A New Anti-Inflammatory Compound, FR167653, Ameliorates Crescentic Glomerulonephritis in Wistar-Kyoto Rats

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Abstract. The pathophysiologic effects of FR167653 were investigated in a model of crescentic glomerulonephritis induced by a small dose of nephrotoxic serum in Wistar-Kyoto rats. The rats developed crescentic glomerulonephritis by 6 d after the administration of serum. The subcutaneous administration of FR167653 (32 mg/kg) markedly decreased the severity of the renal damage. In a group of rats treated with FR167653 daily from day 0 to day 6, glomerular damage, including crescent formation and proteinuria, was virtually absent. FR167653 markedly decreased urinary levels of monocyte chemoattractant protein-1 (MCP-1). In addition, FR167653 reduced production of MCP-1 protein and transcripts in the diseased kidneys. In a group of rats for which treatment was initiated on day 3, shortly after the appearance of glomerular abnormalities, the progression of renal disease was appreciably retarded, with partial inhibition of MCP-1. In contrast, when rats were treated only on the first day, no beneficial effects were observed and severe proliferative and necrotizing glomerulonephritis, with crescent formation, was induced by day 6, with the upregulation of MCP-1. These results suggest that FR167653 may be effective against crescentic glomerulonephritis, possibly via the inhibition of MCP-1. In addition, there was marked reduction in renal injury even when FR167653 treatment was initiated after glomerular inflammation was established, suggesting that the therapeutic application of FR167653 may be clinically useful for human renal diseases.

In Wistar-Kyoto (WKY) rats, a very small dose of nephrotoxic serum induces severe proliferative and necrotizing glomerulonephritis with crescent formation that leads to glomerulosclerosis and interstitial fibrosis, resembling human crescentic glomerulonephritis (1). The pleiotropic cytokines interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α), which are produced mainly by activated monocytes/macrophages, lymphocytes, and resident renal cells, are believed to play an important role in the pathogenesis of human and experimental glomerulonephritis, especially crescentic glomerulonephritis (2–7). Although the two cytokines are structurally distinct and function through separate receptors, they share various biologic activities, i.e., the induction of mesangial cell proliferation and the recruitment and activation of monocytes and lymphocytes in diseased kidneys, directly or through increased synthesis of chemokines and lipid mediators by resident renal cells (2).

After IL-1 and TNF-α are produced, they induce various other inflammatory mediators, including chemokines, which may be involved in the inflammatory process (8). In addition, recent studies revealed that the C-C chemokine monocyte chemoattractant protein-1 (MCP-1) is involved in the pathogenesis of experimental crescentic glomerulonephritis (1) and human nephritis, including crescentic glomerulonephritis (9–11). Therefore, it is reasonable to speculate that the upregulation of IL-1β and TNF-α, followed by MCP-1, may be an important target of interventions to treat crescentic glomerulonephritis.

Various attempts to block the actions of proinflammatory cytokines and chemokines have been evaluated in animal models. Indeed, the blocking of IL-1 had tremendous effects on crescent formation in crescentic glomerulonephritis. IL-1 receptor antagonist reduced crescentic lesions via a decrease in macrophage accumulation (3). In addition, an absence of TNF-α has been demonstrated to have an inhibitory effect on the pathogenesis of crescentic glomerulonephritis (12). Antibodies against MCP-1 remarkably decreased crescent formation and proteinuria in experimental crescentic glomerulonephritis (1). However, whether single or simultaneous suppression of cytokines/chemokines would be more beneficial for the treatment of crescentic glomerulonephritis remains to be investigated. To elucidate the therapeutic possibilities for renal diseases, we examined the effects 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-phenylethanediene sulfate monohydrate), a new anti-inflammatory compound (13–16), on renal inflammation in an experimental crescentic glomerulonephritis model in WKY rats. Materials and Methods Animals

Inbred male WKY rats (10 wk of age; Charles River Japan, Atsugi, Kanagawa, Japan) were quarantined for 1 wk at the Animal Research Center of Kanazawa University, to confirm the absence of diseases before use. All procedures used in the animal experiments complied with the standards described in the Guidelines for the Care and Use of Laboratory Animals in Takara-machi Campus of Kanazawa University. Preparation of Anti-Rat Glomerular Basement Membrane Antibodies

Rat glomerular basement membrane was prepared using the method of Krakower and Greenspon (17). The preparation of anti-rat glomerular basement membrane antibodies was described previously (1). Specificity was confirmed by in vitro indirect immunofluorescence assays, using FITC-conjugated anti-rabbit IgG, on frozen sections of normal Wistar rat kidneys. Sharp linear immunofluorescence was observed only along the glomerular basement membrane. Experimental Design

Three separate experiments were performed.

