Preventive and therapeutic effects of imatinib in Wistar-Kyoto rats with anti-glomerular basement membrane glomerulonephritis

Masayuki Iyoda[1], Takanori Shibata[1], Mio Kawaguchi[2], Toshimitsu Yamaoka[3] and Tadao Akizawa[1]

[1]Division of Nephrology, Department of Internal Medicine, Showa University School of Medicine, Tokyo, Japan; [2]Department of Respiratory Medicine, Institute of Clinical Medicine, University of Tsukuba, Tsukuba, Japan and [3]Division of Respiratory and Allergy, Department of Internal Medicine, Showa University School of Medicine, Tokyo, Japan

Imatinib is a selective tyrosine kinase inhibitor that can block activity of the platelet-derived growth factor receptor (PDGFR) and that has immunomodulatory effects on various cell types. Here we measured the protective effects of imatinib in Wistar-Kyoto rats with nephrotoxic serum nephritis, a kidney disease model where CD8+ T cells and macrophages play pathogenetic roles. Groups of animals were given imatinib from one day before up to 13 days following induction of nephritis and from day 7 to 20 following disease induction. Compared to control rats, at each time point imatinib treatment caused significantly less proteinuria, lowered serum blood urea nitrogen and creatinine, and decreased the number of glomeruli with necrosis, crescents, and fibrin deposits. Imatinib-treated rats had a significant reduction in glomerular macrophage accumulation and reduced renal cortical PDGFR-β and M-CSF receptor mRNA expression. Using colocalization we found that glomerular macrophages had reduced IL-1β and MCP-1 protein expression. Late imatinib treatment significantly reduced proteinuria, serum blood urea nitrogen, and creatinine, and reversed renal histopathological changes. We show that imatinib has renoprotective and therapeutic properties and provide pre-clinical work that will need to be confirmed in patients with crescentic glomerulonephritis.

Kidney International (2009) **75,** 1060–1070; doi:10.1038/ki.2009.43; published online 25 February 2009

KEYWORDS: anti-GBM disease; cytokines; imatinib; macrophage

Correspondence: *Masayuki Iyoda, Division of Nephrology, Department of Internal Medicine, Showa University School of Medicine, 1-5-8 Hatanodai, Shinagawa-ku, Tokyo 142-8666, Japan. E-mail: iyoda@med.showa-u.ac.jp*

Received 14 July 2008; revised 30 December 2008; accepted 7 January 2009; published online 25 February 2009

Crescentic glomerulonephritis (GN) is characterized clinically by the rapid deterioration of renal function and histologically by mononuclear cell infiltration in the glomeruli and tubulointerstitium, glomerular cell proliferation, necrotizing glomerular lesions, crescent formation, and eventual glomerulosclerosis. In Wistar-Kyoto (WKY) rats, a small dose of rabbit anti-rat glomerular basement membrane (GBM) antiserum (nephrotoxic serum, NTS) induces severe proliferative and necrotizing GN, with crescent formation resembling human crescentic GN. It has been considered that the cardinal pathological features of crescentic GN are mediated largely by the infiltration of monocytes/macrophages and T cells.[1,2] In addition, several studies have suggested that some types of growth factors, such as platelet-derived growth factor (PDGF), transforming growth factor-β, and connective tissue growth factor, may, at least in part, contribute to the process of glomerular crescent formation.[3–5]

Imatinib (Gleevec, Novartis Pharmaceuticals Co., Basel, Switzerland), a selective tyrosine kinase inhibitor that inhibits c-Abl, c-Kit, and PDGF receptors (PDGFRs), has been shown to be highly active in patients with Philadelphia chromosome-positive chronic myeloid leukemia and acute lymphoblastic leukemia, as well as in gastrointestinal stromal tumors.[6] The therapeutic benefit of imatinib in animal models of kidney diseases, such as mesangial proliferative glomerulonephritis,[7] chronic allograft nephropathy,[8] diabetic nephropathy,[9] lupus nephritis,[10,11] and unilateral obstructive nephropathy,[12] has been reported. In total, these studies have shown that the beneficial effects of imatinib therapy are the result of its inhibitory action on PDGF, leading to a reduction of glomerular cell proliferation and extracellular matrix accumulation.

Recently, imatinib has been shown to have potential immunomodulatory effects on various cell types, including monocytes/macrophages and T cells. Treatment with imatinib significantly inhibited glomerular macrophage infiltration in mouse models of diabetic nephropathy[9] and lupus nephritis.[10] Although the precise mechanism of the macrophage inhibition by imatinib remains obscure, there is evidence that imatinib has an inhibitory effect on

macrophage colony-stimulating factor (M-CSF)/c-fms signaling in macrophage development.[13,14] M-CSF, acting through its receptor c-fms, is the major macrophage growth factor, and it has been shown to play a key role in macrophage accumulation during renal tissue injury.[15] Recently, Paniagua *et al.*[14] reported that imatinib significantly inhibited c-fms and the activity of its downstream signaling molecule, Akt, in macrophage culture. On the other hand, imatinib also has inhibitory effects on T-cell proliferation and response. When stimulated through the T cell receptor and CD28, by mitogens or by antigens imatinib reduced the expansion of CD4+ and CD8+ T cells.[16–18] Sinai *et al.*[19] recently showed that imatinib induced the apoptosis of naive and memory CD8+ T cells *in vitro* and diminished memory CD8+ T cells' response to *Listeria monocytogenes* infection *in vivo*.

In this study, we showed that *in vivo* treatment with imatinib had preventive and therapeutic effects on rat anti-GBM glomerulonephritis induced by NTS in WKY rats by reducing glomerular macrophage influx and associated cytokine production in glomeruli, but not by reducing glomerular CD8+ cells.

RESULTS

Imatinib treatment reduces proteinuria in rats with NTS-N

The experimental design is described in Figure 1. The rats treated with vehicle developed proteinuria starting on day 4. In the early treatment study, urinary protein excretion in rats treated with imatinib was significantly decreased compared with that in controls at each time point (day 4, $P<0.01$; day 7, $P<0.05$; and day 13, $P<0.001$) (Figure 2a). In the late treatment study, urinary protein excretion of control rats reached 143.55 ± 15.17 mg/day on day 20, which was significantly higher than that on day 7 ($P<0.01$) (Figure 2b). Late imatinib treatment, which was performed from days 7 to 20, also significantly reduced the urinary protein level on day 20 ($P<0.01$) (Figure 2b).

Imatinib treatment preserves and reverses renal function in rats with NTS-N

Vehicle-treated rats with NTS nephritis (NTS-N) (WKY-Vehicle) had high serum blood urea nitrogen (BUN) and creatinine levels starting on day 4. In the early treatment study, treatment with imatinib significantly reduced serum BUN and creatinine levels in imatinib-treated rats compared with those similarly treated in control rats on days 4, 7, and 13 (BUN: $P<0.05$, $P<0.001$, and $P<0.05$; creatinine: $P<0.05$, $P<0.0001$, and $P<0.001$, respectively, Table 1). In the late treatment study, the serum BUN and creatinine levels of control rats with NTS-N (WKY-Vehicle) were 29.40 ± 0.96 and 0.34 ± 0.02 mg/100 ml, respectively, on day 20 (Table 1). On the other hand, the serum BUN and creatinine levels on day 20 were significantly lower in imatinib-treated rats than those in the control group (WKY-Vehicle) (BUN: 25.86 ± 1.57 mg/100 ml, $P<0.05$; creatinine: 0.25 ± 0.01 mg per 100 ml, $P<0.001$; Table 1).

