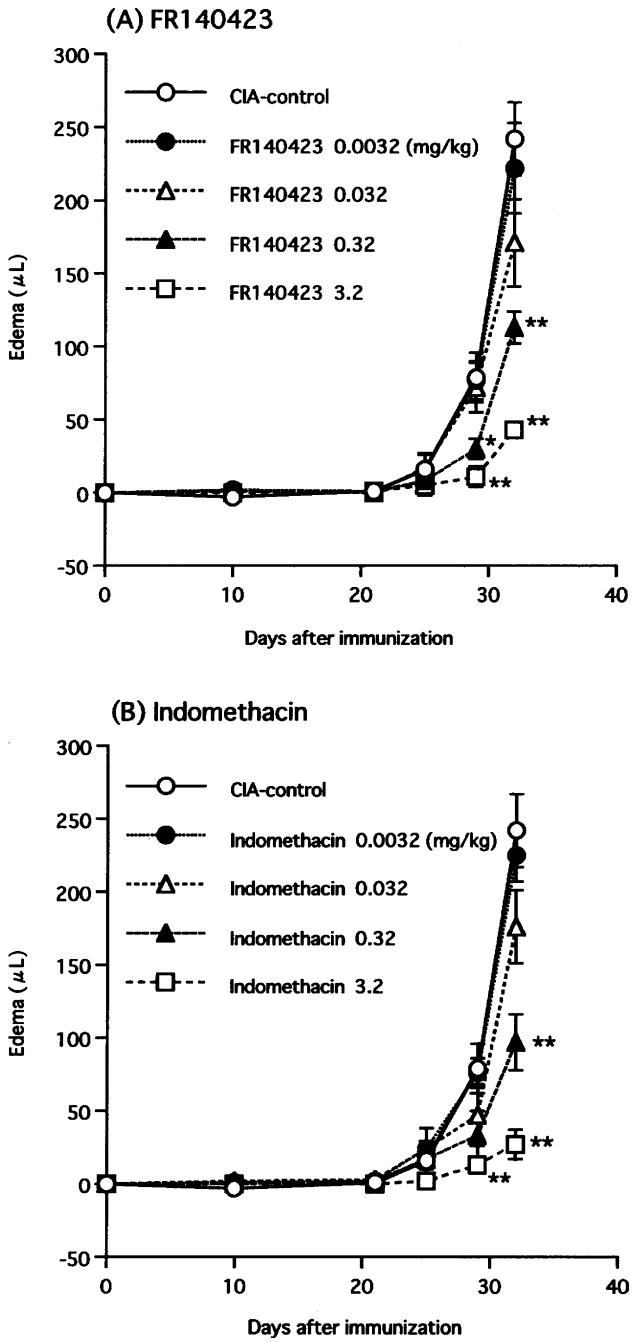

Fig. 2. Anti-inflammatory effect of FR140423 on type II collagen-induced arthritic mouse paw edema. FR140423 (A) and indomethacin (B) at doses 0.0032 mg/kg (closed circles), 0.032 mg/kg (open triangles), 0.32 mg/kg (closed triangles) and 3.2 mg/kg (open squares) and vehicle-treated CIA-control (open circles) were given orally once a day from day 21 to day 32 after the first CII immunization. Significantly different from the CIA-control, $^{*}P < 0.05$, $^{**}P < 0.01$. Values are mean ± SEM, N = 5.

were sustained through day 32. Production of PGE_2 and TXB_2 in the CIA mouse paws was associated with paw edema. In the CFA alone group and ICFA plus CII group, the levels of PGE_2 and TXB_2 in either mouse paws did not increase during the experiment. No significant change was found in the levels of LTB_4 and LTC_4 in the CIA mouse paws.

