FR167653, a Cytokine Synthesis Inhibitor, Exhibits Anti-Inflammatory Effects Early in Rat Carrageenin-Induced Pleurisy but No Effect Later

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ABSTRACT

We prepared a pharmacological profile of FR167653 (1-[7-(4-fluorophenyl)-1,2,3,4-tetrahydro-8-(4-pyridyl) pyrazolo[5,1-c][1,2,4]triazin-2-yl]-2-phenylethanedion sulfate monohydrate), a cytokine synthesis inhibitor, on early (5 h after irritation) and late (14–24 h after irritation) phases of rat carrageenin-induced pleurisy and on mediator-induced plasma exudation, in comparison with that of dexamethasone. In the early phase, FR167653 (30 mg/kg) and dexamethasone (0.3 mg/kg) equally suppressed plasma exudation and leukocyte infiltration. Furthermore, both agents significantly lowered the prostaglandin levels in the exudate. Expression of cyclooxygenase-2 protein on leukocytes in the early phase of inflammation was not affected by dexamethasone, but it was suppressed by FR167653. However, FR167653 did not significantly affect the leukocyte mRNA level of cyclooxygenase-2. Both agents significantly suppressed the levels of both tumor necrosis factor-α and interleukin-1β. FR167653 had a different pharmacological profile from dexamethasone in the late phase of this model in that, unlike dexamethasone, it did not affect cyclooxygenase-2 expression in mesothelial cells, the 6-keto-prostaglandin F1α level in the exudate or hyperplasia of mesothelium. Furthermore, unlike dexamethasone, FR167653 did not consistently inhibit mediator-induced plasma exudation. These results suggest that FR167653 or one of its analogs may be new candidates for therapy with a spectrum of activity distinct from that of current anti-inflammatory steroids.

It has been widely accepted that cytokines such as tumor necrosis factor-α and interleukin-1, and also arachidonic acid metabolites, have an important role in inflammatory diseases such as rheumatoid arthritis, systemic lupus erythematosus, asthma, inflammatory bowel disease, psoriasis, and other chronic inflammatory and autoimmune diseases. Glucocorticoids are potent anti-inflammatory agents widely used in those inflammatory conditions. Glucocorticoids exert their effects by binding to a cytoplasmic glucocorticoid receptor within the target cells. The glucocorticoid-receptor complex then translocates to the nucleus and modulates the expression of specific target genes in a positive or negative manner, interfering with multiple signaling pathways. Consequently, a wide variety of genes are regulated by glucocorticoids (Barnes, 1998; McKay and Cidlowski, 1999). Therefore, although glucocorticoids are effective for treatment of inflammatory diseases, their use is limited by their severe adverse effects, such as increased susceptibility to infection, osteoporosis, delayed wound healing, and so on (Stein and Pincus, 1997).

In recent years, pyridinyl imidazole derivatives have been reported as a novel class of cytokine synthesis inhibitors (Lee et al., 1993, 1994). These agents inhibit the production of specific cytokines including tumor necrosis factor-α, interleukin-1, interleukin-6, and interleukin-8 in an in vitro system (Lee et al., 1993). It is reported that the target of these inhibitors is a pair of closely related protein kinases, which are human homologs of p38 mitogen-activated protein kinase, termed cytokine-suppressive anti-inflammatory drug-binding protein (CSBP). The binding of these drugs inhibits CSBP kinase activity and could be directly correlated with their ability to inhibit cytokine production (Lee et al., 1994). FR167653 is a pyridinyl isomidazole derivative and is shown to inhibit cytokine production (Yamamoto et al., 1996). It inhibits the expression of tumor necrosis factor-α, interleukin-1β, and cyclooxygenase-2 in human blood monocytes and

ABBREVIATIONS: CSBP, cytokine-suppressive anti-inflammatory drug-binding protein; PCR, polymerase chain reaction; FR167653, 1-[7-(4-fluorophenyl)-1,2,3,4-tetrahydro-8-(4-pyridyl) pyrazolo[5,1-c][1,2,4]triazin-2-yl]-2-phenylethanedion sulfate monohydrate; SB203580, 4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)-imidazole; SK&F 86002, 6-(4-pyridyl)-2,3-dihydroimidazo-(2,1-b)-thiazole.
alveolar macrophages (Kawano et al., 1999) and also can exert protective effects on lipopolysaccharide-induced disseminated intravascular coagulation (Yamamoto et al., 1996) and endotoxin-induced shock (Yamamoto et al., 1997) in animal models.