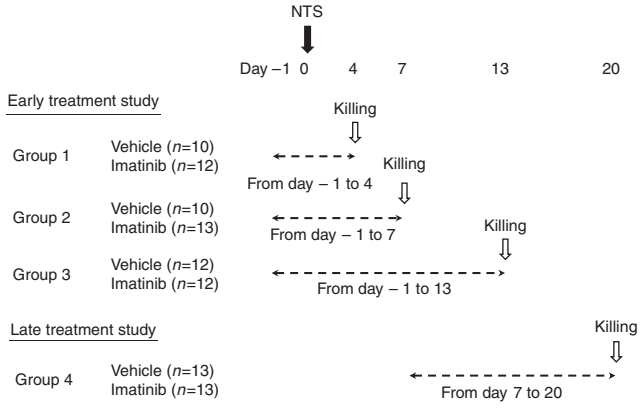

Figure 1 | Experimental design. A total of 95 female WKY rats were injected intravenously with 20 μl of NTS on day 0. Groups of animals were given either imatinib (50 mg/kg) or vehicle daily by an intraperitoneal injection from day −1 to day 4, 7, or 13 in the early treatment study, and from days 7 to 20 in the late treatment study; the rats were then killed. Vehicle-treated groups received an equal volume of sterile water.

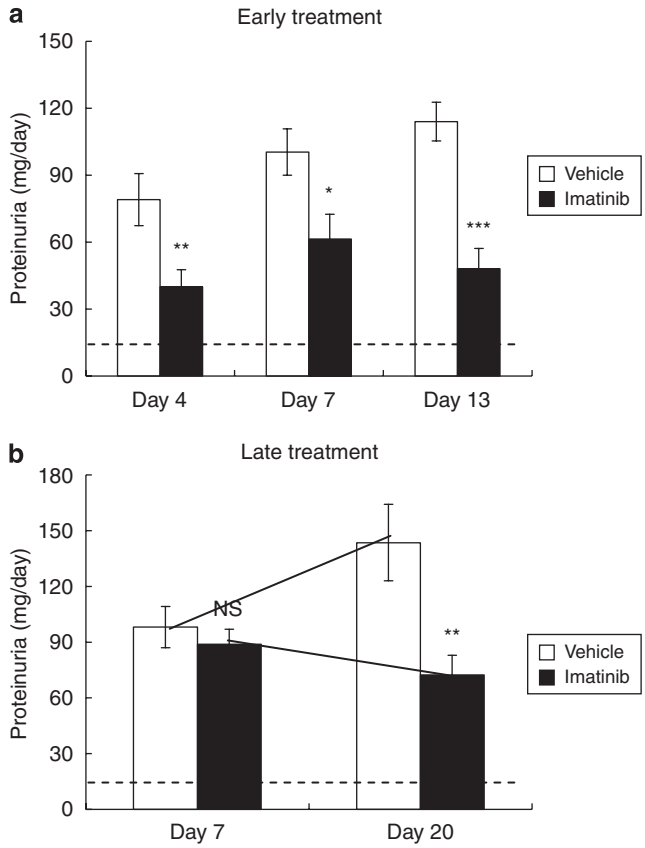

Figure 2 | Imatinib treatment reduces proteinuria in rats with NTS-N. Urinary protein levels in the (**a**) early treatment study and (**b**) late treatment study of vehicle-treated and imatinib-treated rats with NTS-N on the day indicated. The horizontal dotted lines show the level of proteinuria in normal WKY rats. Data are expressed as mean ± s.e.m. The Mann–Whitney test: $^{*}P<0.05$, $^{**}P<0.01$, and $^{***}P<0.001$ vs NTS-N rats with the same duration of vehicle treatment. NS, not significant.

Table 1 | Effects of imatinib on renal function by the study group

Group	BUN (mg/100 ml)	Cr (mg/100 ml)
WKY-NTS(−) (*N*=6)	22.75 ± 1.02	0.20 ± 0.00
Early treatment[a]		
WKY-Vehicle		
Day 4 (*N*=12)	32.48 ± 1.63	0.29 ± 0.02
Day 7 (*N*=13)	31.68 ± 0.85	0.34 ± 0.01
Day 13 (*N*=12)	32.00 ± 1.75	0.33 ± 0.01
WKY-Imatinib		
Day 4 (*N*=10)	20.89 ± 1.91**	0.24 ± 0.01*
Day 7 (*N*=10)	22.84 ± 1.38**	0.23 ± 0.01#
Day 13 (*N*=12)	25.43 ± 0.89*	0.24 ± 0.01***
Late treatment[b]		
WKY-Vehicle		
Day 20 (*N*=13)	29.4 ± 0.96	0.34 ± 0.02
WKY-Imatinib		
Day 20 (*N*=13)	25.86 ± 1.57*	0.25 ± 0.01***

BUN, blood urea nitrogen; Cr, creatinine; WKY, Wistar-Kyoto rats.
Data are mean ± s.e.m.
Mann–Whitney test: *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, #$P < 0.0001$ vs NTS-N rats with the same duration of vehicle treatment.
[a]The rats were treated with either vehicle or imatinib from day −1 to day 4, 7, or 13.
[b]The rats were treated with either vehicle or imatinib from day 7 to 20.

Imatinib reduces the percentage and the absolute number of peripheral CD8+ cells but not CD3+ and CD4+ cells in rats with NTS-N

The effect of imatinib on the circulating T-cell subsets was analyzed in the acute phase of NTS-N (days 4 and 7). When compared with vehicle treatment on day 7, imatinib treatment reduced significantly both the percentage and the absolute number of peripheral CD8+ cells (percentage: 27.92 ± 1.00 vs 22.49 ± 1.00%, $P<0.01$; absolute number: 6.91 ± 0.86 × 10^2/μl vs 3.55 ± 0.57 × 10^2/μl, $P<0.01$, vehicle-treated vs imatinib-treated rats, respectively) but not CD3+ and CD4+ cells (data not shown). Thus, there was a significant increase in the CD4+/CD8+ cell ratios (1.48 ± 0.08 vs 1.97 ± 0.15, $P<0.01$). However, there was no significant difference in the CD8+ cell percentage (24.65% ± 1.76% vs 26.04% ± 1.70%, NS, not significant) and number (8.22 ± 1.31 vs 6.77 ± 0.98 × 10^2/μl, NS) between vehicle- and imatinib-treated rats on day 4.

Early and late treatment with imatinib reduces fibrin deposition in rats with NTS-N

Prominent glomerular fibrin deposition was observed in control rats starting on day 4, with a peak on day 7 (Figure 3a). The semiquantitative evaluation of fibrin deposition showed a significantly reduced fibrin score in imatinib-treated rats compared with that in control rats at each time point (Figure 3e). Rabbit immunoglobulin G (IgG) was detected in an intense linear pattern along the glomerular capillaries in vehicle- and imatinib-treated rats with NTS-N (Figure 3c and d). Rat IgG was also detected along the glomerular capillaries on days 7, 13, and 20. Rat C3 staining