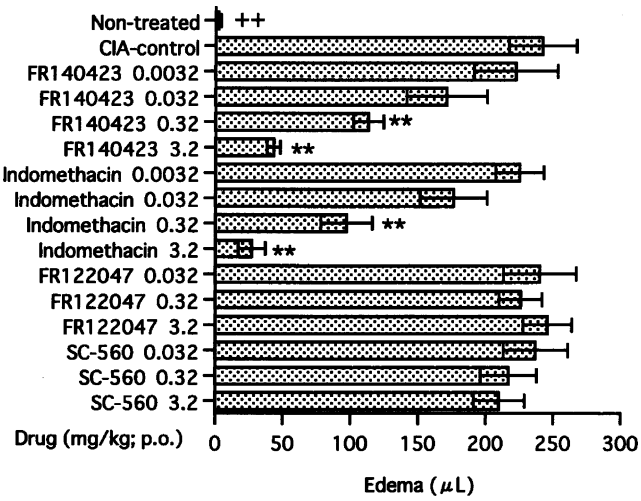

Fig. 3. Anti-inflammatory effect of selective inhibitors of COX-1 and COX-2 on type II collagen-induced arthritic mouse paw edema. Drugs were given orally once a day from day 21 to day 32 after the first CII immunization. Significantly different from the CIA-control, $^{++}P < 0.01$, $^{**}P < 0.01$. Values are mean ± SEM, N = 5.

3.4. Effect of drugs on the formation of prostanoids in the CIA mouse paws

Oral administration of FR140423 (0.0032–3.2 mg/kg) dose dependently reduced the formation of PGE_2 and TXB_2 in the CIA mouse paws with ED_{50} values (95% CL) of 0.20 (0.022–3.9) and 0.12 (0.010–2.3) mg/kg, respectively (Fig. 5). Indomethacin also showed a dose-dependent inhibition of the formation of PGE_2 and TXB_2 in the CIA mouse paws with ED_{50} values (95% CL) of 0.092 (0.0027–2.4) and 0.13 (0.019–0.81) mg/kg, respectively. In contrast, FR122047 and SC-560 at doses up to 3.2 mg/kg had no effect the levels of these prostanoids in the CIA mouse paws.

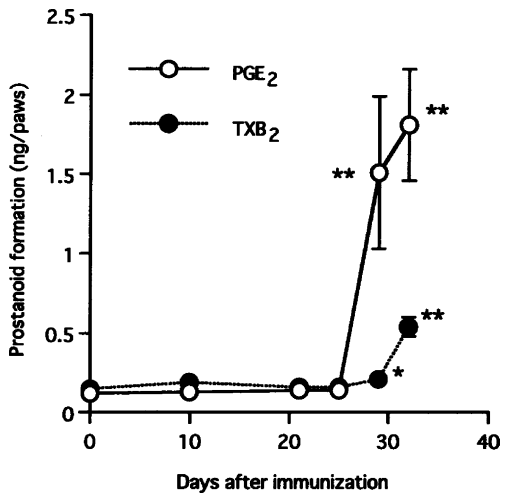

Fig. 4. Time course of PGE_2 and TXB_2 formation in type II collagen-induced arthritic mouse paws. Mice were euthanized by CO_2 inhalation at various times, and PGE_2 (open circles) and TXB_2 (closed circles) in the CIA mouse paws were extracted and analyzed by radioimmunoassay. Significantly different from day 0, $^{*}P < 0.05$, $^{**}P < 0.01$. Values were corrected for recovery efficiency and expressed as ng/paws ± SEM, N = 5.

