Imatinib ameliorates renal disease and survival in murine lupus autoimmune disease

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Platelet-derived growth factor (PDGF) has been proved to play an important role in progressive glomerular disease of systemic lupus. The present study investigated the tyrosine kinase inhibitor of PDGF receptor, imatinib, as a novel therapeutic approach for the cure of lupus nephritis in New Zealand Black/White (NZB/W)F1 hybrid mice with established disease. Two groups of NZB/W mice ($N = 30$ each group), starting at 5 months of age, were given by gavage vehicle or imatinib (50 mg/kg b.i.d). Fifteen mice for each group were used for the survival study. The remaining were killed at 8 months. Imatinib significantly ($P = 0.0022$) ameliorated animal survival with respect to vehicle. The drug significantly delayed the onset of proteinuria (% proteinuric mice, 7 and 8 months: 33 and 47 vs vehicle, 80 and 87, $P < 0.05$) and limited the impairment of renal function. Imatinib protected the kidney against glomerular hypercellularity and deposits, tubulointerstitial damage, and accumulation of F4/80-positive mononuclear cells and α-smooth actin-positive myofibroblasts. The abnormal transforming growth factor-β mRNA expression in kidneys of lupus mice was reduced by imatinib. In conclusion, findings of amelioration of animal survival and renal manifestations in NZB/W lupus mice with established disease by imatinib suggests the possibility to explore whether imatinib may function as steroid-sparing drug in human lupus nephritis.

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New Zealand Black/White (NZB/W)F1 hybrid mice spontaneously develop an autoimmune disease with immune complex glomerulonephritis, proteinuria, and progression to renal insufficiency, reminiscent of human systemic lupus erythematosus.[1,2] Although many different immunologic and nonimmunologic factors contribute to disease expression in lupus nephritis, the production of autoantibodies against nuclear and endogenous antigens and the formation of glomerular immune deposits are typically initial events.[3] Factors leading to autoantibody production include breakdown of B- and T-cell tolerance, defects in removal of apoptotic cells, and abnormal presentation of autoantigens (e.g., nucleosomes, DNA complexed to histones).[4,5] Immune deposits seem to form *in situ* through the direct binding of autoantibodies to intrinsic constituents of the glomerular basement membrane or to glomerular cells,[3] resulting in complement activation and recruitment of inflammatory cells, along with activation of renal parenchymal cells. Cytokines and chemoattractants generated by renal resident and infiltrating cells amplify and perpetuate immune complex-mediated injury. Therapies include immunosuppressants to reduce immune complexes, associated with steroids[6] that potently limit upregulation of cytokines and chemokines in activated cells.[7] Both categories of drugs, however, cause significant side –effects, including susceptibility to infection and cancer.[8,9] This has resulted in a quest to identify less toxic drugs to modulate immune complex formation and deposition, as well as molecules directed against the inflammatory reaction.[10–15]

Overproduction of platelet-derived growth factor (PDGF), particularly the PDGF-B isoform, has been consistently implicated in the cell proliferation and extracellular matrix accumulation that characterize progressive inflammatory glomerular disease of systemic lupus.[16–18] Elevated levels of PDGF-B chain mRNA were found in the kidneys of both NZB/W[19,20] and MRL-lpr[21] lupus mice as well as of patients with lupus nephritis,[22] in correlation with the progression of the nephritis. PDGFs are a pleiotropic group of peptide growth factors that can be synthesized both by inflammatory cells and resident renal cells.[16,17] Biological effects of PDGF include promotion of cell proliferation and extracellular matrix synthesis, chemotaxis, modulation of synthesis of various growth factors, and regulation of inflammatory

responses. PDGF isoforms signal through cell surface PDGF receptor tyrosine kinase, the α- and β-receptors, with different affinities.[17] Ligand binding induces receptor dimerization and autophosphorylation, leading to activation of cytoplasmic SH2 domain-containing signal transduction molecules, thereby initiating various signaling pathways, including tyrosine phosphorylation and c-fos mRNA induction. There are different potential strategies to block PDGF overactivity. The administration of neutralizing antibodies or oligonucleotide aptamer antagonist against PDGF ameliorated experimental glomerulonephritis.[23,24] Monoclonal PDGF receptor antibodies that block ligand binding and receptor activation have also been described.[17] However, these maneuvers remain an uncertain possibility for human use. Inhibitors of PDGF receptor tyrosine kinases have been developed more recently. Imatinib mesylate (Glivec, formerly known as STI571), a 2-phenylaminopyrimidine derivative, is a selective inhibitor of the tyrosine kinases ABL, PDGF receptors, and c-kit.[25,26] Because of its capacity to block BCR-ABL oncoprotein, it is used clinically in chronic myeloid leukemia.[26] Imatinib has been reported to prevent experimental mesangial proliferative glomerulonephritis[27] and to confer renoprotection in a model of accelerated diabetic nephropathy.[28]

In the present study, we sought to investigate whether the use of imatinib may represent a new therapeutic approach to lupus nephritis. Specifically, we evaluated whether imatinib could mitigate established renal disease and prolong survival of NZB/W lupus mice possibly by virtue of the drug's antiproliferative and antifibrogenic actions.