Experiment I. Twelve male WKY rats were given intravenous injections of 0.1 ml of nephrotoxic serum on day 0. Six of 12 rats were treated with FR167653 subcutaneously, at a dose of 32 mg/kg in emulsion with methylcellulose, and the remaining six rats were treated with vehicle (methylcellulose) alone on day 0. The injections were administered at the time of induction of glomerulonephritis. Rats were euthanized on day 6, and blood samples were collected. Experiment II. Six rats were treated with FR167653 subcutaneously, at a dose of 32 mg/kg, and six rats were treated with vehicle alone, daily from day 0 to day 5. All animals were euthanized on day 6. Experiment III. Six rats were treated with FR167653 subcutaneously, at a dose of 32 mg/kg, and six rats were treated with vehicle only, starting on the third day after the induction of nephritis; all animals were euthanized on day 6.

In each experiment urine samples were collected, using a metabolic cage, from day −1 to the time of injection of nephrotoxic serum on day 0 and again from day 5 to the time of death. Histopathologic Studies

A portion of the renal tissue was fixed in 10% buffered formalin, followed by embedding in paraffin and staining with hematoxylin and eosin as well as periodic acid-Schiff reagent. Each section was evaluated by two independent observers with no prior knowledge of the experimental design. Total cell numbers were measured in at least 10 glomeruli for each rat and were expressed as the number per glomerular cross-sectional area. The number of crescentic formations was measured in at least 30 glomeruli for each rat and expressed as a percentage of total glomeruli. Another portion of renal tissue was rapidly frozen and stained with FITC-conjugated antiproliferative cell nuclear antigen (PCNA) (no. 033L245; Leinco Technologies, St. Louis, MO). To analyze the cell populations infiltrating glomeruli, cryostat sections were stained either with a mouse monoclonal antibody against rat tissue monocytes and macrophages (ED1, IgG1; BMA Biomedicals Ltd., Augst, Switzerland) or with a mouse monoclonal antibody against rat CD8 molecules (IgG1, no. 0412; Cedarlane Laboratories, Hornby, Ontario, Canada), followed by FITC-conjugated rabbit antimouse IgG (Organon Teknika Corp., Durham, NC). To determine the total number of PCNA/ED1-positive or PCNA/CD8-positive cells in each glomerulus, double-staining was performed using FITC-conjugated anti-PCNA antibodies and murine antibodies against ED1 or CD8, followed by Texas Red-conjugated rabbit anti-mouse IgG (Organon Teknika). Positive cells were counted in at least 30 randomly chosen glomeruli, and the results were expressed as the number of positive cells per glomerular cross section. Renal tissue specimens obtained from six normal WKY rats were used as negative controls. FITC-conjugated anti-rabbit IgG (no. 38236; Organon Teknika), FITC-conjugated anti-rat C3 (no. 38810; Organon Teknika), and FITC-conjugated anti-rat IgG (no. 38731; Organon Teknika) were used. The extent of fluorescence was evaluated in at least 50 glomeruli and was graded on a scale from 0 to 3 (0, negative; 1, scattered; 2, weakly diffuse; 3, strongly diffuse).

Determination of Urinary Protein Concentrations

Urinary protein concentrations were determined using the pyrogallol red method (18). Urinary protein excretion was expressed as the total amount excreted in a 24-h period. Detection of MCP-1 Protein in Diseased Kidneys

The presence of antigenic MCP-1 protein was demonstrated immunohistochemically in frozen tissue specimens by using the indirect avidin-biotinylated alkaline phosphatase complex method, with goat anti-rat MCP-1 antibodies, as described previously (1). Two independent observers, with no prior knowledge of chemokine levels or the experimental protocols, also examined the immunohistochemical findings.

