ABSTRACT

Antibody-mediated glomerulonephritis, including that resulting from immune complexes, is an important cause of renal failure and is in need of more specific and effective treatment. Binding of antibody or immune complexes to Fc receptors activates intracellular signal transduction pathways, including spleen tyrosine kinase (Syk), leading to the production of inflammatory cytokines. We examined the effect of R788 (fostamatinib disodium), an oral prodrug of the selective Syk inhibitor R406, in nephrotoxic nephritis in Wistar-Kyoto rats. Treatment with R788 reduced proteinuria, tissue injury, glomerular macrophage and CD8+ cell numbers, and renal monocyte chemoattractant protein-1 (MCP-1) and IL-1β, even when we started treatment after the onset of glomerulonephritis. When we administered R788 from days 4 to 10, glomerular crescents reduced by 100% (P < 0.001) compared with the vehicle group. When we administered R788 treatment from days 7 to 14, established glomerular crescents reversed (reduced by 21%, P < 0.001), and renal function was better than the vehicle group (P < 0.001). In vitro, R406 downregulated MCP-1 production from mesangial cells and macrophages stimulated with aggregated IgG. These results suggest that Syk is an important therapeutic target for the treatment of glomerulonephritis.

Glomerulonephritis is an important cause of renal failure and chronic kidney disease. Current treatment often involves the use of nonspecific immunosuppressive therapies, which may lead to severe side effects, including life-threatening sepsis and reduced fertility. More specific and effective therapy is clearly needed. Binding of antibody or immune complexes to Fc receptors is important in the pathogenesis of many types of glomerulonephritis. Mice with genetic defects in activating Fc receptors (FcyRI and FcyRIII) showed reduced severity of induced glomerulonephritis. Fc receptors have been shown to be important in accumulation of macrophages in experimental glomerulonephritis in Wistar-Kyoto (WKY) rats. Immunoreceptor tyrosine-based activation motif (ITAM)-containing Fc receptors have been shown to be important in the pathogenesis of experimental glomerulonephritis. Activation of spleen tyrosine kinase (Syk) is an important early step of FcR activation, leading to downstream inflammatory events. In this study, we examined the effect of R788 (fostamatinib disodium), a selective Syk inhibitor (a prodrug of active metabolite R406), in both prevention and treatment of experimental glomerulonephritis. The well-characterized model of nephrotoxic nephritis (NTN) in WKY rats was studied. This model has a rapid onset of disease, with macrophage infiltration reaching a maximum between days 4 and 7, fibrin deposition, and tissue destruction. By day 7, most of the glomeruli are affected by cellular crescents. In experiment 1, we examined the effect of R788 in prevention of glomerular injury compared with vehicle at day 7 (n = 8). The first dose of R788 was given by oral gavage 1 h before induction of glomerulonephritis. Twice-daily treatment with R788 at 15 mg/kg (n = 8) or 40 mg/kg (n = 8) reduced the severity of glomerular injury as shown by proteinuria (96% reduction, P < 0.05; 98% reduction, P < 0.001, respectively), glomerular fibrinoid necrosis (98% reduction, P < 0.01; 100% reduction, P < 0.01, respectively), glomerular macrophage number (82% reduction, P < 0.05; 99% reduction, P < 0.001, respectively), and glomerular CD8+ cells (59% reduction, not significantly different; 93% reduction, P < 0.001, respectively; Figure 1).