Rat carrageenin-induced pleurisy is the most widely accepted model suitable for collection and analysis of cells and exudate from the inflammatory site. Although, carrageenin pleurisy is a neutrophil-dominated model, a number of reports have demonstrated that neutrophils also have the ability to synthesize and release cytokines (Cassatella, 1995). Using this model, we have previously shown that a high level of cyclooxygenase-2 is detectable from 3 to 7 h in neutrophils and mononuclear leukocytes (Harada et al., 1994, 1996), and from 9 to 24 h in pleural mesothelial cells (Hatanaka et al., 1999). In the early phase, selective cyclooxygenase-2 inhibitors suppress plasma exudation and preferentially reduce the prostaglandin E2 level, but not the level of 6-keto-prostaglandin F1α or of thromboxane B2 in the pleural exudate, suggesting that prostaglandin E2 generated via cyclooxygenase-2 by leukocytes in the exudate may play an important role in plasma exudation. In addition, a lower dose of dexamethasone (0.3 mg/kg) does not affect the cyclooxygenase-2 level, even showing potent anti-inflammatory activity (Kawamura et al., 2000). In the late phase, selective cyclooxygenase-2 inhibitors lower the intrapleural level of 6-keto-prostaglandin F1α, a stable metabolite of prostaglandin I2, and inhibit hyperplasia of the pleural matrix, suggesting that cyclooxygenase-2 expressed in mesothelial cells may play a role in the synthesis of extracellular matrix through formation of prostaglandin I2 (Hatanaka et al., 1999).

In the present study, we evaluated the effects of FR167653 on the early and late phases of rat carrageenin-induced pleurisy and on mediator-induced plasma exudation, comparing them with those of dexamethasone, to characterize its pharmacological profile.

Materials and Methods

Carrageenin Pleurisy. All experiments were performed according to the Guideline for Animal Experimentation of Kitasato University. Pleurisy was induced in male Sprague-Dawley rats (9–10 weeks old, specific pathogen free), purchased from Nippon SLC (Hamamatsu, Japan), by intrapleural injection of 0.2 ml of 2% α-carrageenin (Zushi Chemical, Zushi, Japan) under light ether anesthesia according to the method described previously (Harada et al., 1996). For early phase experiments, FR167653 (3, 10, 30 mg/kg; Fujisawa Pharmaceutical Co., Ltd., Osaka, Japan) suspended with 1% carboxymethyl cellulose in saline had been administered orally 1 h before, and dexamethasone (0.3 mg/kg; Banyu Pharmaceutical Co., Ltd., Tokyo, Japan) had been intraperitoneally injected 2 h before the injection of carrageenin. For late phase experiments, FR167653 (30 mg/kg, p.o.) and dexamethasone (0.3 mg/kg, i.p.) were administered 9 h after the induction of pleurisy, unless otherwise stated.

The rats were exsanguinated under ether anesthesia at given times. The pleural exudate was harvested, and its volume was measured. The harvested cells were washed twice with 10 mM phosphate-buffered saline (pH 7.2) containing 1 mM EDTA by centrifugation at 200g for 5 min at 4°C, and the resulting cell pellet was immediately processed for cyclooxygenase-2 mRNA measurement and stored at −80°C prior to Western blot analysis for cyclooxygenase protein. The cells in the exudate were counted using an improved Neubauer cell count plate (Erma, Tokyo, Japan) after fixation with Turk's solution (Wako Pure Chemicals, Osaka, Japan) and were classified under a light microscope after being smeared on a glass slide and stained by the Wright-Giemsa method. The surface of the parietal pleura of 14 h-pleurisy rats was scraped with a stainless steel spatula with 2 ml of the phosphate-buffered saline containing 1 mM EDTA, and the lavage was harvested (Hatanaka et al., 1999). The harvested cells were washed twice with the phosphate-buffered saline containing 1 mM EDTA by centrifugation at 200g for 5 min and were stored at −80°C for Western blotting analysis. A part of the parietal pleura was dissected from 24-h pleurisy rats or normal control rats and fixed with 10% formalin solution for histological observation of the pleura. Cross-sections (5-μm thickness) of paraffin-embedded pleural tissue were stained with hematoxylin-eosin and examined under a light microscope.

