Inhibition of p38 Mitogen-Activated Protein Kinase Is Effective in the Treatment of Experimental Crescentic Glomerulonephritis and Suppresses Monocyte Chemoattractant Protein-1 but Not IL-1β or IL-6

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Activation of p38 mitogen-activated protein kinase (MAPK) is known to be important in cytokine production and cell survival in inflammation. This study examined the effect of inhibiting p38 MAPK after onset of renal injury in an experimental model of crescentic glomerulonephritis. Furthermore, this study investigated whether p38 MAPK inhibition would cause widespread suppression of the cytokine network in vivo or uncontrolled apoptosis. In the in vivo studies, daily treatment with a p38 MAPKα/β inhibitor was started 1 h (early treatment study) or 4 d (late treatment study) after induction of nephrotic nephritis in Wistar Kyoto rats. The treated rats remained healthy with normal weight gain during the study. Both early and late treatment with p38 MAPK inhibitor reduced renal monocyte chemoattractant protein-1 (MCP-1) levels, the number of glomerular macrophages, the severity of tissue injury, and proteinuria compared with the vehicle group. Unexpected, treatment with p38 MAPK inhibitor did not suppress renal levels of IL-1β or IL-6. In the in vitro study, the p38 MAPKα/β inhibitor reduced production of MCP-1 and IL-6 by TNF-α- or IL-1β-stimulated mesangial cells without any effect on cell viability or apoptosis. In conclusion, p38 MAPK inhibition is effective in reducing the severity of crescentic glomerulonephritis even when treatment is started after onset of disease. The therapeutic effect is associated with selective suppression of MCP-1, without widespread suppression of cytokine production or increased apoptosis. Therefore, p38 MAPK therapeutic blockade is a promising strategy in the treatment of antibody-mediated glomerulonephritis.


Proinflammatory cytokines and chemokines are important in the pathogenesis of glomerulonephritis. Inhibiting renal synthesis of these cytokines may reduce glomerular inflammation and prevent long-term tissue injury. p38 mitogen-activated protein kinase (MAPK) was initially identified in macrophages that were stimulated with LPS (1) and was subsequently found in most cells, including renal cells (2,3). The p38 MAPK family includes four isoforms—α, β, γ, and δ—that have heterogeneous expression in different cell types. α, β, and δ isoforms are widely expressed: p38α is highly expressed in leukocytes and endothelial cells; p38β is highly expressed in endothelial cells (4); p38γ is expressed predominantly in skeletal muscle; and p38δ gene expression is found in the lungs, kidneys, testis, pancreas, and small intestine.

Extracellular stimuli of the p38 MAPK pathway include a variety of cytokines, such as IL-1 and TNF-α (5). p38 MAPK has been shown to stabilize cytokine mRNA in many different cell types (6). Activation of p38 MAPK augments the production of TNF-α and IL-1 in stimulated monocytes in vitro (7). Therefore, p38 MAPK inhibitors are expected to inhibit not only the production of proinflammatory cytokines but also their downstream effects, thereby potentially ameliorating inflammatory and immune-mediated diseases. SB 203580, a widely used p38 MAPK inhibitor, has been investigated in vivo in several models of disease and has shown activity in TNF-α–mediated inflammation (8). This compound inhibited LPS-induced TNF production in a mouse model of endotoxin-induced shock. It was also effective in a variety of inflammatory models, such as DBA/1 collagen–induced arthritis and adjuvant arthritis.

In normal human kidney, phosphorylated p38 (p-p38) MAPK has been detected in the nuclei of a small number of podocytes, parietal epithelial cells, and tubular cells. Increased expression of p-p38 MAPK can be detected in both intrinsic renal cells and infiltrating leukocytes in glomerulonephritis, and this correlated with severity of renal dysfunction and histopathology (9). There are promising reports on the effect of p38 MAPK inhibition in prevention of experimental glomerulonephritis. Initially, Wada et al. (10) found that FR167653, a
novel inhibitor of IL-1β and TNF-α of unknown molecular mechanism, was effective against experimental crescentic glomerulonephritis when given within 3 days of induction of the disease. There was marked reduction in renal injury with downregulation of monocyte chemoattractant protein-1 (MCP-1), which plays an important role in the inflammatory process of crescentic glomerulonephritis (11,12). Subsequently, FR167653 was shown to inhibit p38α MAPK selectively (13).

Stambe et al. (14) found that in nephrotoxic nephritis (NTN) in rats, there was an increase in p38 activation within glomerular endothelial cells, podocytes, and infiltrating neutrophils, suggesting an important role for p38 MAPK in acute inflammation. Accumulation of glomerular macrophages and their activation is critical in pathogenesis of proliferative glomerulonephritis (15). When NPC31145, an inhibitor of p38α MAPK, was given before induction in a short-term model of NTN, treated rats had lower numbers of glomerular neutrophils and platelets but still had a large number of macrophages (16). The effect of NPC31145 on established glomerulonephritis has not been examined.

p38 MAPK also has a critical role in various cellular functions. For example, p38β MAPK has been reported to protect rat mesangial cells from TNF-α-induced apoptosis (17). Therefore, it is important to know whether inhibition of p38 MAPK would have undesirable side effects in vivo. Furthermore, because of the central role of p38 MAPK in cytokine synthesis and action, we were interested in the effect of inhibition of p38 MAPK on the levels of multiple cytokines that are produced in vivo. The main hypothesis in this project was that RO4399247-000, a specific inhibitor of p38 MAPKα/β, would reduce renal injury and downregulate MCP-1 and other proinflammatory cytokines in NTN. We therefore investigated whether inhibition of p38 MAPK was effective in reducing renal injury in both early and late treatment of crescentic glomerulonephritis in vivo and evaluated the impact of p38 MAPK inhibitor on the production of proinflammatory cytokines and on cell survival of glomerular mesangial cells in vitro.