Experiment 2 was designed to examine the relevance of Syk inhibitor after the onset of disease, to model the clinical situation. NTN was induced in four groups of rats. In group I (to assess injury at the time of the start of treatment), rats received no treatment. Histology taken on day 4 showed increased numbers of glomerular macrophages (n = 4; Figure 2). In group II (control), rats were treated with vehicle from day 0 to day 10 (n = 8). All rats from group II developed severe crescentic glo-
merulonephritis, with crescents in 94 ± 1% of the glomeruli (Figure 2). In group III (prevention), rats received treatment with R788 at 40 mg/kg twice daily from day 0 to day 10 (n = 8). The severity of glomerulonephritis was reduced in the prevention group: 99% reduction of proteinuria (P < 0.001), 100% reduction in glomerular crescents, 99% reduction in glomerular macrophages (P < 0.01), 89% reduction in glomerular CD8+ cells (P < 0.001), and 33% reduction in serum creatinine (P < 0.001). In group IV (treatment), rats received R788 at 40 mg/kg twice daily from day 4 to day 10 (n = 8). The severity of glomerulonephritis was reduced in the treatment group: 98% reduction of proteinuria (P < 0.05), 99% reduction in glomerular crescents (P < 0.01), 99% reduction in glomerular macrophages (P < 0.01), 81% reduction in glomerular CD8+ cells (P < 0.01), and 31% reduction in serum creatinine (P < 0.01). NTN was then induced in another 12 rats to study the effect of treatment on renal cytokines. Six rats received vehicle, and another six rats received R788 at 40 mg/kg twice daily from day 4 to day 6. Renal monocyte chemoattractant protein-1 (MCP-1; 89% reduction, P < 0.05) and IL-1β (60% reduction, P < 0.005), but not TNF-α, were reduced by treatment with R788 (Figure 2). Differential effects of kinase inhibitors in renal
cytokines in vivo, which have also been observed in other studies, may be due to interaction of multiple cell types in vivo.\textsuperscript{13,18}

Experiment 3 was designed to examine whether Syk inhibitor was effective at even later stages of crescentic glomerulonephritis. NTN was induced in four groups of rats. In group I (to assess injury at the time of the start of treatment), rats did not receive any treatment ($n = 4$). Renal histology on day 7 showed that cellular crescents were present in $89 \pm 1.9\%$ of the glomeruli. In group II (control), rats received vehicle twice daily from day 7 to day 14 ($n = 8$). These rats had severe proteinuria and a high percentage of cellular crescents, and glomerular macrophages were detected on day 14. Rats in group III (low-dose treatment) received treatment with R788 at $15\text{ mg/kg}$ twice daily from day 7 to day 14 ($n = 8$). On day 14, late treatment of rats with R788 at $15\text{ mg/kg}$ showed reduction of the severity of glomerular injury: reduction of proteinuria (16%, $P < 0.05$), glomerular crescents (20%, $P < 0.05$), glomerular macrophages (54%, $P < 0.05$), and serum creatinine (24%, $P < 0.05$), but no changes in CD8+ cells (Figure 3). Rats in group IV (high-dose treatment) received treatment with R788 at $40\text{ mg/kg}$ twice daily from day 7 to day 14 ($n = 8$). On day 14, late treatment of rats with R788 at $40\text{ mg/kg}$ twice daily showed dose-dependent reduction of the severity of glomerular injury: reduction of proteinuria (23%, $P < 0.05$), glomerular crescents (21%, $P < 0.001$), glomerular macrophages (93%, $P < 0.001$), glomerular CD8+ cells (74%, $P < 0.01$), and serum creatinine (28%, $P < 0.001$; Figure 3).

We also compared the amount of antibody deposits in the glomeruli. In experi-
ment 1 (prevention), rats receiving Syk inhibitor had an increased binding of rabbit antibody to glomerular basement membrane (Figure 3). Increased rabbit antibody binding in the treated groups of rats is likely to be seen because antibody binding is easier to detect in the intact anatomy of the less-inflamed glomeruli. Rat antibody binding can be detected at 7 d after induction. The binding was similar in the vehicle and 15 mg/kg treatment group. At 40 mg/kg, there was a reduction of rat antibody binding (P < 0.05) in association with a lower level of circulating antibody to rabbit IgG (Figure 1). Indeed, Syk is known to be involved in B cell receptor signaling.19,20 In experiment 2, rats treated with Syk inhibitor from day 4 to day 10 showed similar glomerular deposition of rat IgG to the vehicle group. Therefore, in the treatment of established disease, the effect of Syk inhibitor is not dependent on altering antibody deposition.