In separate experiments, rats were intravenously injected with pontamine sky blue (50 mg/kg; Tokyo Kasei, Tokyo, Japan) 20 min before exsanguination for assessment of the plasma exudation rate (Harada et al., 1996). The amount of dye in the pleural exudate was measured spectrophotometrically by absorption at 630 nm and normalized according to the concentration of the dye in the serum. The detection limit for the dye was approximately 2.5 μg/sample. For the prostaglandin assay, 2 ml of saline solution containing 20 μM indomethacin and 15.4 mM EDTA were injected into the pleural cavity immediately after exsanguination. The pleural fluid was then collected and immediately frozen at −80°C until clean-up. To determine the cytokines, pleural exudate was harvested into a tube containing heparin. The harvested pleural exudate was centrifuged at 200g for 5 min. The supernatant was stored at −80°C until assay.

Prostanoid Assay. The prostaglandin level was determined as described previously (Harada et al., 1996). Briefly, the frozen pleural fluid was thawed and centrifuged at 2000g for 10 min. The supernatant was acidified to pH 3 with 1 N HCl and again centrifuged at 2000g for 10 min. The resulting supernatant was applied to a Sep-Pak C18 column (Waters Associates, Milford, MA). After the separation of prostaglandin E2, thromboxane B2, and 6-keto-prostaglandin F1α by high-performance liquid chromatography, prostaglandin was assayed by enzyme-immunoassay kits (Cayman Chemical, Ann Arbor, MI). The overall recovery rates assessed by addition of authentic prostaglandin to the sample were 52.8 ± 2.2% (n = 13), 48.9 ± 1.8% (n = 13), and 43.0 ± 2.2% (n = 13) for prostaglandin E2, thromboxane B2, and 6-keto-prostaglandin F1α, respectively. The detection limit for each prostaglandin was approximately 0.1 to 0.06 ng/sample.

Measurement of Tumor Necrosis Factor-α and Interleukin-1β. The frozen cell-free supernatant of the exudate was thawed, and tumor necrosis factor-α and interleukin-1β were determined by enzyme-immunoassay kits (BioSource, Camarillo, CA).

Western Blot Analysis. Western blot analysis for cyclooxygenase-1 and cyclooxygenase-2 was performed by the method described previously (Harada et al., 1994). In brief, the frozen cells collected from the exudate and parietal pleura were thawed and suspended in 20 mM Tris-HCl buffer (pH 7.4), containing 5 mM tryptophan and 2 mM phenylmethyl sulfonyl fluoride (Wako Pure Chemicals), and then sonicated for 1 min at 4°C. The homogenate was then solubilized in 0.5% Tween 20 and centrifuged at 140,000 g for 1 h at 4°C. The resulting supernatant was diluted with an equal volume of a sampling buffer of the following composition: 0.1 M Tris-HCl (pH 6.8), 20% glycerol, 0.1 mg/ml methyl green, and 2% sodium dodecyl sulfate. The solubilized protein (40–50 μg of protein/lane) was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and then transferred to a polyvinylidene difluoride membrane (Millipore Corporation, Bedford, MA). After blocking with Block Ace (Dainippon Pharmaceutical Co., Osaka, Japan), the blot membrane was incubated with rabbit antivine cyclooxygenase-1 antiserum (Ishimura et al., 1993) or rabbit antimurine cyclooxygenase-2 antiserum (Cayman Chemical). Then, after incubation with goat anti-rabbit immunoglobulin conjugated with horseradish peroxidase (Organon Teknika N.V.-Cappel Products, Durham, NC), the membrane...
was stained with Konica immunostain HRP-1000 (Konica, Tokyo, Japan).