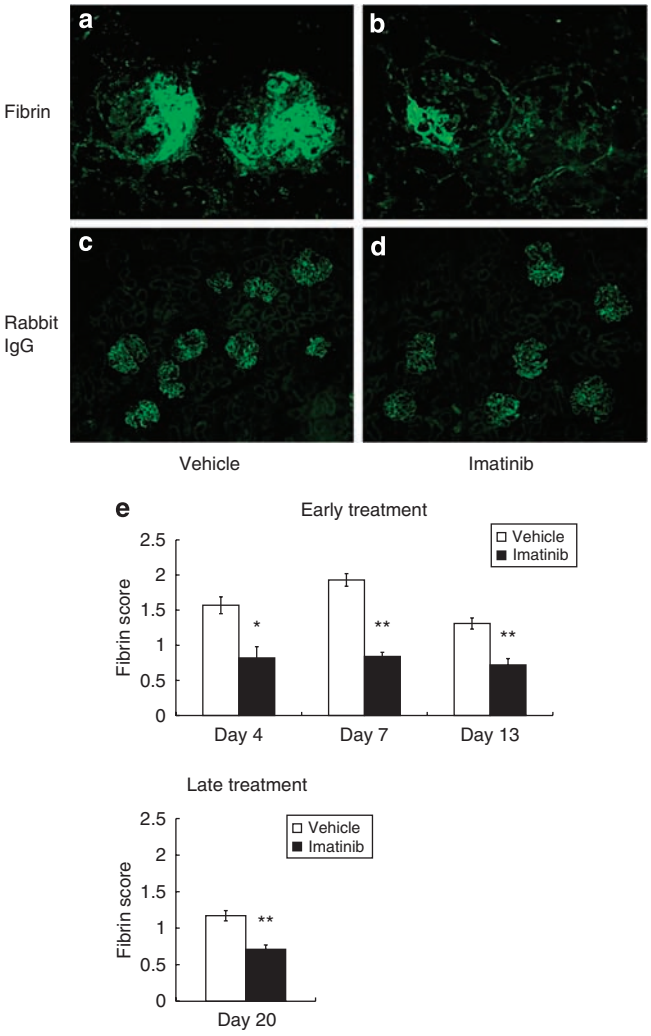

Figure 3 | Representative fibrin and rabbit IgG staining by immunofluorescence microscopy. Immunofluorescent staining for (**a**, **b**) fibrin and (**c**, **d**) rabbit IgG in (**a**, **c**) vehicle-treated NTS-N and (**b**, **d**) imatinib-treated NTS-N rats on day 7. (**e**) Semiquantitative assessment of fibrin deposition in each group. Each group contained 10–13 rats, and 50 glomeruli per rat were evaluated in a blind manner. Data are expressed as mean ± s.e.m. The Mann–Whitney test: *$P<0.01$ and **$P<0.001$ vs vehicle-treated NTS-N rats.

was faint at each time point. There was no significant difference in rabbit IgG, rat IgG, and rat C3 glomerular staining between vehicle- and imatinib-treated rats with NTS-N (data not shown). Furthermore, there was no significant difference in the levels of serum anti-rabbit IgG antibody between vehicle- and imatinib-treated rats with NTS-N at each time point (OD value, day 4: 0.37 ± 0.03 vs 0.36 ± 0.04, NS; day 7: 0.39 ± 0.03 vs 0.39 ± 0.03, NS; day 13: 0.31 ± 0.05 vs 0.30 ± 0.03, NS; and day 20: 0.76 ± 0.13 vs 0.90 ± 0.15, NS, vehicle-treated rats with NTS-N vs imatinib-treated rats with NTS-N; Normal WKY rats: 0.20 ± 0.002). These findings suggest that imatinib treatment does not significantly affect the process of heterologous antibody deposition or autologous antibody production.

Early and late treatment with imatinib ameliorates NTS-N

In control rats with NTS-N, the glomeruli on days 4 and 7 exhibited endocapillary proliferation, severe fibrinoid necrosis, and cellular crescent formation. Figure 4a and b shows typical silver-stained sections of glomeruli on days 4 and 7, respectively, in this group of rats. The crescentic glomeruli started to transform from cellular to fibrocellular on day 13, and there was a mixture of fibrocellular and fibrous crescents on day 20 (Figure 4c). Consistent with the urinary protein and serum creatinine data, crescent formation and fibrinoid necrosis were markedly reduced in imatinib-treated NTS-N rats (Figure 4d–f). The morphological analysis of renal tissue damage revealed that glomerular injury was ameliorated by early imatinib treatment, as well as by late imatinib treatment. Imatinib-treated NTS-N rats developed fewer crescents and less necrosis, and had a lower crescent score than did control rats at each time point (Figure 4g–i).

Early and late treatment with imatinib reduces macrophage accumulation but not CD8+ cell influx in NTS-N

To identify the cell phenotype of leukocytes affected by imatinib treatment, ED1+ and CD8+ cells in glomeruli were examined. Immunostaining for ED1 in control NTS-N rats showed intense glomerular staining, whereas significantly less staining was observed in imatinib-treated NTS-N rats at each time point (Figure 5a–d and g). The CD8+ cell numbers per glomerular cross-section in imatinib-treated NTS-N rats on days 4 and 7 were identical to those in vehicle-treated NTS-N rats (Figure 5e, f, and h).

Early and late treatment with imatinib reduces pro-inflammatory cytokine expression in NTS-N

As pro-inflammatory cytokines are fundamental in the pathogenesis of NTS-N, they were examined by immunohistochemistry. Immunostaining for interleukin (IL)-1β and monocyte chemoattractant protein 1 (MCP-1) showed a strong expression in the glomeruli of control rats. In contrast, significantly decreased glomerular staining was observed in imatinib-treated rats (Table 2, Figure 6a–d). Double immunostaining of ED1 with either IL-1β or MCP-1 was performed to confirm that a significant reduction of these molecules in glomeruli is associated with the inhibition of macrophage accumulation by imatinib treatment. A large number of ED1+ macrophages exhibited double staining for either IL-1β or MCP-1 in control rats with NTS-N (Figure 7a and c). In imatinib-treated rats, there was a potent reduction of glomerular macrophage accumulation, along with an inhibition of IL-1β or MCP-1 expression that was colocalized with glomerular macrophages (Figure 7b and d).

Early and late treatment with imatinib reduces PDGFR-β and c-fms expressions in NTS-N

To determine the importance of PDGFR signaling as a target of imatinib, the expressions of PDGFR-β and c-fms, which is known as a family of protein tyrosine kinase receptors, and their ligands PDGF-B and M-CSF, were studied. Imatinib treatment attenuated the PDGFR-β mRNA upregulation at each time point. On the other hand, a significant difference in the PDGF-B mRNA expression was seen only on day 13 ($P<0.01$). M-CSF is a fundamental chemokine for macrophage accumulation and local proliferation in NTS-N.[20] The ability of imatinib to inhibit M-CSF receptor c-fms activity is generally accepted.[13] In this study, imatinib treatment significantly attenuated the c-fms mRNA expression on day 7 ($P<0.001$), day 13 ($P<0.05$), and day 20 ($P<0.01$), whereas a significant difference in the M-CSF mRNA expression was found only on day 13 ($P<0.01$) (Figure 8a–d).

DISCUSSION

In this study, early and late imatinib treatment decreased urinary protein excretion, serum BUN and creatinine levels, glomerular crescent formation, and the necrotizing glomerular lesions, along with a dramatic reduction of glomerular macrophage accumulation and macrophage-associated pro-inflammatory cytokine protein expressions, in an experimental model of crescentic GN.

Our study's most substantial finding was that the administration of imatinib potently reduced glomerular macrophage influx, which in turn suppressed the protein expression of macrophage-associated cytokines, MCP-1 and IL-1β, in the glomeruli. The double staining of ED1 and these cytokines indicated that glomerular staining of these cytokines was macrophage-dependent and hence, the double staining was abolished, along with macrophage reduction, after imatinib treatment in NTS-N rats. Imatinib has been shown to reduce glomerular macrophage accumulation in animal models of kidney diseases, such as diabetic nephropathy[9] and lupus nephritis.[10] However, little evidence is available regarding the potential mechanisms of macrophage reduction by imatinib treatment in these animal models. Contrary to these reports, we found recently that imatinib treatment resulted in an increased accumulation of macrophages in the glomeruli in cryoglobulinemic membranoproliferative glomerulonephritis (MPGN).[21] This finding is consistent with the result of a study of imatinib treatment in the rat anti-Thy 1.1 model of GN.[22] Thus, the effect of imatinib on macrophages is pleiotrophic among experimental models of renal diseases. The explanation for this pleiotrophic effect awaits further studies.