It is not known whether Syk may also be important in production of cytokines by intrinsic glomerular cells. Therefore, we examined the effect of a Syk inhibitor (R406, the active drug) on cultured WKY mesangial cells. We found that Syk inhibitor had a dose-dependent effect in reducing MCP-1 synthesis in mesangial cells stimulated by heat-aggregated IgG (Figure 4). Syk is known to be important in macrophage production of proinflammatory cytokines.6,21 Glomerular macrophages have been shown to express FcR in NTN in WKY rats.5 We found that R406 inhibited production of MCP-1 from macrophages stimulated with aggregated IgG (Figure 4). Furthermore, Syk is required for monocyte/macrophage chemotaxis to fractalkine (CX3Cl1).22 Therefore, Syk inhibition may affect initial chemokine production, sensitivity of the macrophage to chemotactic signals, and secondary chemokine production from recruited macrophages.

There were no ill effects of the treatment in any of the experiments. We only detected weight loss (10 g) in the rats receiving 40 mg/kg R788 from day 0 to day 7 in experiment 1. The liver showed normal histology at the end of these experiments (Supplemental Figure 1). The reported side effects of R788 are neutropenia, diarrhea, hypertension, and elevation of liver enzymes in a small proportion of patients. These side effects are dose dependent and reversible on stopping the medication.23

R406 inhibited Syk-dependent cell assays, including activation of Fc receptor signaling in human macrophages, neutrophils, and mast cells, and B cell receptor signaling in B cells.9 Moreover, R406 potently inhibited Syk biochemical kinase activity. Furthermore, using anti-phosphopeptide Western blots, R406 was shown to inhibit most potently Syk activity, followed by the activity of Flt3, Jak, Lck, and c-Kit, albeit less potently (>5-fold).9

Figure 3. Treatment of NTN with Syk inhibitor 7 d after induction of disease is shown. NTN was induced in four groups of rats: I, nephrotoxic serum only (culled on day 7 for assessment of renal morphology); II, vehicle; III, low-dose late treatment (receiving 15 mg/kg R788 by oral gavage twice daily from day 7 to day 14); and (IV) high-dose late treatment group (receiving 40 mg/kg R788 twice daily from day 7 to day 14). (A) Proteinuria was detected in the vehicle group and was significantly reduced in the group receiving 40 mg/kg R788. (B) Cellular crescents were detected in approximately 90% of glomeruli. On day 14, severe crescentic glomerulonephritis persisted in the vehicle group, and crescents were reduced by 19 and 21% in the groups receiving late treatment with 15 and 40 mg/kg R788, respectively. (C) On day 14, there was a dose-dependent reduction of the number of glomerular macrophages. (D) On day 14, there was a significant reduction of CD8+ cells in the group receiving 40 mg/kg R788, in comparison with the vehicle group. (E) On day 14, there was a dose-dependent reduction of serum creatinine.
In summary, we found that Syk inhibitor is effective in reducing the severity of antibody-mediated crescentic glomerulonephritis in WKY rats, even when treatment is started after the onset of disease. These results are relevant to clinical situations in which patients present with proteinuria and microscopic hematuria, and renal biopsy shows crescentic glomerulonephritis. It is remarkable that the treatment is effective in reversing the histologic features of established crescentic glomerulonephritis, even when treatment was started 7 d after induction of disease. The in vitro study showed that Syk inhibitor has effects on mesangial cells in addition to the known effects on macrophages.24 Syk has been reported previously to mediate MCP-1 production via FcγR in mesangial cells.