We chose mesangial cells for the in vitro studies for several reasons. First, mesangial cells have a central position in the glomerular structure, with close proximity to the renal circulation through their cytoplasmic processes and proximity to glomerular basement membrane. Second, after intravenous injection of heat-aggregated Ig into rats in vivo, accumulation of aggregated Ig can be detected in mesangial cells within 10 min by immunoelectron microscopy (18). Third, mesangial cells express the relevant receptors for immune complexes and inflammatory cytokines that are responsible for initiation and progression of crescentic glomerulonephritis. Fourth, through the stimulation of these cell surface receptors, mesangial cells produce a range of chemokines and inflammatory mediators that are relevant to the pathogenesis of crescentic glomerulonephritis (19). Fifth, the mechanisms learned from cultured mesangial cells have been shown to be important in subsequent intervention studies in crescentic glomerulonephritis in vivo and in clinical studies in humans (20–23). Therefore, examination of glomerular mesangial cells is relevant to understanding the molecular mechanisms of crescentic glomerulonephritis.

The results of these studies show that treatment with a p38 MAPK inhibitor reduced renal MCP-1, glomerular macrophage number, and renal injury but to our surprise did not suppress renal IL-1β or IL-6.

Materials and Methods

Animals

Male Wistar Kyoto (WKY) rats that weighed between 190 and 260 g were used. Rats had free access to standard laboratory diet and water. All procedures were performed in accordance with the United Kingdom Animals (Scientific Procedures) Act.

Reagents

RO4399247-000 (molecular weight 414.4), a pyridinyl imidazole derivative that inhibits p38 MAPKα/β, was provided by (Roche Palo Alto LLC, Palo Alto, CA) (24). The relative specificity of RO4399247-000 was examined using a series of different assay panels, including binding affinity to a panel of 180 kinases using recently described technology (25). When tested at 10 μM in a panel of 180 kinases, RO4399247-000 was found to bind significantly to only a small number of kinases. RO4399247-000 bound with high affinity to the α and β but not to the γ and δ isoforms of p38 MAPK (Kd = 0.12 and 4.9 nM, respectively). RO4399247-000 did not display significant binding to other kinases, except for the c-Jun N-terminal kinase (JNK) family members, with JNK2 representing the next lowest Kd of 1.2 nM. In cell-free enzyme assays, RO4399247-000 inhibited the α and β isoforms of p38 MAPK with an IC50 of 1 to 2 nM and inhibited JNK activity only at very high concentration (IC50 = 284 nM). In TNF-α-stimulated SW1353 chondrosarcoma cells, RO4399247-000 inhibited phosphorylation of p38 substate Hsp27 with an IC50 of 9 nM and phosphorylation of JNK2 substrate c-Jun with an IC50 of 9000 nM. RO4399247-000 was dissolved in a carboxymethylcellulose solution that contained 0.9% benzyl alcohol (Aldrich, Poole, UK), 3 mM polyoxyethylenesorbitan monooleate (Tween 80 Sigma P8074), 0.15 M sodium chloride, and 5 μM food-grade low-viscosity sodium carboxymethylcellulose (Sigma, Poole, UK) pH 3.5 ± 0.1 in distilled water.

Induction of Experimental Glomerulonephritis

Rabbit nephrotoxic serum was prepared as described previously (26). NTN was induced in WKY rats by an intravenous injection of 0.1 ml of nephrotoxic serum. Rats were killed 1 h after intraperitoneal injection of bromodeoxyxuridine (BrdU) to assess glomerular cell proliferation.

Assessment of Renal Disease

In all experiments, urine was collected by housing rats in metabolic cages for 24-h periods with free access to food and water. Urinary protein was measured by the sulfosalicylic acid method (27). Rats were killed under isoflurane general anesthesia, and serum and kidneys were collected. Serum and urine creatinine was measured using an Olympus AU600 analyser (Olympus, Eastleigh, UK). Samples of kidney were fixed in both 10% formal saline and methyl Carnoy solution and then embedded in paraffin. Kidney tissue was also snap-frozen in isopentane, immersed in liquid nitrogen, and stored at −70°C.