In addition to suppressing pro-inflammatory cytokines, IL-1β, and MCP-1, imatinib inhibits PDGFR-β and c-fms expressions. Although several reports have suggested the upregulation of PDGFRs in human crescentic glomerulonephritis, little evidence is available about the profile of PDGFRs in this model. We found increased PDGFR-β and PDGF-B mRNA levels already on day 4 in NTS-N rats compared with normal WKY rats, and they remained upregulated thereafter, indicating that PDGF signaling pathway may be contributing to the pathogenesis of this model of glomerulonephritis. This increased PDGFR-β mRNA level was significantly reduced by imatinib treatment. Therefore, it is tempting to speculate that imatinib may prevent renal

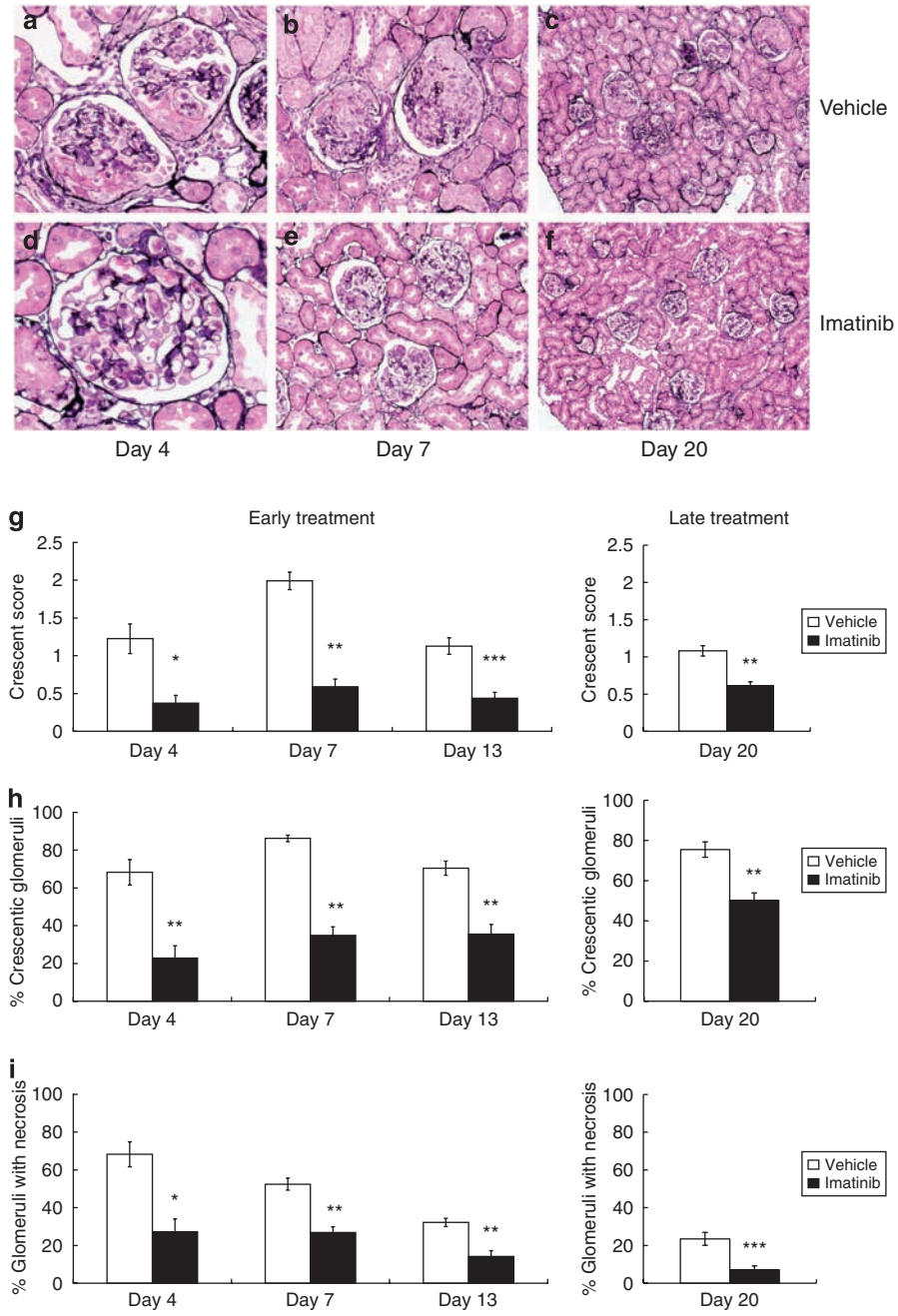

Figure 4 | Representative renal histology and quantification of crescent formation and necrotizing glomerular lesions. Renal histology on (**a**, **d**) day 4, (**b**, **e**) day 7, and (**c**, **f**) day 20 in (**a**–**c**) vehicle-treated and (**d**–**f**) imatinib-treated rats with NTS-N. Original magnification, ×400 (**a**, **d**), ×200 (**b**, **e**), and ×100 (**c**, **f**). (**g**–**i**) Semiquantitative assessment of the crescent score and quantitative assessment of crescent formation and fibrinoid necrosis in each group. Each group contained 10–13 rats, and 100 glomeruli per rat were evaluated in a blind manner. Data are expressed as mean ± s.e.m. The Mann–Whitney test: $*P<0.01$, $**P<0.001$, and $***P<0.0001$ vs vehicle-treated NTS-N rats.

damage at least in part through PDGF signaling blockade, consistent with other experimental models of glomerulonephritis. However, the manner by which the decrease of the PDGFR-β mRNA expression contributed to macrophage influx reduction, an essential feature of this study, remains unclear. On the other hand, this study provided *in vivo* evidence that imatinib treatment reduced renal cortical M-CSF receptor c-fms mRNA expression that was significantly upregulated compared with normal controls (WKY-NTS(-)) rats. M-CSF is a fundamental chemokine for macrophage accumulation and local proliferation in animal models of NTS-N and unilateral ureteral obstruction,[20] and its receptor c-fms belongs to the same family of protein tyrosine kinase receptors as PDGFR-α and PDGFR-β.[23,24] Thus, imatinib has the potential to inhibit the c-fms signaling pathway. Recently, Dewar *et al.*[13] showed that imatinib also