Cyclooxygenase-2 mRNA Measurement. The reverse transcription and competitive polymerase chain reaction (PCR) was used to measure cyclooxygenase-2 mRNA. The total RNA of the exudate cell pellet (approximately 4 x 10⁷ cells) from a 5-h pleurisy rat was extracted in Isogen (Nippon Gene, Tokyo, Japan), a mixture of guanidinium isothiocyanate and phenol (Chomczynski and Sacchi, 1987). The yield of RNA extracted was determined spectrophotometrically. Two micrograms of each sample was reverse-transcribed at 42°C for 1 h in buffer (50 mM Tris-HCl, pH 8.3, 75 mM KCl, 8 mM MgCl₂, and 10 mM dithiothreitol) containing 2.5 μM random hexamer oligonucleosides (TaKaRa Biomedicals, Shiga, Japan), 10 units of reverse transcriptase (RAV-II; TaKaRa Biomedicals), each of the 2'-deoxynucleoside 5'-triphosphates at 1 mM and 20 units of RNase inhibitor (TaKaRa Biomedicals).

Competitive PCR amplification was performed in buffer (10 mM Tris-HCl, pH 8.3, 50 mM KCl, and 1.5 mM MgCl₂) containing 200 μM each 2'-deoxynucleoside 5'-triphosphate, 0.4 μM each primer, and 0.625 units of Taq polymerase (TaKaRa Biomedicals), cDNA from the reverse transcription reaction and mimic DNA (10-fold serial dilutions of mimic DNA; 10⁵ to 10⁶ copies/tube). The sequences of cyclooxygenase-2 primers were 5'-TAC CAC TGG CAT TGT GAT GG-3' (sense) and 5'-TTA ATG TCA CGC ACG ATT TC-3' (antisense) (Chen et al., 1999). The PCR product was 198 base pairs. PCR was performed for 35 cycles, each cycle consisting of 30 s at 94°C, 30 s at 55°C, and 1 min at 72°C. Mimic DNAs were DNA fragments, which were used as competitors in PCR amplification. The mimic DNAs for cyclooxygenase-2 and β-actin gene were prepared using a competitive DNA construction kit (TaKaRa Biomedicals) according to the manufacturer's instructions. The PCR

Fig. 1. Effects of FR167653 (FR; 3, 10, 30 mg/kg) and dexamethasone (DEX; 0.3 mg/kg) on pleural exudate volume (upper panel) and the amount of dye exuded over 20 min (lower panel) 5 h after carrageenin injection. Each value indicates the mean ± S.E.M. of 4 to 11 rats.

was stained with ethidium bromide. The unknown quantity of target cDNA was semiquantitatively determined from the amounts of target cDNA and mimic cDNA present with equal band intensity in each lane by assessing the intensity of the bands (Kishimoto et al., 1997). The gene for β-actin was used as an internal control. The sequences of β-actin primers were 5'-TAC CAC TGG CAT TGT GAT GG-3' (sense) and 5'-TTA ATG TCA CGC ACG ATT TC-3' (antisense), respectively (Kishimoto et al., 1997). The PCR product was 198 base pairs. PCR was performed for 35 cycles, each cycle consisting of 30 s at 94°C, 30 s at 55°C, and 1 min at 72°C. Mimic DNAs were DNA fragments, which were used as competitors in PCR amplification. The mimic DNAs for cyclooxygenase-2 and β-actin gene were prepared using a competitive DNA construction kit (TaKaRa Biomedicals) according to the manufacturer’s instructions. The PCR

Fig. 2. Effects of FR167653 (FR; 30 mg/kg) and dexamethasone (DEX; 0.3 mg/kg) on numbers of total leukocytes (top panel), neutrophils (middle panel), and mononuclear leukocytes (bottom panel) in the pleural exudate 5 h after carrageenin injection. Each value indicates the mean ± S.E.M. of five to seven rats.
products were 362 base pairs for mimic cyclooxygenase-2 and 237 base pairs for mimic /H9252-actin.