Histology and Immunohistochemistry

Sections were stained with periodic acid-Schiff reagent and hematoxylin and eosin for determination of glomerular cellularity and glomerular fibrinoid necrosis. Fibrinoid necrosis was quantified in 100 consecutive glomeruli by scoring of the number of quadrants of each glomerulus. The percentage of glomeruli affected by crescents was
counted by examination of 100 consecutive glomeruli in periodic acid-Schiff–stained sections. The severity of glomerular crescents was assessed further by a scoring system. The glomerular crescent severity score was determined by examination of 100 consecutive glomeruli per section. Glomeruli were scored 0 for no crescent, 1 for a crescent that involved <50% of the glomerular circumference, and 2 for a crescent that involved >50% of the glomerular circumference. The glomerular crescentic severity score was the result of the following equation: [number of crescents score 1 + 2 × (number of crescents score 2)]/3. Tubulointerstitial injury was assessed and scored from 0 to 4.

Total monocytes/macrophages; monocytes/macrophages that expressed sialoadhesin (CD169), a marker of activation; and CD8+ cells were immunostained with mAb ED1, ED3, and OX8, respectively (Serotec, Oxford, UK) and quantified as we previously described (27,28). Renal expression of MCP-1 was detected using a goat polyclonal antibody (R-17) raised against a C-terminal peptide of rat MCP-1 (Santa Cruz Biotechnology, Santa Cruz, CA). Briefly, formalin-fixed, paraffin-embedded rat tissues were cut at 2 μm and dried overnight onto glass microscope slides. Sections were then dewaxed by placing the slides in xylene and rehydrated through a series of graded alcohols to water. The sections were microwaved in sodium citrate buffer for antigen retrieval. The following blocking steps were performed: Peroxide block for 30 min and avidin and biotin block for 15 min each with washing in PBS/T in between each step. The sections were incubated with 20% normal swine serum for 30 min at room temperature in a humidified chamber. After this, the primary antibody to MCP-1 was added at a dilution of 1:50 + 5% rat serum, and the slides were left at 4°C overnight. The slides were then washed and incubated in the secondary antibody, biotinylated swine anti-goat (Dako, Ely, UK), at a dilution of 1:200 for 45 min. The positive signal was amplified further by incubation of the slides in avidin and biotinylated enzyme complex (Dako) for 30 min at room temperature. The positive staining was visualized using diaminobenzidine (Sigma), and sections were counterstained with hematoxylin. The relative contribution of intrinsic glomerular cells and macrophages to expression of MCP-1 was assessed by two-color immunohistochemical staining for MCP-1 and macrophages. Paraffin sections were dewaxed and taken down to water. Endogenous peroxidase was blocked for 15 min. The slides were rinsed in tap water, and then antigen retrieval was performed as described previously. The slides were then blocked with 20% normal rabbit serum for 10 min. An avidin-biotin blocking step was then performed. After washing the slides in PBS, the first primary antibody to MCP-1 (Santa Cruz) was applied at a 1:50 dilution for 1 h at room temperature. The slides were then washed in PBS, and a rabbit anti-goat biotinylated (Dako) secondary antibody, diluted 1:200, was applied for 30 min at room temperature. The slides were then washed in PBS and the third layer, alkaline phosphatase–conjugated streptavidin (Roche Diagnostics, Lewes, East Sussex, UK), was applied for 30 min at room temperature. The slides were again washed in PBS, and the color was developed using a fast blue substrate kit (Vector Laboratories, Burlingame, CA) as per instructions. Once a suitable blue color had been achieved, the slides were rinsed in PBS and antigen retrieval was performed as before. The slides were rinsed in PBS, and an avidin-biotin blocking step was performed again. The slides were washed in PBS and incubated with the second primary antibody, ED-1 (Serotec, Oxford, UK), at a dilution of 1:500 overnight at 4°C. The slides were then again washed in PBS, and a secondary antibody, rabbit anti-mouse biotinylated (Dako) diluted 1:200, was applied for 30 min at room temperature. The slides were then washed in PBS, and the third layer, peroxidase-conjugated streptavidin (Roche Diagnostics), was applied for 30 min at room temperature. The slides were then washed in PBS, and the red color was developed using an AEC kit (Launch Diagnostics, Longfield, Kent, UK). Once a suitable red color had been developed, the slides were washed in PBS and coated in aquaperm (Thermo-Shandon, Astmoor, Cheshire, UK) and allowed to dry completely, after which the slides were mounted in Pertex (BDH, Poole, UK).

Intercellular adhesion molecule-1 (ICAM-1) expression was detected by immunohistology of 5-μ cryostat sections using a mouse anti-rat ICAM-1 mAb (clone 1A29) at 1:50 dilution (Serotec). Biotinylated goat anti-mouse (Dako) at a dilution of 1:200 was the secondary antibody. The positive signal was amplified further by incubation of the slides in avidin and biotinylated enzyme complex (Dako) for 30 min at room temperature. The positive staining was visualized using diaminobenzidine (Sigma), and sections were counterstained with hematoxylin. For quantification of any differences in immunostaining, glomeruli were examined under ×20 magnification, using an Olympus BX40 microscope (Olympus Optical, London, UK) mounted with a Photonic Science Color Coolview digital camera (Photonic Science, East Sussex, UK). Digital images were then captured and analyzed using Image-Pro Plus software (Media Cybernetics, Silver Spring, MD), and color segmentation was performed for each slide, individually defining pixels that contained the appropriate coloration. For each slide, 20 consecutive glomeruli were defined as an “area of interest,” and the percentage of each cross-sectional area that stained with the defined color was calculated. The final value for each slide was shown as the mean percentage area stained from 20 glomeruli.