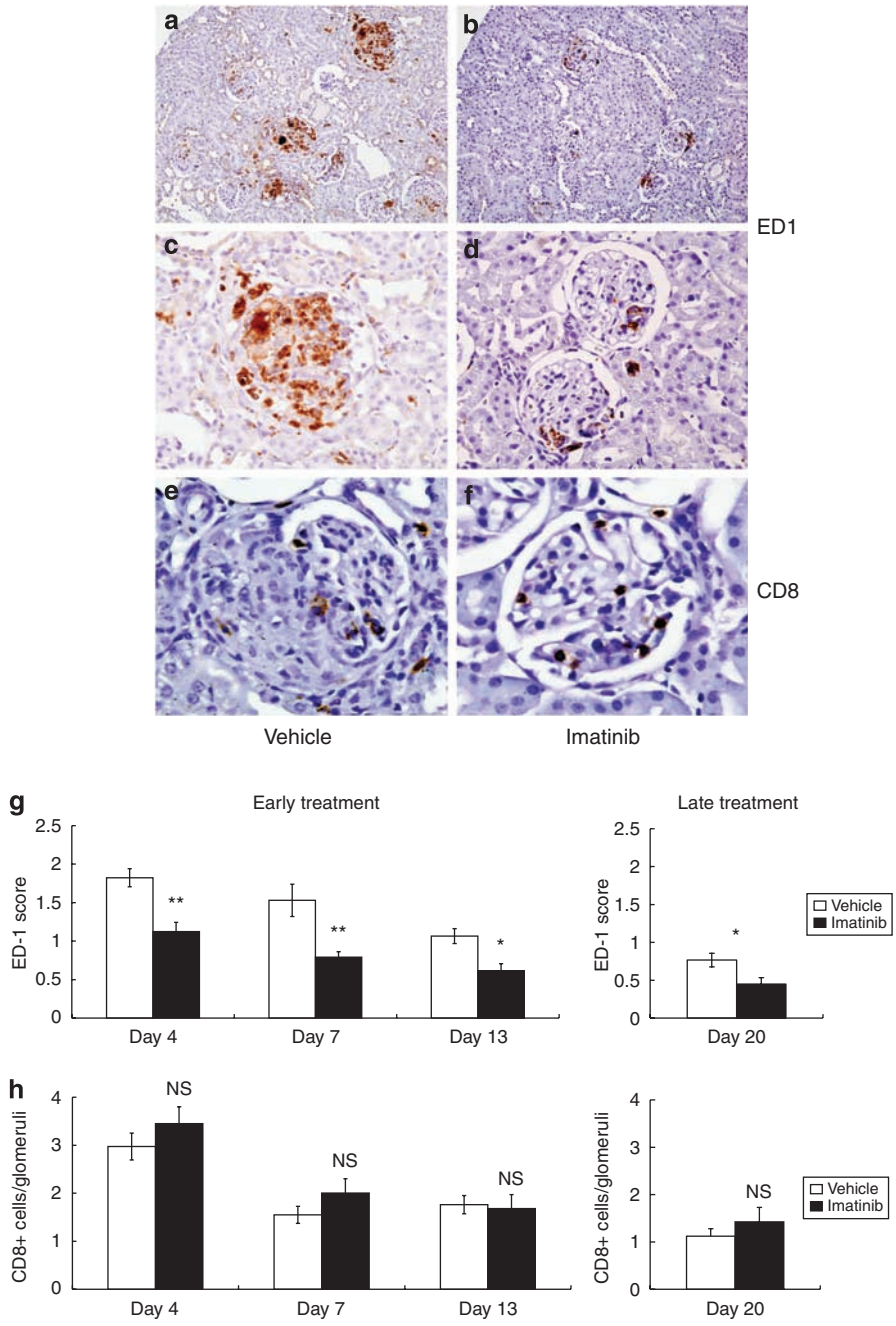

Figure 5 | Representative photomicrograph and quantification of macrophage infiltration in vehicle-treated and imatinib-treated rats with NTS-N. Immunostaining for (**a**–**d**) ED1 and (**e**, **f**) CD8 in (**a**, **c**, **e**) vehicle-treated and (**b**, **d**, **f**) imatinib-treated rats with NTS-N on day 7. (**g**) Semiquantitative assessment of the ED1 score and (**h**) quantitative assessment of CD8+ cells per glomerular cross-section in each group. Each group contained 10–13 rats, and 50 glomeruli per rat were evaluated in a blind manner. Data are expressed as mean ± s.e.m. The Mann–Whitney test: $^{*}P<0.01$ and $^{**}P<0.001$ vs vehicle-treated NTS-N rats.

specifically targets c-fms at therapeutic concentrations. Moreover, they found that the abrogation of c-fms tyrosine phosphorylation by imatinib was a direct mechanism for the inhibitory effect of imatinib on monocyte and/or macrophage growth and function *in vitro*.[25,26] These lines of evidence suggest that M-CSF/c-fms signaling inactivation may have been one of the fundamental factors in the mechanism of macrophage reduction by imatinib treatment in this study. The fact that imatinib did not reduce the c-fms mRNA expression on day 4 indicates that not only c-fms reduction but also multiple factors, such as chemokines, adhesion molecules, and co-stimulatory factors, play a part in glomerular macrophage reduction, especially in the early phase of this experimental model of GN.

Another important finding of this study is that imatinib treatment had a therapeutic effect in NTS-N even when it was

Table 2 | Results of IL-1β and MCP-1 staining

Group	IL-1β score	MCP-1 score
Early treatment[a]		
WKY-Vehicle		
Day 4 (*N*=12)	2.35 ± 0.24	2.32 ± 0.27
Day 7 (*N*=13)	1.68 ± 0.19	1.16 ± 0.16
Day 13 (*N*=12)	1.65 ± 0.14	1.05 ± 0.07
WKY-Imatinib		
Day 4 (*N*=10)	1.17 ± 0.12*	1.41 ± 0.21*
Day 7 (*N*=10)	0.90 ± 0.10*	0.45 ± 0.07*
Day 13 (*N*=12)	0.79 ± 0.12*	0.46 ± 0.08*
Late treatment[b]		
WKY-Vehicle		
Day 20 (*N*=13)	1.27 ± 0.15	0.78 ± 0.06
WKY-Imatinib		
Day 20 (*N*=13)	0.57 ± 0.05*	0.32 ± 0.08*

IL-1β, interleukin-1β; MCP-1, monocyte chemoattractant protein 1.
Data are mean ± s.e.m.
Mann–Whitney test: *$P < 0.01$ vs NTS-N rats with the same duration of vehicle treatment.
[a]The rats were treated with either vehicle or imatinib from day −1 to day 4, 7, or 13.
[b]The rats were treated with either vehicle or imatinib from day 7 to 20.

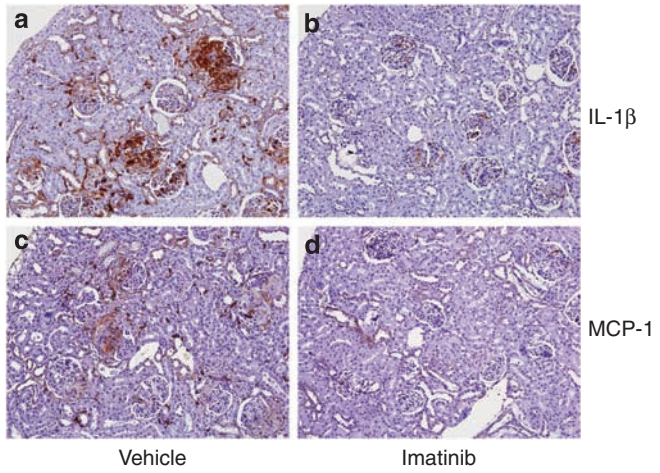

Figure 6 | Representative photomicrograph of immunostaining for IL-1β and MCP-1 in vehicle-treated and imatinib-treated rats with NTS-N. Immunostaining for (**a**, **b**) IL-1β and (**c**, **d**) MCP-1 in (**a**, **c**) vehicle-treated and (**b**, **d**) imatinib-treated rats with NTS-N on day 4.

administered after crescent formation and necrotizing lesions had been fully established. Late treatment with imatinib significantly accelerated glomerular injury repair and reversed renal dysfunction based on proteinuria and the serum BUN and creatinine levels. This suggests that imatinib has potential as a novel therapeutic agent for human crescentic glomerulonephritis. However, the molecular mechanism of the therapeutic effect remains obscure. The first possible mechanism is the reduction of macrophages and macrophage-associated cytokines, similar to the preventive effects of imatinib. In fact, glomerular macrophage accumulation was also attenuated, and the expressions of IL-1β and MCP-1 in glomeruli that were colocalized with macrophages were significantly suppressed by late treatment with imatinib. An interaction between macrophage accumulation and renal fibrosis in NTS-N has been suggested in earlier reports using an anti-MCP-1 antibody.[27,28] The second possible mechanism of action is PDGFR inhibition. Recently, we attenuated the established cryoglobulinemic MPGN in Thymic Stromal lymphopoietin (TSLP) transgenic mice using a PDGFR-β-specific inhibitor, APB5.[29] APB5 treatment significantly attenuated established extracellular matrix accumulation, but did not affect immunological factors, such as circulating cryoglobulins, serum Ig levels, or glomerular complement deposition. This result strongly suggests that the PDGFR-β inhibition is involved in the therapeutic effects of imatinib.

In experimental crescentic glomerulonephritis, the depletion of circulating CD8+ cells and the subsequent reduction of glomerular CD8+ cells with an anti-CD8 monoclonal antibody (OX8) have been shown to prevent the initiation and progression of renal injury in both WKY[2] and Sprague-Dawley rats.[30,31] In this study, the lymphocyte subpopulations determined by flow cytometry indicated a decrease in the peripheral CD8+ cell percentage and number, whereas the peripheral CD3+ and CD4+ cell numbers remained unchanged after imatinib treatment. The results of this study are consistent with recent reports that indicate the inhibitory effect of imatinib on CD8+ T cells in experimental[18,19] and clinical studies.[32,33] CD8+ cells appear to be indirectly involved in glomerular injury by accumulating monocytes/macrophages through the induction of cytokines and the upregulation of ICAM-1 in NTS-N.[30] Unexpectedly, imatinib treatment did not reduce glomerular CD8+ cell accumulation in NTS-N, indicating that the renal protective effect of imatinib in this crescentic nephritis model is independent of a decrease in circulating CD8+ cell number and percentage.

In conclusion, imatinib treatment showed renal preventive and therapeutic effects in rats with NTS-N, with a dramatic reduction of glomerular macrophage infiltration, which is an indispensable factor in the pathogenesis of this GN *in vivo*. These results strongly suggest a promising therapeutic use of imatinib in patients with necrotizing and crescentic GN, both during the initiation of injury and after the necrotizing and crescentic GN has been well established.