**Mediator-Induced Plasma Exudation**. Male Sprague-Dawley rats (9–10-weeks old, specific pathogen-free; Nippon SLC) were anesthetized with pentobarbital (50 mg/kg, s.c.; Abbott Lab., North Chicago, IL) and were intravenously injected with pontamine sky blue (50 mg/kg; Tokyo Kasei). Intradermal injection of various doses of histamine or bradykinin in 0.1 ml of Tyrode’s solution were started 5 min after injection of the dye into the shaved abdominal skin at 8 to 10 sites for each rat. The rats were sacrificed by exsanguination 40 min after the end of the injection of mediators. FR167653 (30 mg/kg, p.o.) or dexamethasone (0.3 mg/kg, i.p.) was administered 30 min before injection of the mediators.

**Data Analysis**. Results were expressed as the mean ± S.E.M. from n experiments. Fisher’s or Scheffe’s test was used to evaluate significant differences between means. A P value of less than 0.05 was considered statistically significant and is indicated by an asterisk in the figures.

**Results**

**Effects in the Early Phase of Pleurisy**. The amount of pleural fluid collected from normal control rats was 0.04 ± 0.01 ml (n = 6) and that of pontamine sky blue that leaked
into the pleural cavity after intravenous injection over 20 min was 2.89 ± 0.32 μg (n = 7). The total count of leukocytes harvested from the lavage fluid from the pleural cavity of normal control rats was 11.40 ± 0.94 × 10⁶ cells (n = 12). Mononuclear leukocytes accounted for 72.6 ± 2.6%. The remaining cells included eosinophils (12.8 ± 1.1%), mast cells (8.0 ± 0.9%), and neutrophils (3.5 ± 0.7%). These parameters were not significantly affected by pretreatment with FR167653 or dexamethasone (data not shown). Intrapleural injection of carrageenin caused the accumulation of a volume of pleural exudate that reached 1.57 ± 0.12 ml (n = 11), and the plasma exudation rate, estimated from the exuded dye amount, reached 167 ± 15 μg (n = 11) 5 h after the injection. The total leukocyte count was 157 ± 15 × 10⁶ (n = 6), with 91.4 ± 1.3% of neutrophils and 6.0 ± 0.5% of mononuclear leukocytes, 5 h after pleurisy induction. FR167653 (3, 10, 30 mg/kg) dose dependently suppressed both the accumulation of pleural exudate and the plasma exudation rate 5 h after carrageenin injection, as did dexamethasone (0.3 mg/kg) (Fig. 1). Furthermore, FR167653 (30 mg/kg) significantly decreased the number of neutrophils, and consequently the total number of leukocytes, in the exudate 5 h after carrageenin injection, as potentially as dexamethasone (0.3 mg/kg) (Fig. 2).

The levels of prostaglandin E₂, thromboxane B₂, and 6-κeto-prostaglandin F₁α, in the lavage fluid from normal control rats were 0.16 ± 0.06, 0.49 ± 0.13, and 0.94 ± 0.24 ng/rat (n = 6), respectively, and these levels were significantly increased to 0.96 ± 0.11, 2.97 ± 0.27, and 5.68 ± 0.77 ng/rat (n = 19), respectively, 5 h after pleurisy induction. FR167653 suppressed significantly and almost equally all of the prostaglandins measured, as did dexamethasone (Fig. 3).

In pleural lavage cells harvested from normal control rats, only cyclooxygenase-1, but not cyclooxygenase-2, was detectable, as previously reported (Harada et al., 1994). In the exudate cells 5 h after the induction of pleurisy, both cyclooxygenase-1 and cyclooxygenase-2 were detectable at high levels (Fig. 4A). Western blot analysis showed that pretreatment with FR167653 (30 mg/kg) reduced the level of cyclooxygenase-2 below the detection limit, but dexamethasone (0.3 mg/kg) had no effect. Reverse transcription-competitive PCR analysis indicated that FR167653 slightly, but not significantly, reduced the level of cyclooxygenase-2 mRNA, but dexamethasone did not (Fig. 4B).

Tumor necrosis factor-α and interleukin-1β were detectable in the pleural exudate 1 h after carrageenin injection. Their level peaked 2 and 5 h, respectively, after the irritation and then declined (Fig. 5A). The effects of FR167653 and dexamethasone were assessed 2 h after carrageenin injection. Both drugs significantly suppressed the levels of tumor necrosis factor-α and interleukin-1β (Fig. 5B).