Methyl Carnoy fixed tissue was used to detect BrdU (mAb BrdU; Dako). The protocol was similar to that used for formalin-fixed sections. An additional step after endogenous peroxidase block was required. This was to dissociate histones and partially denature the DNA. The sections were immersed in a pot that contained 1 M HCl, preheated to 60°C for 5 min. The primary antibody was used in 1:50 dilution, followed by detection with rabbit anti-mouse biotinylated secondary antibody at 1:200 dilution and streptavidin-biotin-peroxidase complex.

**Direct Immunofluorescence Microscopy**

Detection of rat and rabbit IgG in glomeruli was assessed on frozen sections by direct immunofluorescence microscopy as we have described previously (27).

**Homogenization of Frozen Kidney Tissues**

A stock concentration of homogenizing solution (PBS [pH 7.4] that contained 1 mM PMSF; Sigma-Aldrich) was prepared by dissolving 348 mg of PMSF in 10 ml of ethanol. This gave 10 ml of 200 mM PMSF (stock concentration), which can be stored for months at 4°C. On the day of homogenization, 10 ml of the homogenizing solution (1200 dilution of the stock concentration) was prepared by addition of 50 μl of PMSF to 10 ml of PBS (pH 7.4) on ice. Snap-frozen renal tissues were homogenized (1 ml/0.1 g kidney tissues) and sonicated with homogenizing buffer. The homogenate was then centrifuged to remove tissue debris. The supernatant was aliquotted and stored at −20°C.

**Sandwich ELISA for Rat MCP-1, TNF-α, IL-1β, and IL-6**

Sandwich ELISA was performed to assess the presence of cytokines in renal tissue homogenate, urine, serum, and cell culture supernatants. MCP-1 ELISA was performed using matched paired antibodies from BD Pharmingen (San Diego, CA) according to the manufacturer’s instructions. The sensitivity of the ELISA was 0.13 ng/ml. A similar protocol was used for rat TNF-α (R&D Systems, Abingdon, UK; sensitivity 0.25 ng/ml), rat IL-1β (R&D Systems; sensitivity 0.13 ng/ml), and rat IL-6 (R&D Systems; sensitivity 0.25 ng/ml).
Measurement of Serum Concentration of RO4399247-000

RO4399247-000 and internal standard were separated from serum by protein precipitation with the addition of acetonitrile. The samples were centrifuged, and an aliquot of the supernatant was diluted with 20 mM ammonium formate buffer (pH 4.4). RO4399247-000 and internal standard were analyzed on an Applied Biosystems Sciex API 4000, ionized by positive electrospray ionization, and detected by multiple reaction monitoring. The sensitivity of the assay is 0.0012 μM.

Cultured Rat Mesangial Cells

Mesangial cells from WKY rats were cultured in 24-well plates at approximately 0.5 × 10⁶ cells per well in RPMI (Invitrogen, Paisley, UK), 20% FCS, 5 ml insulin transferase, selenium ITS (Sigma), and 1000 U penicillin and streptomycin. Experiments were performed on cells at passages 8 through 10. Cells were changed to serum-free medium 24 h before stimulation. Then mesangial cells were incubated either with serum-free medium that contained p38 MAPK inhibitor in 0.2% DMSO with or control medium that contained 0.2% DMSO, for 1 h before addition of TNF-α or IL-1β. After 24 h, the supernatants were collected for measurement of MCP-1.

Cell viability and numbers were assessed by trypan blue exclusion assay of trypsinized cells and cell counting using a hemocytometer. Cell viability was assessed further by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). Apoptotic mesangial cells were assessed using Hoechst 33342 stain (Sigma, Poole, UK) and propidium

![Figure 1](image.png)

Figure 1. Effect of early treatment with p38 mitogen-activated protein kinase (MAPK) inhibitor on renal histology. Treatment with RO4399247-000, at a dosage of 30 mg/kg per d, was given by oral gavage, starting 1 h after induction of nephrotoxic nephritis (NTN). Renal tissues from day 6 after induction of NTN were examined. (A and B) Morphology of glomerular injury was assessed in hematoxylin- and eosin-stained, formalin-fixed, paraffin-embedded tissue sections. (C and D) Macrophage infiltration was assessed by immunoperoxidase staining using ED-1 mAb. In vehicle-treated rats, glomeruli were affected with fibrinoid necrosis and early cellular crescents (A) and showed intense macrophage infiltration (C). In p38 MAPK inhibitor–treated rats, the severity of glomerulonephritis (B) and macrophage infiltration (D) was reduced. The differences in glomerular fibrinoid necrosis score and cellular crescents are shown in E and F, respectively. Data are means ± SEM.
### Table 1. Effect of early treatment with p38 MAPK inhibitor on renal injury and cytokines in NTN in WKY rats