MATERIALS AND METHODS

NTS

NTS was prepared as described earlier.[34] The activity and specificity of NTS were tested by indirect immunofluorescence microscopy of frozen sections of a normal rat kidney.

Experimental protocol

The experimental protocol for this study was reviewed and approved by the Animal Care Committee of Showa University in Tokyo. Seven-week-old female WKY rats weighing 150 g were purchased from Charles River Japan (Atsugi, Kanagawa, Japan) and used in all the experiments. The animals were housed in the animal care facility of Showa University (25 °C, 50% humidity, 12-h dark/light cycle)

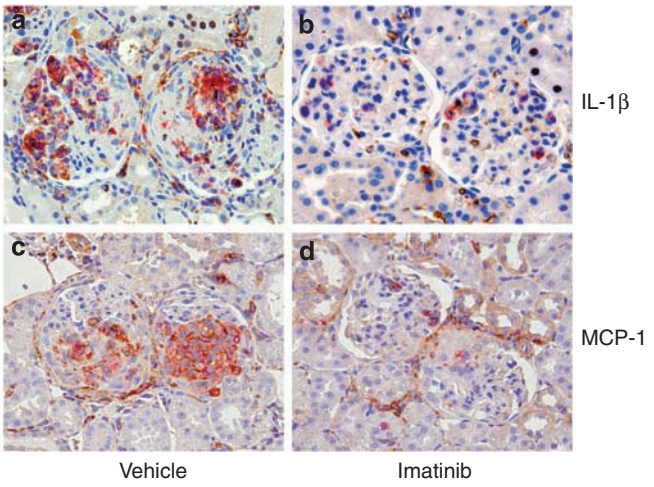

Figure 7 | Double immunostaining for ED1 with either IL-1β or MCP-1 in vehicle-treated rats with NTS-N on day 7. Kidney sections were stained using two-color immunohistochemistry with ED1 being stained red, and (**a**, **b**) IL-1β and (**c**, **d**) MCP-1 stained brown in (**a**, **c**) vehicle-treated and (**b**, **d**) imatinib-treated rats. A large number of ED1+ macrophages in the glomeruli were double stained by IL-1β and MCP-1.

with free access to food and water. A total of 95 female WKY rats were injected intravenously with 20 µl of NTS on day 0. Groups of animals were given either imatinib (50 mg/kg; Novartis Pharmaceuticals, Basel, Switzerland) or vehicle daily by an intraperitoneal injection, from day −1 to day 4, 7, or 13 in the early treatment study, and from days 7 to 20 in the late treatment study; the rats were then killed (Figure 1). Vehicle-treated groups received an equal volume of sterile water. Six female WKY rats at the age of 7 weeks were used as normal controls. At the end of the study, the rats were anesthetized, their blood was collected by cardiac puncture, and their organs were collected. Renal tissue was divided; some portions were snap-frozen in liquid nitrogen and some portions were fixed in 2% paraformaldehyde/ phosphate-buffered saline (PBS) for later use.

Proteinuria and creatinine determination

For analysis of proteinuria, the rats were housed individually in metabolic cages for the 24-h urine collection. Urine samples were collected on the day before killing. Urinary protein was determined using the Biuret method. BUN and creatinine levels were measured using an automated analyzer (Hitachi, Tokyo, Japan) according to the manufacturer's instructions.

Measurement of circulating anti-rabbit IgG antibody

The level of circulating anti-rabbit IgG antibody in rats with NTS-N was measured by ELISA, according to the ELISA procedure as described earlier.[34] Microtiter plates (Nunc, Roskilde, Denmark) were coated with 5 µg/ml of purified rabbit IgG (ZYMED Laboratories, San Francisco, CA, USA). Plates were then incubated with 1:400 diluted serum samples from nephritic rats. The assay was developed with an alkaline phosphatase-conjugated rabbit anti-rat IgG antibody (Sigma Chemical Co., St Louis, MO, USA). The absorbance at 405 nm was analyzed with microplate reader.

Flow cytometry

Isolated lymphocytes from peripheral blood were labeled with specific monoclonal antibodies (CD3-PE, CD4-FITC, and CD8-PE; BD Pharmingen, San Diego, CA, USA). The proportion of cells positive for these molecules was determined by flow cytometry (FACScan, BD Pharmingen), and the results were analyzed using Cell Quest software.

Light microscopic study

Tissues fixed in 2% paraformaldehyde/PBS were embedded in paraffin using routine protocols. The paraffin-embedded tissues were sectioned at 4 µm for routine staining with hematoxylin and eosin and periodic-acid Schiff. Two-µm-thick sections were used for periodic-acid methenamine silver stains (silver). The number of crescentic or necrotizing glomeruli per 100 glomeruli of each rat was calculated and expressed as a percentage. The percentage of area occupied by crescents in each glomeruli was estimated and assigned one of the following scores: 0, absent; 1, $<1/4$; 2, between 1/4 and 1/2; 3, between 1/2 and 3/4; and 4, more than 3/4 of the whole glomerulus.[35] The mean score of 50 glomeruli was then calculated as the crescent score.

Immunohistochemistry

The monoclonal antibodies used in this study were mouse anti-rat ED1 antibody (BMA, Augst, Switzerland) as a macrophage marker; mouse anti-rat IL-1β antibody (clone number: SILK 6; Cosmo Bio, Tokyo, Japan); and mouse anti-rat CD8 antibody (clone number: X8; Antigenix America, Huntington Station, NY, USA). In addition, polyclonal antibodies against goat anti-rat MCP-1 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and goat anti-rat IL-1β (Santa Cruz Biotechnology) were also used. Biotinylated rabbit anti-mouse IgG or anti-goat IgG antibody and peroxidase-conjugated streptavidin (LSAB 2 Kit/HRP) were purchased from Dako (Glostrup, Denmark).

Immunohistochemical staining for ED1, CD8, IL-1β, and MCP-1 was performed as follows: the paraffin sections of renal tissues were dewaxed, washed in PBS, drained, and incubated overnight at 4 °C, with the anti-ED1, CD8, IL-1β, and MCP-1 antibodies as the primary antibodies. They were then washed thrice in PBS and incubated with a secondary antibody, the biotinylated rabbit anti-mouse IgG or the anti-goat IgG antibody. After the endogenous peroxidase was inactivated by incubation with 0.3% H_2O_2 in methanol, the sections were incubated with an LSAB 2 Kit/HRP and developed using diaminobenzedine (DAKO) as the substrate to produce a brown stain, and the sections were counterstained with hematoxylin.

Two-color immunostaining was used to detect the colocalization of IL-1β/ED1 and MCP-1/ED1. The paraffin sections were dewaxed, washed in PBS, drained, and incubated overnight at 4 °C, with the goat anti-IL-1β (1:10 dilution) or MCP-1 (1:15 dilution) antibodies as the primary antibodies. They were then washed thrice in PBS and incubated with a secondary antibody, the biotinylated rabbit anti-goat IgG antibody. After the endogenous peroxidase was inactivated by incubation with 0.3% H_2O_2 in methanol, the sections were incubated with LSAB 2 Kit/HRP and developed using diaminobenzedine (DAKO) as the substrate to produce a brown stain. To denature all antibodies bound to the tissue, the sections were microwaved twice for 5 min. The sections were then washed in PBS and incubated with the second mouse monoclonal antibody, ED1 (1:50 dilution). The slides were once again washed in Tris-buffered saline (TBS) and incubated with the secondary antibody, rabbit