**Effects in the Late Phase of Pleurisy.** We have previously demonstrated that a high level of cyclooxygenase-2 is detectable in the cells scraped off the parietal pleura 14 h after the induction of pleurisy (Hatanaka et al., 1999). These results were confirmed in the present study (Fig. 6). Dexamethasone administered 9 h after the induction inhibited the cyclooxygenase-2 expression in the scraped cells, but FR167653 did not. In addition, dexamethasone significantly suppressed 6-keto-prostaglandin F₁α levels, but FR167653 did not (Fig. 7).

In normal control rats, the pleura was less than 10 μm in thickness, and the underlying basement membrane was too thin to detect in cross-section under the light microscope before irritation by carrageenin. The pleura had thickened, reaching a thickness of approximately 40 to 50 μm, and the fibrous matrix, with its typical collagen-like features, had developed markedly, so that it was detectable after 24 h of pleurisy (Hatanaka et al., 1999). As shown in Fig. 8, these results were confirmed. Moreover, dexamethasone, administered 8, 14, and 20 h after irritation (0.3 mg/kg in each time), inhibited the thickening of the pleura and the development of a fibrous matrix, but FR167653 administered in 30 mg/kg doses 9, 15, and 21 h after irritation did not (Fig. 8).

**Effects on Mediator-Induced Plasma Exudation.** Intradermal injection of bradykinin (0.1–10 nmol/site) and histamine (0.5–500 nmol/site) caused dose-dependent amounts of plasma exudation in the rat. Dexamethasone significantly suppressed the plasma exudation induced by both mediators. However, FR167653 did not suppress it, except at the threshold dose of bradykinin (Fig. 9).

**Discussion**

We performed a pharmacological characterization of FR167653 in relation to the early and late phases of rat
carrageenin-induced pleurisy and mediator-induced plasma exudation in comparison with dexamethasone. Glucocorticoids up- or down-regulate the expression of a wide variety of genes (Barnes, 1998; McKay and Cidlowski, 1999). In fact, even though we chose a lower dose of dexamethasone (Kawamura et al., 2000), the drug suppressed all of the changes that we tested here, except for the leukocyte cyclooxygenase-2 expression in the early phase. In contrast, FR167653 exhibited its effects, including those on mediator-induced plasma exudation, only in the early phase, not in the late phase.

In recent years, an increasing number of compounds have been implicated in the suppression of cytokine synthesis (Lee et al., 1993). SK&F 86002 [6-(4'-pyridyl)-2,3-dihydroimidazo-(2,1-b)-thiazole, a pyridinyl imidazole], is the prototype of a novel class of cytokine synthesis inhibitors that bind CSBP kinase and inhibit its activity (Lee et al., 1993, 1994). In the present study, FR167653 significantly suppressed the levels of tumor necrosis factor-α and interleukin-1β (Fig. 5B). Thus, FR167653 showed a cytokine synthesis inhibitory activity in this acute exudative inflammatory model as potent as in human alveolar macrophages and peripheral blood monocytes (Kawano et al., 1999), in a rat lipopolysaccharide-induced disseminated intravascular coagulation model (Yamamoto et al., 1996), and in a rat lipopolysaccharide-induced lung injury model (Yoshinari et al., 2001). Recently, it is reported that FR167653 reduces the expression of p38 mitogen-activated protein kinase (Otani et al., 2000) and the phosphorylation of p38 mitogen-activated protein kinase (Yoshinari et al., 2001) in the rat lung tissue. Thus, FR167653 may exert anti-inflammatory effects interfering with p38 mitogen-activated protein kinase pathway.

We showed here that dexamethasone suppressed cyclooxygenase-2 expression in mesothelial cells (Fig. 6) but did not affect that in leukocytes (Fig. 4). We have previously reported that a lower anti-inflammatory dose of dexamethasone (0.3 mg/kg) did not affect the level of cyclooxygenase-2 protein expressed in leukocytes in this model, whereas higher doses of the drug suppressed it (Kawamura et al., 2000). The present results confirm the profile of the lower dose of dexamethasone on leukocyte cyclooxygenase-2 expression in the levels of protein (Fig. 4A) and mRNA (Fig. 4B). In contrast, FR167653 suppressed cyclooxygenase-2 expression in leukocytes (Fig. 4) but did not affect that in mesothelial cells (Fig.