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<td>Body weight (g)</td>
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<td>233 ± 6.34</td>
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<td>All macrophage/gcs</td>
<td>15.1 ± 1.5</td>
<td>4.3 ± 2.4b</td>
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<td>CD8⁺ cells/gcs</td>
<td>1.6 ± 0.3</td>
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<td>Rabbit IgG (arbitrary unit)</td>
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<td>Serum creatinine (µmol/L)</td>
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<td>Renal homogenate IL-1β (pg/mg tissue protein)</td>
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<td>Renal homogenate IL-6 (pg/mg tissue protein)</td>
<td>608 ± 180</td>
<td>1776 ± 322</td>
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<td>Renal homogenate TNF-α (pg/mg tissue protein)</td>
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<td>Urine TNF-α (pg/d)</td>
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<td>Serum TNF-α (pg/ml)</td>
<td>74 ± 3</td>
<td>37.5 ± 5.1</td>
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aTreatment with p38 MAPK inhibitor (30 mg/kg per d orally) or vehicle was started 1 h after induction of nephrotoxic nephritis (NTN). Histology and cytokine measurements were performed 6 d after induction of nephritis. Serum and urinary concentrations of IL-1β and IL-6 were below the sensitivity of the ELISA used. Data are means ± SEM. gcs, glomerular cross-section; MAPK, mitogen-activated protein kinase.

bP < 0.05.

Statistical Analyses

Statistical analysis was performed using GraphPad Prism (GraphPad Software, San Diego, CA). Data are expressed as means ± SEM. Comparison between treated and vehicle groups was by two-tailed Mann-Whitney U test.

Results

Effect of p38 MAPK Inhibitor on NTN In Vivo

**Experiment 1: Early Treatment.** NTN was induced in 12 male WKY rats, which were randomized into two groups. RO399247-000 at a daily dosage of 100 mg/kg was shown to reduce severity of experimental arthritis in human TNF-transgenic mice (24). On the basis of pharmacokinetic studies (Stacie A. Dalrymple, Roche Palo Alto LLC, personal communication, February 10, 2003), a lower dosage of 30 mg/kg per d was chosen as the starting dosage for this study. In this experiment, the dosage of 30 mg/kg per d was found to be effective in reducing renal injury and therefore was used throughout the study. Six rats were treated with p38 MAPK inhibitor at a dosage of 30 mg/kg per d by oral gavage starting 1 h after induction of nephritis and continued daily up to day 5. The other six rats were given carboxymethylcellulose vehicle. Rats were housed in metabolic cages from day 5 to day 6 for 24-h urine collection. All rats were in good condition and survived until the end of the experiment.

Treated rats showed 71.5% lower glomerular macrophage counts (P < 0.05; Figure 1, Table 1), 78.3% reduction of glomerular fibrinoid necrosis (P < 0.005; Figure 1), 74% reduction of early cellular crescent (P < 0.05; Figure 1), and 93% reduction in proteinuria (P < 0.05; Figure 2) as compared with rats that received vehicle alone. There was no significant difference in the number of CD8⁺ cells in the glomeruli (Table 1). No difference was found in glomerular deposition of rabbit IgG between the groups using direct immunofluorescence staining (Table 1).

For examination of the effect of p38 MAPK inhibitor on inflammatory mechanisms, cytokines in the renal tissues, urine, and serum were measured by specific ELISA. In the p38 MAPK inhibitor–treated rats, the major effect was a 72% reduction in urinary MCP-1 (31.6% reduction; P = 0.13) and serum MCP-1 (38.3% reduction; P < 0.05; Figure 3). We also found a smaller effect of p38 MAPK inhibitor on levels of urinary MCP-1 (the major effect was a 72% reduction in MCP-1 in renal homogenates (P < 0.05; Figure 3). We also found a smaller effect of p38 MAPK inhibitor on levels of urinary MCP-1 (31.6% reduction; P = 0.13) and serum MCP-1 (38.3% reduction; P < 0.05; Figure 3). The amounts of TNF-α, IL-1β, and IL-6 in the renal tissue homogenates were not significantly different between treatment and vehicle groups (Table 1). The amount of renal IL-1β in nephritic rats was 12-fold higher than in normal renal tissue. The amount of IL-6 was approximately three times that of normal rats. For TNF-α, the amount was within the range seen in normal rats. There was no significant difference in the amount of TNF-α detected in the urine and serum of treated and untreated rats (Table 1). Neither IL-1β nor IL-6 was detectable in either serum or urine.
Experiment 2: Late Treatment. This experiment was designed to study the effect of p38 MAPK inhibitor on NTN when treatment was started after the disease was established. NTN was induced in 16 rats, which then were randomized into two groups. Eight rats were given 30 mg/kg per d p38 MAPK inhibitor by oral gavage starting at day 4. The other group of eight control rats was given vehicle. One rat from the treated group died a few hours after administration of the first oral gavage. This was likely to be related to oral gavage procedure rather than effect of the inhibitor. The other treated animals remained healthy throughout the study, with similar weight gain to the control group (Table 2). Rats were housed in metabolic cages from day 6 to day 7 and from day 13 to day 14 for 24-h urine collection. Rats were killed 1 h after intraperitoneal injection of BrdU. This experiment was repeated on another 16 rats to provide serum samples for measurement of RO4399247-000 and renal tissues for additional immunohistology.

On day 14 of this model, the main features of renal injury are severe cellular crescent formation with macrophage infiltration and early tubulointerstitial nephritis. Late treatment with p38 MAPK inhibitor starting at day 4 ameliorated glomerulonephritis significantly on day 14: 72.3% reduction in glomerular macrophages, 2.35% reduction in CD8+ cells, and 32.1% reduction in TNF-α.