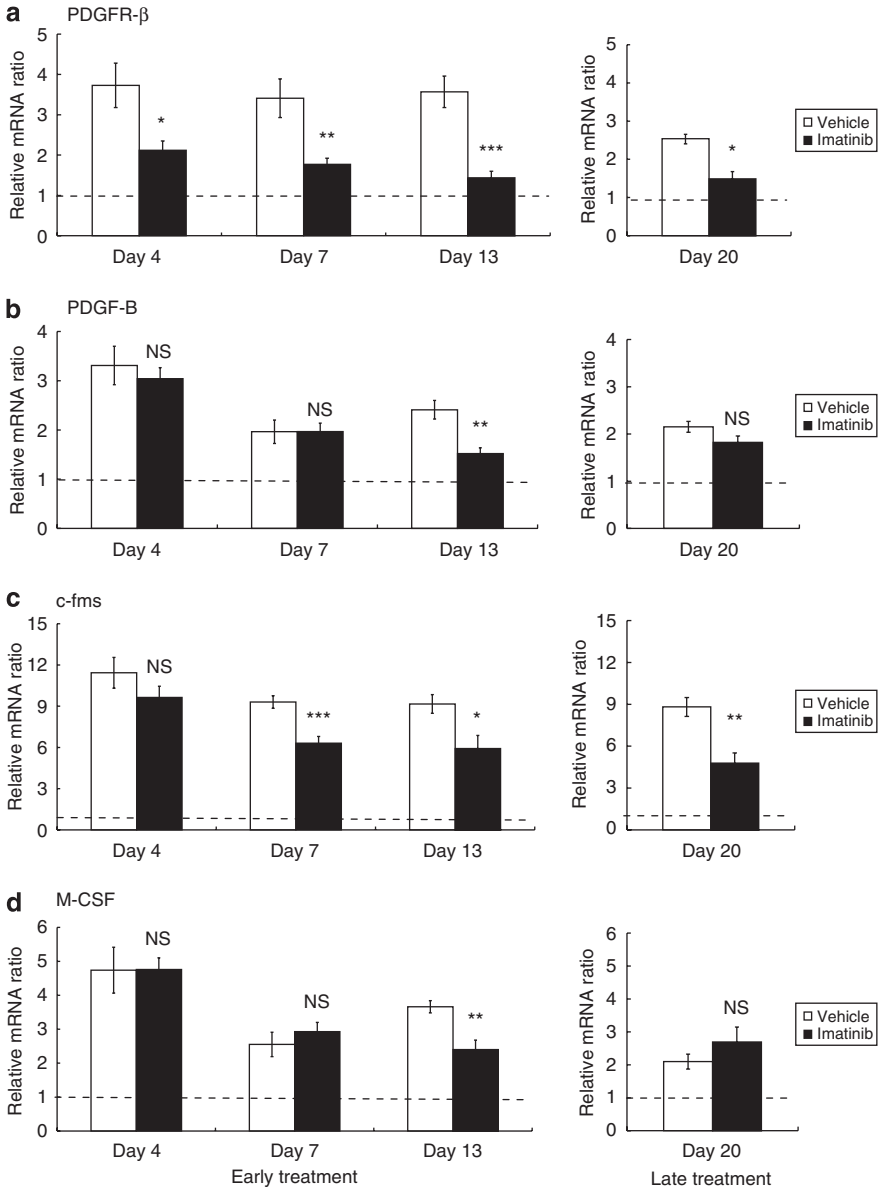

Figure 8 | mRNA expressions for PDGFR-β, PDGF-B, c-fms, and M-CSF in vehicle-treated and imatinib-treated rats with NTS-N as determined by real-time RT-PCR. Real-time RT-PCR for (**a**) PDGFR-β, (**b**) PDGF-B, (**c**) c-fms, and (**d**) M-CSF in each group. Each group contained 10–13 rats. The horizontal dotted lines show the expression levels in normal WKY rats. Data are expressed as mean ± s.e.m. The values were normalized to the GAPDH values and then expressed as relative quantification. The Mann–Whitney test: $^{*}P<0.05$, $^{**}P<0.01$, and $^{***}P<0.001$ vs vehicle-treated NTS-N rats.

anti-mouse biotinylated (diluted 1:500, DAKO), which was applied for 30 min at room temperature. The slides were then washed in TBS, and the third layer, streptavidin alkaline phosphatase (DAKO), was applied for 45 min at room temperature. The slides were then washed in TBS, and the red color was developed using a Fuchsin substrate kit (DAKO). Once a suitable red color had developed, the slides were washed in water and then coverslipped with an aqueous mounting medium.

CD8+ cells were estimated by counting the numbers of these cells within 50 glomeruli and dividing the total number by 50. The extent of ED1, IL-1β, and MCP-1 staining of each glomerulus was graded for 50 glomeruli on a 4-point scale: 0, absent; 1, weak; 2, moderate; and 3, severe.[36,37] The mean score was then calculated as the score of each staining. All the histological analyses were performed by two investigators without knowledge of the origin of the slides, and the mean values were calculated.

Immunofluorescence

The tissues were snap-frozen in liquid nitrogen and cut into 4-μm-thick sections. The deposits of rabbit IgG, rat C3, rat fibrin, and rat IgG in the kidney sections were evaluated by staining with fluorescein-isothiocyanate-conjugated goat anti-rabbit IgG, anti-rat C3, anti-rat fibrinogen (Cappel; Organon Teknika, Durham, NC, USA) and rabbit anti-rat IgG (Sigma Chemical Co.) using a method earlier described.[33] The extent of fibrin deposition in each glomerulus was graded

for 50 glomeruli using a 4-point scale: 0, absent; 1, $<1/3$; 2, between 1/3 and 2/3; and 3, more than 2/3 of the whole glomerulus. The mean score of 50 glomeruli was then calculated as the fibrin score.

Real-time RT-PCR

Gene expressions of rat PDGFR-β, PDGF-B, c-fms, M-CSF, and GAPDH (glyceraldehyde-3-phosphate dehydrogenase) were analyzed using real-time reverse transcriptase-PCR (RT-PCR). Briefly, kidney tissues (cortex) were homogenized using a TissueLyser (QIAGEN, Hilden, Germany), and total RNA was isolated using an RNeasy Fibrous Tissue Mini kit (QIAGEN), in accordance with the manufacturer's instructions. cDNA synthesis was carried out using the SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, CA, USA). Pre-designed TaqMan probe sets for the targets indicated above were purchased from Applied Biosystems (Foster City, CA, USA). Each probe has a fluorescent reporter dye (FAM) linked to its 5′-end and a downstream quencher dye (TAMRA) linked to its 3′-end. Each reaction consisted of 25 μl containing 2 × Universal Master Mix (Applied Biosystems), primers, labeled probes, and cDNA. The amplification conditions consisted of 40 cycles at 95 °C for 15 s and 60 °C for 1 min after incubation at 95 °C for 10 min. Amplification and fluorescence measurements were performed using the MicroAmp Optical 96-Well Reaction Plate on the ABI PRISM 7700 Sequence Detection System (Applied Biosystems). mRNA expressions were normalized using GAPDH as an endogenous control to correct for the differences in the amount of total RNA added to each reaction.

Statistical analysis

Data were recorded as means ± s.e.m. The Mann–Whitney test was performed, and values of $P<0.05$ were considered statistically significant.

DISCLOSURE

All the authors declared no competing interests.

ACKNOWLEDGMENTS

This work was supported in part by Health and Labor Sciences Research Grants from the Research Group on Progressive Renal Disease in Japan. TA is supported by a research grant from Kyowa Hakko Kirin Co. Ltd. and Chugai Pharmaceutical Co. Ltd. We thank Ms Tomoko Suzuki, Ms Naoko Ono, and Ms Fumiko Kondo for their excellent technical assistance. Imatinib was a gift from Novartis Pharmaceuticals (Basel, Switzerland), which did not otherwise participate in the design, execution, or funding of this study.