![Fig. 6. Western blot analysis showing the effect of FR167653 (FR; 30 mg/kg) and dexamethasone (DEX; 0.3 mg/kg) on the levels of cyclooxygenase (COX)-1 (upper panel) and COX-2 (lower panel) in cells scraped from the parietal pleura 14 h after carrageenin injection. The position of the 70-kDa molecular marker protein is indicated. Similar results were obtained from two additional sets of experiments.](image)

![Fig. 7. Effects of FR167653 (FR; 30 mg/kg) and dexamethasone (DEX; 0.3 mg/kg) on levels of prostanoids at 14 h after carrageenin injection. Each value indicates the mean ± S.E.M. of 6 to 11 rats.](image)
These are similar to the results seen with SB203580 [4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)-imidazole], another pyridinyl imidazole class of cytokine synthesis inhibitor that inhibits murine macrophage cyclooxygenase-2 expression, whereas the inhibitor had no significant effect on cyclooxygenase-2 expression in bovine chondrocytes (Patel et al., 1999). Furthermore, FR167653 lowered the level of cyclooxygenase-2 protein, but did not significantly affect the cyclooxygenase-2 mRNA level in leukocytes (Fig. 4), suggesting the involvement of translational events. In contrast, the drug lowered the cyclooxygenase-2 mRNA level in lipopolysaccharide-stimulated human peripheral blood monocytes and alveolar macrophages, suggesting the involvement of transcriptional or mRNA stability events or both (Kawano et al., 1999). In relation to the contrasting results obtained by us for the effects of FR167653 on the cyclooxygenase-2 mRNA level, SK&F 86002 suppressed expression of tumor necrosis factor-α and interleukin-1 at a translational level in human peripheral blood monocytes stimulated with lipopolysaccharide (Lee et al., 1993; Young et al., 1993) and the THP-1 human monocytic cell line stimulated with lipopolysaccharide (Prichett et al., 1995). However, it caused a decrease in the cyclooxygenase-2 mRNA level in human peripheral blood monocytes stimulated with serum-treated zymosan (Pouliot et al., 1997). Therefore, the level at which proinflammatory gene expression is affected by this class of cytokine synthesis inhibitors is still controversial. The level would vary depending on cell types and on the circumstances that cells encounter.

Tumor necrosis factor-α and interleukin-1β were detectable in the pleural exudate (Fig. 5A). In addition, intrapleural injection of tumor necrosis factor-α induces cyclooxygenase-2 expression in leukocytes (Hatanaka et al., 1996). These facts suggest that tumor necrosis factor-α may be a candidate for inducing cyclooxygenase-2 in this model. FR167653 suppressed the expression of cyclooxygenase-2 (Fig. 4A) as well as tumor necrosis factor-α and interleukin-1β (Fig. 5B). Pyridinyl imidazoles suppress cyclooxygenase-2 expression induced by tumor necrosis factor-α and interleukin-1β (Pouliot et al., 1997). Therefore, it may well be that FR167653 suppresses cyclooxygenase-2 expression through direct interference with cyclooxygenase-2 gene expression but not by blocking cytokine production.