Recently, oral administration of R788 also has been found to delay progression of renal disease and prolong survival in a murine model of spontaneous lupus.25 R788 also has been shown to be effective in treatment of patients with rheumatoid arthritis in a phase II clinical trial.23 Tyrosine kinases are important therapeutic targets; for example, imatinib, an inhibitor of tyrosine kinases, has been shown to reduce the severity of established experimental glomerulonephritis, including NTN, in association with reduction of glomerular IL-1β and MCP-1.18,26 Our results suggested that Syk inhibition has potential in the treatment of patients with glomerulonephritis.

**CONCISE METHODS**

**Syk Inhibitors**
The inhibitors R406 and R788 were provided by Rigel Pharmaceuticals (San Francisco, CA). The details of these two molecules have been reported previously.9,25

**Induction of Experimental Glomerulonephritis**
Rabbit nephrotoxic serum was prepared as described previously.10 NTN was induced in WKY rats by an intravenous injection of 0.1 ml of nephrotoxic serum.10 All procedures were performed in accordance with the United Kingdom Animals (Scientific Procedures) Act.

**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 and serum creatinine were quantified as described previously.13

**Histology and Immunohistochemistry**
Sections were stained with periodic acid–Schiff reagent and hematoxylin and eosin for assessment of glomerular crescents and glomerular fibrinoid necrosis. Fibrinoid necrosis on day 7 was quantified in 100 consecutive glomeruli by scoring the number of quadrants with fibrin deposition in each glomerulus. The percentage of glomeruli affected by crescents on days 10 and 14 was counted by examining 100 consecutive glomeruli in periodic acid–Schiff–stained sections. Total monocytes/macrophages and CD8+ cells were immunostained with monoclonal antibodies ED1 and MRC OX8, respectively (Serotec, Oxford, United Kingdom), and quantified by our previously described method.16 Detection of rat and rabbit IgG in glomeruli was assessed on frozen sections by direct immunofluorescence microscopy as described previously.16 Circulating antibodies to rabbit IgG were detected by ELISA.16

**Measurement of Renal IL-1β, TNF-α, and MCP-1**
Renal tissues were homogenized in lysis buffer containing 20 mM Tris-HCl (pH 7.5), 150 mM sodium chloride, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM sodium orthovanadate, and supplemented protease inhibitor cocktail (P8340; Sigma-Aldrich, St. Louis, MO). The cytokines were measured by specific sandwich ELISA as described previously.13 The sensitivity of the ELISA was 0.13 ng/ml for IL-1β, 0.25 ng/ml for TNF-α, and 0.13 ng/ml for MCP-1.

**Cultured Rat Mesangial Cells and Bone Marrow–Derived Macrophages**
Mesangial cells from WKY rats were cultured in RPMI (Invitrogen, Paisley, United Kingdom), 20% FCS, 5 ml of insulin transferase, selenium ITS (Sigma-Aldrich), 500 U/ml penicillin, and 5000 µg/ml streptomycin (Invitrogen) and 2 mM L-glutamine (Invitrogen). Experiments were performed on cells at passages 7 to 10. Bone marrow–derived macrophages were prepared by incubating bone marrow cells in L929 cell-conditioned media for 7 d. Cells were changed to serum-free medium 24 h before stimulation. Then, mesangial cells or macrophages were incubated with serum-free medium containing either R406 inhibitor in 0.05% DMSO, or control medium containing 0.05% DMSO, for 1 h before addition of heat-aggregated rat IgG (Sigma).11 After 24 h, the supernatants were collected for measurement of MCP-1. The cell viability was assessed by Trypan blue stain (Invitrogen).
Statistical Analysis
Statistical analysis was performed using GraphPad Prism (GraphPad Software, San Diego, CA). Data are expressed as mean ± SEM. Comparison between treated and vehicle groups was by Kruskal-Wallis test with Dunn multiple comparison test and Mann-Whitney U test.

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REFERENCES
5. Supplemental information for this article is available online at http://www.jasn.org/.