REFERENCES

1. Isome M, Fijinaka H, Adhikary LP *et al.* Important role for macrophages in induction of crescentic anti-GBM glomerulonephritis in WKY rats. *Nephrol Dial Transplant* 2004; **19**: 2997–3004.
2. Kawasaki K, Yaoita E, Yamamoto T *et al.* Depletion of CD8 positive cells in nephrotoxic serum nephritis of WKY rats. *Kidney Int* 1992; **41**: 1517–1526.
3. Fujigaki Y, Sun DF, Fujimoto T *et al.* Cytokines and cell cycle regulation in the fibrous progression of crescent formation in antiglomerular basement membrane nephritis of WKY rats. *Virchows Arch* 2001; **439**: 35–45.
4. Fujigaki Y, Sun DF, Fujimoto T *et al.* Mechanisms and kinetics of Bowman's epithelial-myofibroblast transdifferentiation in the formation of glomerular crescent. *Nephron* 2002; **92**: 203–212.
5. Kanemoto K, Usui J, Tomari S *et al.* Connective tissue growth factor participates in scar formation of crescentic glomerulonephritis. *Lab Invest* 2003; **83**: 1615–1625.
6. Buchdunger E, O'Reilly T, Wood J. Pharmacology of imatinib (STI571). *Eur J Cancer* 2002; **38**: S28–S36.
7. Gilbert RE, Kelly DJ, Mckay T *et al.* PDGF signal transduction inhibition ameliorates experimental mesangial proliferative glomerulonephritis. *Kidney Int* 2001; **59**: 1324–1332.
8. Savikko J, Taskinen E, Willebrand EV. Chronic allograft nephropathy is prevented by inhibition of platelet-derived growth factor receptor: tyrosine kinase inhibitors as a potential therapy. *Transplantation* 2003; **75**: 1147–1153.
9. Lassila M, Jandeleit-Dahm K, Seah KK *et al.* Imatinib attenuates diabetic nephropathy in apolipoprotein E-knockout mice. *J Am Soc Nephrol* 2005; **16**: 363–373.
10. Zoja C, Corna D, Rottoli D *et al.* Imatinib ameliorates renal disease and survival in murine lupus autoimmune disease. *Kidney Int* 2006; **70**: 97–103.
11. Sadanaga A, Nakashima H, Masutani K *et al.* Amelioration of autoimmune nephritis by imatinib in MRL/lpr mice. *Arthritis Rheum* 2005; **52**: 3987–3996.
12. Wang S, Wilkes MC, Leof EB *et al.* Imatinib mesylate blocks a non-smad TGF-β pathway and reduces renal fibrogenesis *in vivo*. *FASEB J* 2005; **19**: 1–11.
13. Dewar AL, Cambareri AC, Zannettino AC *et al.* Macrophage colony-stimulating factor receptor c-fms is a novel target of imatinib. *Blood* 2005; **105**: 3127–3132.
14. Paniagua RT, Sharpe O, Ho PP *et al.* Selective tyrosine kinase inhibition by imatinib mesylate for the treatment of autoimmune arthritis. *J Clin Invest* 2006; **116**: 2633–2642.
15. Le Meur Y, Tesch GH, Hill PA *et al.* Macrophage accumulation at a site of renal inflammation is dependent on the M-CSF/c-fms pathway. *J Leukocyte Biol* 2002; **72**: 530–537.
16. Seggewiss R, Lore K, Greiner E *et al.* Imatinib inhibits T-cell receptor-mediated T-cell proliferation and activation in a dose-dependent manner. *Blood* 2005; **105**: 2473–2479.
17. Dietz AB, Souan L, Knutson GJ *et al.* Imatinib mesylate inhibits T-cell proliferation *in vitro* and delayed-type hypersensitivity *in vivo*. *Blood* 2004; **104**: 1094–1099.
18. Cwynarski KR, Laylor R, Macchiarulo E *et al.* Imatinib inhibits the activation and proliferation of normal T lymphocytes *in vitro*. *Leukemia* 2004; **18**: 1332–1339.
19. Sinai P, Berg RE, Haynie JM *et al.* Imatinib mesylate inhibits antigen-specific memory CD8 T cell responses *in vitro*. *J Immunol* 2007; **178**: 2028–2037.
20. Isbel NM, Hill PA, Foti R *et al.* Tubules are the major site of M-CSF production in experimental kidney disease: correlation with local macrophage proliferation. *Kidney Int* 2001; **60**: 614–625.
21. Iyoda M, Hudkins KL, Becker-Herman S *et al.* Suppression of cryoglobulinemia and secondary membranoproliferative glomerulonephritis by imatinib. *J Am Soc Nephrol* 2009; **20**: 68–77.
22. Gilbert RE, Kelly DJ, McKay T *et al.* PDGF signal transduction inhibition ameliorates experimental mesangial proliferative glomerulonephritis. *Kidney Int* 2001; **59**: 1324–1332.
23. Yarden Y, Escobedo JA, Kuang WJ *et al.* Structure of the receptor for platelet-derived growth factor helps define a family of closely related growth factor receptors. *Nature* 1986; **323**: 226–232.
24. Classon-Welsh L, Eriksson A, Westermark B *et al.* cDNA cloning and expression of the human A-type platelet-derived growth factor (PDGF) receptor establishes structural similarity to the B-type PDGF receptor. *Proc Natl Acad Sci USA* 1989; **86**: 4917–4921.
25. Dewar AL, Domaschenz RM, Doherty KV *et al.* Imatinib inhibits the *in vitro* development of the monocyte/macrophage lineage from normal human bone marrow progenitors. *Leukemia* 2003; **17**: 1713–1721.
26. Dewar AL, Doherty KV, Hughes TP *et al.* Imatinib inhibits the functional capacity of cultured human monocytes. *Immunol Cell Biol* 2005; **83**: 48–56.
27. Wada T, Yokoyama H, Furuichi K *et al.* Intervention of crescentic glomerulonephritis by antibodies to monocyte chemotactic and activating factor (MCAF/MCP-1). *FASEB J* 1996; **10**: 1418–1425.
28. Fujinaka H, Yamamoto T, Takeya M *et al.* Suppression of anti-glomerular basement membrane nephritis by administration of anti-monocyte-chemoattractant protein-1 antibody in WKY rats. *J Am Soc Nephrol* 1997; **8**: 1174–1178.
29. Iyoda M, Wietecha TA, Hudkins KL *et al.* Platelet-derived growth factor receptor (PDGFR) β inhibition attenuates cryoglobulinemic membranoproliferative glomerulonephritis (MPGN) in mice. *J Am Soc Nephrol* 2007; **18**: 412A (abstract).

30. Fujinaka H, Yamamoto T, Feng L *et al.* Crucial role of CD8-positive lymphocytes in glomerular expression of ICAM-1 and cytokines in crescentic glomerulonephritis of WKY rats. *J Immunol* 1997; **158**: 4978–4983.
31. Huang XR, Tipping PG, Apostolopoulos J *et al.* Mechanisms of T cell-induced glomerular injury in anti-glomerular basement membrane (GBM) glomerulonephritis. *Clin Exp Immunol* 1997; **109**: 134–142.
32. Kantarjian H, Talpaz M, O'Brien S *et al.* High-dose imatinib mesylate therapy in newly diagnosed Philadelphia chromosome-positive chronic phase chronic myeloid leukemia. *Blood* 2004; **15**: 2873–2878.
33. Rea D, Legros L, Raffoux E *et al.* High-dose imatinib mesylate combined with vincristine and dexamethasone (DIV regimen) as induction therapy in patients with resistant Philadelphia-positive acute lymphoblastic leukemia and lymphoid blast crisis of chronic myeloid leukemia. *Leukemia* 2006; **20**: 400–403.
34. Kobayashi K, Shibata T, Sugisaki T. Aggravation of rat nephrotoxic serum nephritis by anti-myeloperoxidase antibody. *Kidney Int* 1995; **47**: 454–463.
35. Zhou A, Ueno H, Shimomura M *et al.* Blockade of TGF-beta ameliorates renal dysfunction and histologic progression in anti-GBM nephritis. *Kidney Int* 2003; **64**: 92–101.
36. Lloyd CM, Minto AW, Dorf ME *et al.* RANTES and monocyte chemoattractant protein-1 (MCP-1) play an important role in the inflammatory phase of crescentic nephritis, but only MCP-1 is involved in crescent formation and interstitial fibrosis. *J Exp Med* 1997; **185**: 1371–1380.
37. Lan HY, Bacher M, Yang N *et al.* The pathogenic role of macrophage migration inhibitory factor in immunologically induced kidney disease in the rat. *J Exp Med* 1997; **185**: 1455–1465.