Detection of MCP-1 Transcripts in Diseased Kidneys

Total RNA was extracted from the cortices and analyses were performed using reverse transcription-PCR, as described previously (19). Reverse transcription for 6 μg of total RNA, from six rats in each group (1 μg RNA/rat), was performed using a reverse transcription-PCR kit (Perkin Elmer, Foster City, CA). The cDNA product (1 μg) was amplified by PCR. Primers (5′ primer, CTCTTCTCCAC-CACTATGC; 3′ primer, CTCTGTCACTGGTCACTTCC) were used to detect MCP-1, as described previously (20). Ten microliters of PCR products was analyzed using 2% agarose gel electrophoresis; gels were stained with ethidium bromide. The housekeeping gene glyceraldehyde-3-phosphate dehydrogenase was used for PCR controls. Determination of Urinary MCP-1 Levels

To determine the effects of FR167653 on MCP-1 excretion in the urine, urinary MCP-1 levels were determined at 1 d with an enzyme-linked immunosorbent assay (ELISA), using a rabbit polyclonal antirat MCP-1 antibody as the capture antibody and a goat polyclonal anti-MCP-1 antibody as the second antibody, as described previously (8). This system is highly specific for MCP-1, because there were no cross-reactivities with other chemokines, including IL-8, platelet basic protein, platelet factor 4, regulated upon activation, normal T cell expressed and secreted (RANTES), and growth-related gene. The recovery rate was confirmed to be more than 95% for concentrations of up to 3 ng/ml in this ELISA system. The possible in vitro gener-
ation of MCP-1 in urine samples containing cells could be excluded with the immediate separation of urinary supernatants by centrifugation. All assays were performed in duplicate. The detection limit of this ELISA system was 40 pg/ml for rat MCP-1. Urinary MCP-1 levels were standardized on the basis of the amount of creatinine in the urine.

Statistical Analyses
The mean and SEM were calculated for all parameters determined in this study. Statistical analyses were performed using the unpaired t test and ANOVA. Values of $P < 0.05$ were considered statistically significant.

Results
Histopathologic Studies
Immunofluorescence analysis revealed no deposition of rabbit IgG in glomeruli from six normal WKY rats (data not shown). Rabbit IgG was detected in an intense linear pattern along the glomerular capillaries in specimens from nephrotoxic serum-injected rats treated with FR167653 or vehicle. Rat IgG and C3 were also detected, but the intensities were faint. Semiquantitative evaluation of deposition revealed no significant differences in the deposition of rabbit IgG, rat IgG, and rat C3 between glomeruli from rats treated with FR167653 and those from rats treated with vehicle only (data not shown).

Glomerular lesions exhibited endocapillary proliferation, severe necrotizing lesions, and crescent formation (Figure 1, C, E, and G). The total number of glomerular cells, which reached a peak on day 6, returned to normal levels in group II with the administration of FR167653 daily after the induction of glomerulonephritis (Figure 1, A and D, Table 1), whereas the number was significantly reduced in group III (Figure 1F, Table 1). In contrast, administration of FR167653 only on the day of induction of glomerulonephritis in group I did not alter the total number of glomerular cells (Figure 1B, Table 1). A considerable number of PCNA- and ED1-positive cells had infiltrated the glomeruli by day 3, and the numbers reached a peak on day 6, as reported previously (1). With the administration of FR167653, numbers of both PCNA- and ED1-positive cells were dramatically reduced in group II, and were reduced by approximately 60% compared with rats treated with vehicle only in group III (Table 1). Similarly, administration of FR167653 drastically reduced severe crescentic lesions and necrotizing lesions, which were observed on day 6 in control animals (Table 1). The administration of FR167653 daily in group II also affected the infiltration of CD8-positive lymphocytes, which are characteristic of this model (Table 1). To determine whether the reduced numbers of PCNA-positive cells were attributable to decreases in proliferation or recruitment, double staining was performed. PCNA/ED1-positive cell levels were particularly reduced by FR167653 treatment in group II. The reduced numbers of PCNA-positive cells were therefore attributable to decreased recruitment of macrophages. In addition, the small numbers of CD8/PCNA-positive cells were affected by FR167653 treatment (Figure 2).