Table 2. Effect of late treatment with p38 MAPK inhibitor on renal injury and cytokines in NTN in WKY rats

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<td>Body weight (g)</td>
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<td>Proteinuria (mg/d)</td>
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<td>Serum creatinine (μmol/L)</td>
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<td>50 ± 2.79c</td>
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<td>All macrophages/gcs</td>
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<tr>
<td>CD8+ cells/gcs</td>
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<td>Glomerular cell proliferation</td>
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<td>2.35 ± 0.3</td>
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<tr>
<td>Rabbit IgG (arbitrary units)</td>
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<td>Rat IgG (arbitrary units)</td>
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<td>Renal homogenate IL-1β (pg/mg tissue protein)</td>
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<td>Serum TNF-α (pg/ml)</td>
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aTreatment with p38 MAPK inhibitor (30 mg/kg per d orally) or vehicle was started 4 days after induction of NTN. Histology and cytokine measurements were performed 14 d after induction of NTN. Data are means ± SEM.

bP < 0.05.

cP < 0.005.
Rhinovirus (P/H11021 0.05; Figure 4, Table 2), 80.5% reduction in sialoadhesin-positive macrophage number (0.4 ± 0.1 versus 2.0 ± 0.5; P < 0.001), 38.8% (P < 0.0005) and 34.4% (P < 0.001) reduction in the frequency and the severity of cellular crescent formation, respectively, 50.3% reduction in tubulointerstitial injury (P < 0.001; Figure 4), and 18.9% reduction in proteinuria (P < 0.05; Figure 5) in comparison with the vehicle group. The treated group had lower serum creatinine levels (50 ± 2.79 versus 83 ± 4.19 μmol/L; P < 0.05) in comparison with the vehicle group. There was no significant difference in the number of CD8 cells in the glomeruli (Table 2). No difference was found in glomerular deposition of either rabbit or rat IgG between the groups using direct immunofluorescence staining. There was no significant difference in glomerular cell proliferation (Table 2).

Diffuse expression of MCP-1 was detected in the glomeruli and tubules in the vehicle-treated group. The intensity of glomerular MCP-1 was reduced in p38 MAPK inhibitor–treated rats. The amount of MCP-1 in the treated rats was lower in the renal tissue homogenate (31.2% reduction; P < 0.05), urine (88.9% reduction; P < 0.005), and serum (53.2% reduction; P < 0.05) in comparison with the vehicle group (Figure 6). Double
immunohistochemical staining for MCP-1 and macrophages showed that there was widespread expression of MCP-1 throughout the glomeruli in the vehicle-treated rats. In the ED-1–negative area, expression of MCP-1, shown as deep blue staining in Figure 7A, can be seen clearly in intrinsic glomerular cells. There was also expression of MCP-1 protein in the cells adjacent to the macrophages and some of the glomerular macrophages (double staining of red and deep blue in Figure 7A).

In the p38 MAPK inhibitor–treated rats, there was general reduction of glomerular expression of MCP-1, including the ED-1–negative areas (Figure 7B). Therefore, the reduction of glomerular MCP-1 protein level was at least partly due to reduction of MCP-1 in the intrinsic glomerular cells.

Surprisingly, the amounts of TNF-α, IL-1β, and IL-6 in the renal tissue homogenates were higher in the treated group than in the vehicle group (Table 2). At this time point, renal IL-1β in the p38 MAPK inhibitor–treated rats was 15-fold higher than in the normal rats. It is puzzling that an excessive amount of IL-1β was detected in renal tissue, whereas the severity of glomerulonephritis was reduced in the treated group. One possibility is that p38 MAPK inhibitor was effective in blocking the actions of IL-1β. Therefore, we examined the expression of ICAM-1, which is one of the downstream molecules from IL-1. Glomerular expression of ICAM-1 was lower in the treated rats (15.4% reduction; \( P < 0.05 \); Figure 8). Therefore, the increased amount of renal IL-1β in the p38 MAPK inhibitor–treated rats was not effective in increasing ICAM-1 expression in the kidneys.

The serum concentration of the p38 MAPK inhibitor was measured to relate this to the dosage-response curve of the cell culture study. On day 14, the peak concentration of RO4399247 was \( 0.85 \pm 0.16 \) μM, and the trough concentration was \( 0.003 \pm 0.0006 \) μM.

Figure 5. Effect of late treatment with p38 MAPK inhibitor on proteinuria. The treatment was started 4 d after induction of NTN. Twenty-four–hour urine was collected using metabolic cages on days 7 and 14. p38 MAPK inhibitor–treated rats had significant reduction of proteinuria on day 14 (\( P < 0.05 \)).