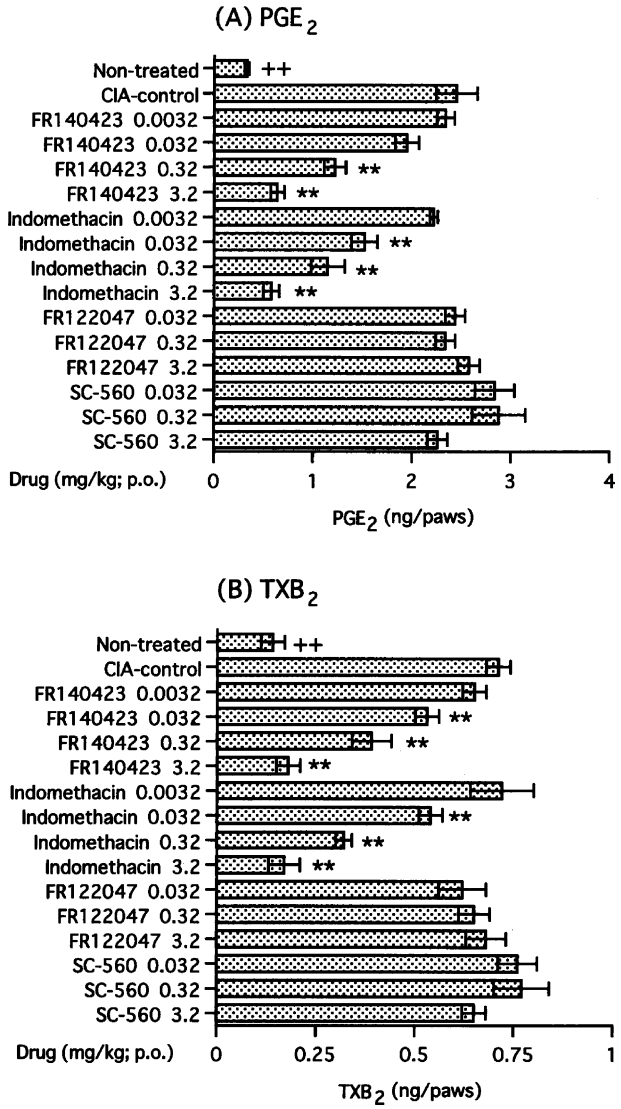

Fig. 5. Effects of selective inhibitors of COX-1 and COX-2 on the formation of PGE_2 and TXB_2 in type II collagen-induced arthritic mouse paws. Drugs were given orally once a day from day 21 to day 32 after the first CII immunization. Mice were euthanized by CO_2 inhalation 32 days after the first CII immunization, and PGE_2 (A) and TXB_2 (B) in the CIA mouse paws were extracted and analyzed by radioimmunoassay. Significantly different from the CIA-control, $^{++}P < 0.01$, $^{**}P < 0.01$. Values were corrected for recovery efficiency and expressed as ng/paws ± SEM, N = 5.

4. Discussion

The present study was designed to elucidate the contribution of two COX isoforms in mediating the chronic inflammation in mouse CIA model. The results obtained in our study define differential effects of COX-1 and COX-2 products in DBA/1J mice with CIA. First of all, we have to inquire into the anti-inflammatory effect of selective inhibitors of COX isozymes in this animal model of arthritis.

We used the selective COX-2 inhibitor FR140423 (COX-1 IC_{50} = 19 μM; COX-2 IC_{50} = 0.13 μM), which is over 150 times more potent as an inhibitor of COX-2 compared with COX-1 in the recombinant human COX enzyme assays [13]. We previously reported that FR140423 reduces rat adjuvant arthritis and inhibits the formation of PGE_2 synthesized by COX-2 in the inflamed paws [13,24]. In this present study, FR140423 produced an anti-inflammatory effect in mouse CIA model in a dose-dependent manner, similar to a conventional NSAID such as indomethacin, which nonselectively inhibits both COX-1 and COX-2. This result suggests the modulation of mouse CIA in paw edema through COX-2 inhibition. Next, using selective COX-1 inhibitors, we examined whether COX-1 modulates to inflammation in the mouse arthritic model. The selective COX-1 inhibitors FR122047 and SC-560 were not effective in mouse CIA model. SC-560 inhibits COX-1-derived platelet TXB_2 and gastric PGE_2 production [15], whereas SC-560 fails to significantly affect the release of COX-2-derived PGE_2 into the inflammatory exudates in the carrageenan-airpouch model [25], indicating that SC-560 potently and selectively inhibits COX-1 *in vivo*. Our previous study has shown that FR122047 was greater 2300 times more selective for COX-1 than COX-2 in the recombinant human enzyme assays [16]. Therefore, these findings clearly demonstrate that COX-2, but not COX-1, contributes to inflammation in mouse CIA model.

