A Spleen Tyrosine Kinase Inhibitor Reduces the Severity of Established Glomerulonephritis

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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 < 0.01) 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.

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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 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 fibri

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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 < 0.05), 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

Figure 1. The effect of preventive treatment with R788 (a Syk inhibitor) on NTN in WKY rats is shown. (A) Treatment with R788 reduced proteinuria on day 7. Normal WKY rats have 2.1 ± 0.2 mg of proteinuria daily. (B) Renal morphology was assessed on day 7 in hematoxylin and eosin–stained renal tissue. In the vehicle group, there was severe glomerulonephritis with fibrinoid necrosis and infiltration of inflammatory cells. Glomerular injury was completely prevented in the R788-treated rats. Treatment with R788 prevented glomerular fibrinoid necrosis. (C) Glomerular macrophages were detected using ED1 mAb. Treatment with R788 resulted in dose-dependent reduction of the number of macrophages per glomerular cross-section (Mφ/gcs). (D) Treatment with R788 resulted in dose-dependent reduction of glomerular CD8+ cells. Normal rats had 0.3 ± 0.05 macrophages per glomerular cross-section and 0.15 ± 0.01 CD8+ cells. (E) Deposition of nephrotoxic antibodies (rabbit IgG) was detected by immunofluorescence. The mean fluorescence index (MFI) for glomerular rabbit IgG (nephrotoxic antibody) deposition was higher in rats treated with R788, in comparison with the vehicle group. (F) Deposition of rat IgG (autologous response to rabbit IgG) in the renal tissues was detected by immunofluorescence. The MFI for glomerular rat IgG deposition was lower in rats treated with 40 mg/kg R788 in comparison with the vehicle group. (G) Treatment with R788 reduced circulating anti-rabbit antibodies. Magnification, ×100 in B and C; ×60 in E and F.
cytokines in vivo, which have also been observed in other studies, may be due to interaction of multiple cell types in vivo.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: I, nephrotoxic serum only (culled on day 4 for assessment of renal morphology; II, vehicle; III, prevention (receiving treatment from day 0 to day 10); and IV, treatment group (receiving R788 from day 4 to day 10). (A) Proteinuria was detected in the vehicle group and was reduced to baseline in both prevention and treatment groups on day 10. (B) Increased numbers of inflammatory cells were detected in the glomeruli 4 d after induction of NTN. The number of glomerular macrophages remained elevated on day 10 in the vehicle group. The numbers of glomerular macrophages were reduced to very low numbers in both prevention and treatment groups on day 10. (D) Glomerular CD8+ cells were reduced to very low numbers in both prevention and treatment groups on day 10. (E) Serum creatinine was lower in both prevention and treatment groups. In normal rats, serum creatinine is 35.3 ± 9 μmol/L. (F) The MFI for glomerular rabbit IgG deposition was higher in rats in the prevention group and treatment group, in comparison with the vehicle group. (G) The MFI for glomerular rat IgG deposition was similar in the treatment group in comparison with the vehicle group. The MFI was significantly lower in the prevention group in comparison with the control group. (H) Renal cytokines were studied in two groups of rats: vehicle (days 4 to 6) and R788 (days 4 to 6). Renal IL-1β and MCP-1, but not TNF-α, were reduced by treatment with R788. Renal IL-1β was 26.1 pg/mg (23.4 to 47.3 pg/mg) total protein in the vehicle group and was reduced by treatment with R788 to 12.1 pg/mg (9.3 to 17.4 pg/mg), P < 0.005. Renal MCP-1 was 1331 pg/mg (739 to 6687 pg/mg) in the vehicle group and was reduced by treatment with R788 to 642 pg/mg (412 to 830 pg/mg), P < 0.05. Results are presented as median (quartiles) and analyzed by two-tailed Mann-Whitney U test.
ment 1 (prevention), rats receiving Syk inhibitor had an increased binding of rabbit antibody to glomerular basement membrane (Figure 1). 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).$^9$
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 effects on mesangial cells in addition to the known effects on macrophages. 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. Rabbit nephrotoxic serum was prepared as described previously. The inhibitors R406 and R788 were provided by Rigel Pharmaceuticals (San Francisco, CA). The cytokines were measured by specific sandwich ELISA as described previously. Circulating antibodies to rabbit IgG in glomeruli were assessed on frozen sections by direct immunofluorescence microscopy as described previously. 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), 5000 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). 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

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