Figure 6. Effect of late treatment with p38 MAPK inhibitor on MCP-1. (A) In vehicle-treated rats, MCP-1 protein was detected in the glomeruli and tubules using goat anti–MCP-1 polyclonal antibody. (B) In the p38 MAPK inhibitor–treated rats, the glomerular MCP-1 protein was reduced. (C through E) MCP-1 protein was measured using sandwich ELISA specific for MCP-1. The horizontal dotted lines show the amounts of MCP-1 from normal rats. (C) Renal tissue was homogenized. The amounts of renal MCP-1 were expressed as a ratio to milligrams of protein. Rats that were treated with p38 MAPK inhibitor had significantly lower levels of MCP-1. (D) Urinary MCP-1 levels on days 7 and 14 were measured in 24-h urine that was collected while rats were in metabolic cages. The amount of urinary MCP-1 in p38 MAPK inhibitor–treated rats was significantly less than in vehicle-treated rats by day 14. (E) Serum MCP-1 concentration was significantly lower in p38 MAPK inhibitor–treated rats.
Effect of p38 MAPK Inhibitor on Mesangial Cells In Vitro

p38 MAPK is known to be involved in macrophage synthesis of cytokines. We were interested in the role of p38 MAPK in intrinsic glomerular cells. Therefore, we studied primary glomerular mesangial cells in vitro. We investigated whether p38 MAPK inhibitor was effective in suppressing MCP-1 and inflammatory cytokine production from glomerular mesangial cells and whether it affected the viability of the mesangial cells. Primary cultures of rat mesangial cells were stimulated with either TNF-α or IL-1β. Addition of the p38 MAPK inhibitor caused a dosage-dependent reduction of MCP-1 and IL-6 (Figure 9, A through D). We also attempted to investigate the effect of p38 MAPK inhibitor on mesangial cell production of IL-1β and TNF-α. We were not able to detect IL-1β- or TNF-α-stimulated mesangial cells, or vice versa. In other experiments, LPS was not able to induce production of IL-1β or TNF-α (data not shown). Incubation with p38 MAPK inhibitor up to a concentration of 10 μM did not cause any difference in cell viability (trypan blue exclusion assay and MTT assay) or total cell number (Figure 9, E and F). Fewer than 2% of mesangial cells were apoptotic (data not shown).

Discussion

In this study, we showed that p38 MAPK inhibitor was effective in downregulating MCP-1 and ICAM-1 and reduced glomerular macrophage infiltration and renal injury, even when treatment was initiated after onset of proteinuria. However, it was surprising that p38 MAPK inhibitor did not suppress renal IL-1β and IL-6. In mesangial cell culture, we found that p38 MAPK inhibitor was effective in reducing production of both MCP-1 and IL-6 at a similar concentration of the inhibitor to that detected in the in vitro studies. The differing effects of the p38 MAPK inhibitor in vitro and in vivo suggest that there may be compensatory mechanisms in vivo that allow IL-6 to escape from the effects of p38 MAPK inhibition. In fact, activation of the extracellular signal-regulated kinase pathway has been reported during blockade of p38 MAPK in a remnant kidney model in rats (29).

One interesting observation was the high concentration of renal IL-1β on day 14 in the treated rats; the amount of IL-1β was 15 times higher than that in normal rats and approximately twice that in the vehicle-treated rats. We investigated this further by examining the downstream effects of IL-1β and found that there was a significant reduction of glomerular ICAM-1 expression in the treated rats. Therefore, the p38 MAPK treatment was effective in blocking a downstream effect of the raised IL-1β levels in renal tissues. We also attempted to investigate the contribution of different cell types to the increased amount of IL-1β in the treated group. However, there was an excessive amount of background staining when we used immunohistology. In the in vitro study, cultured mesangial cells did not produce IL-1β protein when either LPS or TNF-α was added. The amounts of TNF-α in the renal tissue were within the range of normal rats. Therefore, the significance of the lack of suppression of TNF-α by the p38 MAPK inhibitor in vivo remains unclear.

Macrophages are the major effector cells in human and experimental proliferative glomerulonephritis (30–32). MCP-1 and ICAM-1 have been shown to be important in recruitment of macrophages and crescent formation in NTN in WKY rats (22,33). In this study, we demonstrated that treatment with a p38 MAPK inhibitor reduced both renal MCP-1 and ICAM-1 in vivo in association with a significant reduction in the number of glomerular macrophages, less renal injury as shown by renal histology, reduction in proteinuria, and lower serum creatinine. Wada et al. (10) found that administration of FR167653 at the time of induction of
glomerulonephritis reduced the renal cortical mRNA levels for MCP-1, although the protein concentration was not measured. Rovin et al. (3) showed that p38 MAPK inhibitor decreased IL-1β-induced MCP-1 synthesis in stimulated human mesangial cells in vitro. Another interesting issue is how the number of glomerular macrophages was reduced by late treatment with p38 MAPK inhibitor. There is evidence that glomerular macrophages are in dynamic balance between influx of new macrophages and cell proliferation and removal by apoptosis and efflux from the kidneys (34–36). In our study, treatment with p38 MAPK inhibitor suppressed expression of both MCP-1 and ICAM-1, which are key molecules responsible for recruitment of macrophages into inflamed glomeruli. At least a proportion of glomerular macrophages will be removed by apoptosis and efflux, although we did not examine this in our study. SB202190, another selective inhibitor of p38 MAPK, has been shown to induce apoptosis of an LPS-treated macrophage cell line (37).