Effects of FR167653 on Urinary Protein Excretion
Six normal untreated rats excreted minute amounts of protein in the urine (5.4 ± 0.6 mg/24 h) (Figure 3). In contrast, vehicle-treated nephritic rats in groups I, II, and III excreted markedly elevated amounts of protein in the urine on day 6 (23.0 ± 5.8, 22.9 ± 4.7, and 21.3 ± 4.3 mg/24 h, respectively) (Figure 3). Proteinuria was dramatically decreased by the administration of FR167653, with levels reverting to normal on day 6 in group II (5.8 ± 0.7 mg/24 h) (Figure 3). When
FR167653 treatment was delayed until the third day, there was still a significant reduction in proteinuria by day 6 (10.5 ± 0.7 mg/24 h), although the reduction was not as marked as that observed with continuous treatment (Figure 3). However, administration of FR167653 on day 0 in group I did not affect the urinary excretion of protein (22.5 ± 4.8 mg/24 h) (Figure 3).

Effects of FR167653 on MCP-1 Production

To determine the effects of FR167653 on MCP-1 production, renal tissue specimens from six rats in group II were immunohistochemically examined for antigenic MCP-1. MCP-1-positive cells were detected in the diseased glomeruli (particularly in crescentic lesions), vascular endothelial cells, infiltrating mononuclear cells, and tubular epithelial cells in rats treated with vehicle only (Figure 4A). Staining was specific for MCP-1, because neither control isotype-matched rabbit serum nor antibody absorbed with recombinant MCP-1 produced positive staining. In contrast, antigenic MCP-1 was detected faintly at day 6 in kidneys from FR167653-treated rats in group II (Figure 4B).

Effects of FR167653 on MCP-1 Transcript Levels

MCP-1 transcript levels in diseased kidneys were upregulated similarly in rats treated with vehicle only to MCP-1 protein levels (Figure 5). MCP-1 transcript levels were markedly decreased in FR167653-treated rats in group II and were partially decreased in rats in group III (Figure 5). However, MCP-1 transcript levels were not reduced in rats in group I, which were treated with FR167653 for only 1 d (Figure 5).

Effects of FR167653 on Urinary MCP-1 Levels

Urinary MCP-1 levels were determined, using an ELISA, 1 d after the induction of crescentic glomerulonephritis. Urinary MCP-1 levels were significantly elevated in rats treated with vehicle only in groups I and II (n = 12), compared with levels for normal rats (n = 6) (24.0 ± 13.9 versus 1.7 ± 1.0 pg/mg creatinine, P < 0.05) (Figure 6). Urinary MCP-1 levels were dramatically decreased and returned to normal in rats treated with FR167653 (2.0 ± 1.0 pg/mg creatinine; P < 0.05, compared with rats treated with vehicle only) in groups I and II (n = 12) (Figure 6).

Discussion

This study demonstrated that the administration of a new anti-inflammatory compound, FR167653, dramatically reduced proteinuria, prevented histologic changes, and markedly inhibited macrophage infiltration. Semiquantitative evaluation revealed no significant differences in the deposition of rabbit IgG, rat IgG, and rat C3 between glomeruli from rats treated with FR167653 and those from rats treated with vehicle only. These results suggest that the induction of glomerulonephritis was achieved equally. The results of this study, taken together, suggest that FR167653 may be effective in the treatment of crescentic glomerulonephritis.

The administration of FR167653 from day 0 to day 6 returned urinary protein levels to normal. FR167653 has been reported to suppress IL-1β and TNF-α, inducers of MCP-1.
We previously established that the administration of anti-MCP-1 antibodies completely prevented proteinuria by preventing the fusion of epithelial foot processes (1). Supporting this notion, FR167653 decreased MCP-1 transcript and protein levels in diseased kidneys in this study. One possible explanation for decreased proteinuria, as observed in this study, might be the prevention of damage to epithelial foot processes via MCP-1 suppression. In addition to decreased proteinuria, total numbers of glomerular cells and PCNA-positive cells were significantly reduced. Double immunostaining...

Figure 2. Results of double staining in glomeruli. To determine whether the reduced number of proliferating cell nuclear antigen (PCNA)-positive cells was attributable to decreased proliferation or recruitment, double staining was performed. The numbers of PCNA/ED1-positive cells (PCNA+ED-1) per glomerular cross section were markedly reduced by FR167653 treatment in group II. PCNA+CD8, PCNA/CD8-positive cells.