Some inflammatory cytokines play also an important role in the development of CIA [26,27]. Enhancement of serum levels of IL-6 is observed in mice with CIA [28]. The onset of CIA in IL-6-deficient mice is delayed for 2 weeks compared with that in IL-6 wide-type mice [29]. In addition to this, IL-6-deficient mice do not show any histological lesion of the joints [30]. The inhibitor of biosynthesis of IL-1, TNF-α and IL-6, CGP 47969A, is highly effective in mouse CIA [31]. These proinflammatory cytokines cause expression of COX-2 which is involved in the production of PGs in several models of arthritis [32–34].

The next purpose is to explore a little further into the role of COX-2 in the mouse inflamed paws induced by CII. To clarify this question at the site of inflammation, we extracted arachidonic acid metabolites from the CIA mouse paws after treatment of drugs. We investigated the relationship between the development of CIA and the levels of arachidonic acid metabolites, PGE_2, TXB_2, LTB_4 and LTC_4, in the mouse inflamed paws. A gradual increase in the levels of PGE_2 and TXB_2, but not these LTs, occurred in the inflamed paws immunized with CII after the booster immunization, nearly paralleling the increase in paw volume. Oral administration of FR140423 at doses ranging from 0.0032 to 3.2 mg/kg to arthritic animals dose dependently inhibited the formation of PGE_2 and TXB_2 in the CIA mouse paws. The ED_{50} values of FR140423 for the inhibition of PGE_2 and TXB_2 production in the inflamed paws were almost the same as the ED_{50} value of FR140423 for its anti-inflammatory effect in mouse CIA. In our study, a strong correlation was established between the dosing of selective COX-2 inhibitor and a decreased paw edema in mouse CIA, including the formation of these prostanoids produced by COX-2 in the inflammatory site. In contrast, SC-560 and FR122047, highly selective COX-1 inhibitors, at doses up to 3.2 mg/kg had no effect on the formation of PGE_2 and TXB_2 in the CIA mouse paws. Thus, these data presented herein clearly indicate that PGE_2 and TXB_2 formed by COX-2, but not by COX-1, play an essential role at the site of inflammation in mouse CIA model. Our observations agree with the previous conclusion reported by Myers *et al.* [12] indicating that COX-2 enzyme exerts specific physiologic actions without modulating the production of COX-1 in the pathogenesis of mouse CIA using COX-deficient mice.

We have recently reported that both COX-1 and COX-2 play a critical role to inflammation in rat CIA model [17,35]. Surprisingly, the selective COX-1 inhibitor FR122047 produced an anti-inflammatory effect in rat CIA model, whereas FR122047 did not in mouse CIA model. Therefore, we would accept the differential role of COX-1 between two animal models of CIA. One possible reason for this discrepancy is the use of different animal models. For induction of rat CIA, we used CII emulsified with ICFA for the immunization [17,35]. In the present report, DBA/1J mice immunized with CII emulsified in ICFA, on the other hand, did not induce arthritis and the production of PGE_2 and TXB_2 in the paws. For the reason given above, we tried to induce arthritis in mice by injection of CII emulsified in CFA. However, we lack definite information on the difference between these results. Further experiments are necessary to better clarify the mechanisms underlying the difference between these two animal models for arthritis.

In summary, we have evaluated the inhibitory effect of FR140423, a selective inhibitor of COX-2, in DBA/1J mice with CIA *in vivo*. The present study leads to the conclusion that COX-2 plays an important role that is different from that of COX-1 in the development of mouse CIA. These findings suggest that selective blockade of COX-2 may have clinical benefit in the treatment of human rheumatoid arthritis.

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