Figure 8. Effect of late treatment with p38 MAPK inhibitor on intercellular adhesion molecule-1 (ICAM-1). Glomerular ICAM-1 expression was detected by immunoperoxidase technique. (A) Increased glomerular ICAM-1 expression was detected in all of the glomeruli of the vehicle-treated rats. (B) The intensity of glomerular ICAM-1 expression was reduced in p38 MAPK inhibitor–treated rats. (C) The difference in ICAM-1 expression was quantified using Image-Pro Plus software (P < 0.05).

Figure 9. Effect of p38 MAPK on mesangial cells in vitro. Primary cultures of WKY rat mesangial cells were examined. Mesangial cells were incubated with various concentrations of p38 MAPK inhibitors for 1 h, then either recombinant TNF-α or IL-1β was added. Production of cytokines and cell viability were assessed 24 h later. MCP-1, TNF-α, IL-1β, and IL-6 in the cell culture supernatant were measured with specific sandwich ELISA. (A) TNF-α–induced (0.4 ng/ml) production of MCP-1 was suppressed dosage dependently by p38 MAPK inhibitor. (B) IL-1β–induced (10 ng/ml) production of MCP-1 was suppressed dosage dependently by p38 MAPK inhibitor. (C) TNF-α–induced (10 ng/ml) production of IL-6 was suppressed by p38 MAPK inhibitor. IL-1β was not detected in any supernatants. (D) IL-1β–induced (10 ng/ml) IL-6 production was suppressed by p38 MAPK inhibitor. TNF-α was not detected in any supernatant. (E) Cell viability was assessed by trypan blue exclusion assay at various concentrations of p38 MAPK inhibitor. (F) Cell viability was assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay at a wider range of concentrations of p38 MAPK inhibitor.
Iwata et al. (38) used a murine model of lupus nephritis, which is considerably different from NTN. They found that FR167653 reduced the accumulation of macrophages and T cells and prevented kidney pathology, resulting in prolonged survival. This murine model of lupus nephritis is an autoimmune model that shows a gradual onset of the disease. Therefore, it is not possible to dissect the exact effects of treatment on the autoimmune response and subsequent inflammation. In the neutrophil-dependent phase of accelerated NTN in Sprague Dawley rats, Stambe et al. (16) found that NPC31145 reduced the number of glomerular neutrophils and platelets but not macrophages. Their results are not surprising, because there are a relatively small number of macrophages in the early stage of accelerated NTN (30). In the macrophage-dependent phase of glomerulonephritis, FR167653 reduced the number of glomerular macrophages (10).

Although the effect of p38 MAPK inhibitor on experimental glomerulonephritis has been reported previously (10,16,39), the results have varied. FR167653 was not originally designed to be a specific inhibitor of p38 MAPK. For NPC31145, the effect on the progression of glomerulonephritis has not been examined, and initiation of treatment 2 h before induction of nephritis is less relevant to the clinical situation. In this study, we examined the effects of a p38 MAPK inhibitor on experimental crescentic glomerulonephritis in the rat. We have shown that treating WKY rats with p38 MAPK inhibitor 1 h after inducing NTN dramatically reduced renal injury as shown by the significant reduction in glomerular macrophages, glomerular fibrinoid necrosis, and proteinuria. When treatment was started after the disease was already established at day 4, p38 MAPK inhibitor was also effective in reducing glomerular macrophage number, glomerular crescent formation, and proteinuria. These results demonstrate that p38 MAPK inhibitor is effective even when given at a late time point in the course of disease. Therefore, treatment with p38 MAPK inhibitor is likely to be applicable to the clinical situation.

Activation of p38 MAPK has also been investigated in other types of experimental renal disease. The results have varied depending on the exact model used. For example, treatment with p38 MAPK inhibitor reduced proteinuria in Adriamycin-induced nephrotic syndrome (40). However, treatment with FR167653 exacerbated proteinuria in complement-dependent passive Heymann nephritis (41). Treatment with NPC31169 in a remnant kidney model increased proteinuria and progression of renal failure (29). Therefore, the therapeutic application of p38 inhibitor depends on a better understanding of the role of p38 MAPK in specific disease mechanisms in different experimental and clinical situations.

It is a major concern that inhibition of p38 MAPK could disrupt any essential physiology. In our in vivo study, the well-being and healthy weight gain of the p38 MAPK inhibitor–treated rats support the feasibility of p38 MAPK inhibition as a therapeutic approach. This study did not show any differences in glomerular cell proliferation as assessed by quantitative scoring of BrdU-positive cells. The p38 MAPK inhibitor had no harmful effects in vitro, as demonstrated by the cell viability. Furthermore, analysis of renal cytokines in our in vivo study suggested that the p38 MAPK inhibitor used has a selective effect on MCP-1 without causing widespread suppression of cytokine production. Because activation of p38 MAPK is involved in a variety of inflammatory diseases, several inhibitors of cytokine synthesis with p38 MAPK selectivity are now in Phase I and II clinical trials for Crohn’s disease, rheumatoid arthritis, and chronic obstructive pulmonary diseases (4).

Conclusion

p38 MAPK inhibition is effective in reducing the severity of crescentic glomerulonephritis even when treatment is started after onset of disease. It showed selectivity in targeting MCP-1 production and did not downregulate IL-1β and IL-6. Effective late treatment suggests that p38 MAPK inhibitor may be clinically useful for human glomerulonephritis.

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