Figure 3. Effects of FR167653 on urinary protein excretion. Results for normal rats, rats treated with vehicle only, and rats treated with FR167653 in each group are shown. Administration of FR167653 markedly reduced proteinuria, to near normal levels, on day 6 in group II.
ing revealed that the reduced numbers of glomerular cells were primarily attributable to decreased recruitment of macrophages and partially attributable to decreased proliferation of macrophages, CD8-positive cells, and resident renal cells. IL-1β and TNF-α are reported to induce effector molecules, such as platelet-derived growth factor and basic fibroblast growth factor, from activated macrophages and renal parenchymal cells (21). Moreover, MCP-1 activates macrophages, in addition to recruiting macrophages (8). The inhibition of MCP-1 (as well as, in part, basic fibroblast growth factor and platelet-derived growth factor) may be responsible for the decreased numbers of glomerular cells and PCNA-positive cells. Therefore, it is tempting to speculate that the simultaneous blockade of IL-1β, TNF-α, and MCP-1 by FR167653 may prevent renal damage via the inhibition of effector molecules by activated macrophages and renal parenchymal cells.

FR167653 inhibited CD8-positive lymphocyte infiltration, which is characteristic of this glomerulonephritis model. Chemokines such as macrophage inflammatory protein-1α and RANTES, in addition to MCP-1, may be responsible for CD8-positive lymphocyte and macrophage infiltration (11,22).
duction of these chemokines is induced by cytokines such as IL-1α, IL-1β, TNF-α, and interferon-γ (23). FR167653 may therefore inhibit these chemotactic cytokines through the inhibition of IL-1β and/or TNF-α, resulting in complete inhibition of inflammatory cell infiltration into diseased kidneys.

In addition to suppressing cytokines and chemokines, FR167653 suppresses prostaglandin synthesis via the inhibition of cyclooxygenase-2 (COX-2) production; the inhibition of COX-2 mRNA by this drug is independent of IL-1β and TNF-α (16). COX-2 upregulation is a specific finding for patients with active lupus nephritis, and COX-2 and CD68 are often colocalized on the same cells (24). This indicates that monocytes infiltrating the glomeruli contribute to the exaggerated local synthesis of thromboxane A2. In addition, thromboxane A2 levels were increased in diseased kidneys, and selective inhibition of thromboxane A2 synthesis improved renal function (25). The amelioration of renal injury in this model that occurs with FR167653 may therefore be considered to be partly attributable to the inhibition of COX-2. Alternatively, a recent study revealed that in a crescentic glomerulonephritis model in rats, selective COX-2 inhibitors had less influence on MCP-1 expression than did COX-1 inhibitors (26). FR167653 may therefore be effective via the suppression of COX-2 production, as well as the inhibition of macrophage recruitment and activation by cytokines/chemokines. Moreover, recent studies demonstrated the new therapeutic targets and mechanisms of FR167653 in a transplantation model (27), in cardiovascular function (28), and in indomethacin-induced small intestinal lesions (29). Additional studies of the detailed molecular mechanisms of and indications for FR167653 are therefore needed.

IL-1β, TNF-α, and MCP-1 are upregulated in diseased kidneys with crescentic glomerulonephritis, soon after to several days after the injection of nephrotoxic serum (1,6). After subcutaneous injection of FR167653, at least 30 to 60 min are required before the systemic concentration of FR167653 reaches therapeutic levels: the half-life of FR167653 is then nearly 24 h (data not shown). A single injection of FR167653 was therefore not sufficient to prevent subsequent cytokine upregulation in the diseased kidneys in group I up to day 6. Most importantly, FR167653 partially but significantly ameliorated renal damage when administration was initiated on the third day. This result suggests that FR167653 treatment of sufficient duration may be effective even if renal damage has already commenced. This suggests a possible effect on already established human renal diseases for which cytokines and/or chemokines play an important role in pathogenesis. Collectively, these data suggest a promising future for the therapeutic application of FR167653 in human crescentic glomerulonephritis. In summary, we established that FR167653 treatment markedly ameliorated crescentic glomerulonephritis in a WKY rat model.

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