In the early phase, FR167653 lowered the measured levels of prostanoid (Fig. 3). In contrast, the drug did not affect the prostanoid levels in the late phase (Fig. 7). This result suggests that FR167653 may not directly interfere with enzymes involved in prostanoid formation, whereas SK&F 86002 was originally reported to be a dual inhibitor of arachidonic acid metabolism, since it inhibits both cyclooxygenase and 5-lipoxygenase (Griswold et al., 1987). We have previously shown that selective cyclooxygenase-2 inhibitors suppress preferentially the level of prostaglandin E<sub>2</sub> but they did not significantly lower the levels of thromboxane B<sub>2</sub> and 6-keto-prostaglandin F<sub>1α</sub> in the early phase of this model (Harada et al., 1996). However, FR167653 and dexamethasone almost equally lower the levels of these prostanoids (Fig. 3). Thus, there exists a difference in profiles at each prostanoid level. Both FR167653 and dexamethasone inhibited intrapleural infiltration of leukocytes (Fig. 2), which may contribute to prostanoid formation in the inflammatory site. Therefore, the inhibitory effect of FR167653 on the leukocyte infiltration may partly contribute to the lowering of the prostanoid level. In addition, FR167653, and higher doses of dexamethasone,
suppress cyclooxygenase-2 protein expression (Fig. 4A; Kawamura et al., 2000). Murakami et al. (1997) proposed that intracellular translocation of enzymes involved in prostanoid formation may occur after induction of cyclooxygenase-2. These mechanisms might explain the difference in inhibitory profiles between selective cyclooxygenase-2 inhibitors and FR167653.

Both FR167653 and dexamethasone lowered the level of prostanoids and suppressed plasma exudation in the early phase (Figs. 1 and 3). In carrageenin-induced pleurisy, bradykinin (Majima et al., 1993) and prostanoids, especially prostaglandin E2 (Harada et al., 1998), play a crucial role in plasma exudation. Prostanoid itself does not induce plasma exudation but potentiates that induced by bradykinin or other mediators (Williams and Morley, 1973). Dexamethasone suppressed mediator-induced plasma exudation, but FR167653 did not consistently affect it (Fig. 9). These results suggest that FR167653 may exhibit an inhibitory effect on plasma exudation through lowering the level of prostanoids, especially prostaglandin E2, in the inflammatory site.

Proteins are activated by many chemotactic factors. Proinflammatory cytokines, such as tumor necrosis factor-α, interleukin-1β, and interleukin-8, also induce neutrophil activation (Czybulske et al., 1986; Utsunomiya et al., 1996). These cytokines were detectable in this model, and FR167653 suppressed levels of tumor necrosis factor-α and interleukin-1β (Fig. 5). FR167653 is also reported to suppress the level of interleukin-8 (Aiba et al., 2000). It is demonstrated that dexamethasone suppresses mediator-induced leukocyte infiltration (Katori et al., 1990). On the other hand, SKF 86002 does not inhibit LTB4-induced leukocyte chemotaxis, whereas it does inhibit inflammatory cell infiltration induced by carrageenin in the mice (Griswold et al., 1989). All these results suggest that this class of cytokine synthesis inhibitors may inhibit leukocyte infiltration through inhibition of the production of mediator(s), which activate leukocytes, but not through leukocyte migration itself.

FR167653 suppressed plasma exudation and leukocyte infiltration in the early phase of inflammation, as did dexamethasone (Figs. 1 and 2). However, it did not suppress the expression of cyclooxygenase-2 or hyperplasia of the pleural mesothelium in the late phase (Figs. 6 and 8). It is suggested that prostaglandin I2 may be generated via cyclooxygenase-2 and be involved in the hyperplasia of pleural mesothelium, because selective cyclooxygenase-2 inhibitors lowered the level of 6-keto-prostaglandin F1α and suppressed the hyperplasia, simultaneously (Hatanaka et al., 1999). Dexamethasone suppressed the cyclooxygenase-2 expression and preferentially lowered 6-keto-prostaglandin F1α (Figs. 6 and 7). As mentioned above, FR167653 suppressed the cyclooxygenase-2 expression and lowered almost equally all prostanoids measured (Figs. 3 and 4A). These results suggest the presence of a difference between leukocytes and mesothelial cells in regulatory mechanism of prostanoid formation. The role of leukocytes in the late phase of inflammatory response is not clear, but it may participate in the healing process (Mizuno et al., 1997; Shigeta et al., 1998).

FR167653 exhibited potent anti-inflammatory effects and suppressed proinflammatory gene expression, especially in the early phase of carrageenin-induced pleurisy. The selective suppression of gene expression may be beneficial in a safer anti-inflammatory agent. Thus, FR167653 may be the source of new therapeutic candidates with a spectrum of activity distinct from current anti-inflammatory steroids.

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