RESULTS

NZB/W mice treated with imatinib gained weight in a manner similar to that of mice given vehicle (at 8 months of age, body weight: 34 ± 0.8 vs 33 ± 0.5 g). There were no signs of liver dysfunction in treated mice as indicated by levels of serum aspartate aminotransferase and alanine aminotransferase similar to vehicle-mice (at 8 months of age, aspartate aminotransferase: 152 ± 8 vs 151 ± 12 UI/l; alanine aminotransferase: 25 ± 1 vs 23 ± 3 UI/l).

Imatinib ameliorates survival of NZB/W lupus mice

Figure 1 shows the survival curves of NZB/W mice given vehicle or imatinib from 5 months of age. Mice treated with imatinib survived significantly longer than vehicle-mice ($P = 0.0022$). The mean time to death was 9.5 vs 8.3 months, respectively. At 8 months of age, 100% of mice given the drug were alive as compared with 53% of those on vehicle. At 10 months, when all mice died in the vehicle group, the percentage of survival for imatinib group was 20%.

Effect of imatinib on proteinuria and renal function

Cumulative percentage of mice with heavy proteinuria (>4 mg/day) was evaluated at different stages of the disease in the two experimental groups. As shown in Figure 2, imatinib significantly delayed the onset of proteinuria as compared to vehicle. Thus, at 7 and 8 months of age, the percentage of proteinuric mice was 33 and 47% in the treated group vs 80 and 87% in the vehicle-group ($P < 0.05$). At 9 months, the percentage of proteinuric mice in the two groups increased to 86 and 100%, respectively.

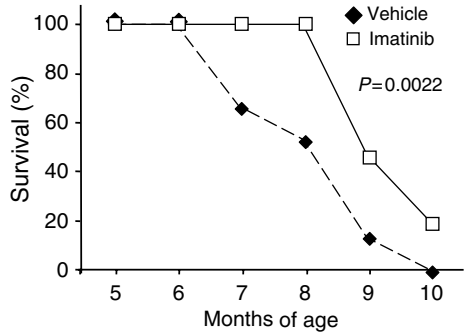

Figure 1 | Survival rate in NZB/W mice orally given imatinib (50 mg/kg b.i.d) or vehicle from 5 months of age, a time point that follows the onset of immune complex deposition. Imatinib significantly ($P = 0.0022$) prolonged life survival.

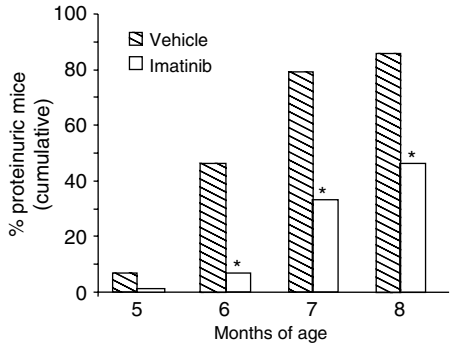

Figure 2 | Effect of imatinib on the cumulative percentage of mice with proteinuria >4 mg/day in NZB/W mice at different months of age. Treatment started at 5 months of age. Each point reflects the current level of proteinuria in surviving mice as well as the last measurement in deceased mice. *$P < 0.05$ vs vehicle.

Basal levels of blood urea nitrogen (BUN) as determined in all mice at 2 months of age were <30 mg/dl. Table 1 shows the percentage of vehicle or imatinib-treated lupus mice with abnormal BUN levels, that is, >30 mg/dl, during the experimental period. In the vehicle group, renal function progressively deteriorated so that at 7, 8, and 9 months of age, 67, 88, and 100% of lupus mice, respectively, had abnormal BUN values. By contrast, in the imatinib group, percentages reached only 27% (7 months) and 47% (8 and 9 months).

Effect of imatinib on anti-DNA antibodies

Serum levels of circulating anti-DNA antibodies measured in 2-month-old NZB/W mice averaged 10 ± 1.4 U/ml. Anti-DNA antibodies significantly increased during time averaging 144 ± 61 U/ml at 5 months of age, before treatment, and reaching 270 ± 125 U/ml at 8 months in NZB/W mice given vehicle ($P < 0.01$ and $P < 0.001$ vs 2 months, respectively). In

Table 1 | Cumulative % mice with BUN > 30 mg/dl

Months of age	2	6	7	8	9	10
Vehicle (%)	0	27	67	88	100	100
Imatinib (%)	0	0	27	47	47	53

BUN, blood urea nitrogen.
BUN levels > 30 mg/dl were considered abnormal (normal range: 14–29 mg/dl).

mice treated with imatinib, anti-DNA formation was slightly retarded (203 ± 92 U/ml).

Effect of imatinib on renal histology

By light microscopy, focal glomerular hypercellularity was found in the kidneys of mice killed at 5 months of age, time at which the treatment was started (score 0.6 ± 0.2). By immunofluorescence, immunoglobulin G (IgG) and complement 3 (C3) deposits were found in glomeruli with predominant mesangial distribution (Figure 3). The presence of immune complex disease was confirmed by electron microscopy analysis showing both mesangial and subendothelial electron dense deposits (Figure 3). No or very sparse glomerular deposits of IgG and C3 nor glomerular electron dense deposits were detected in 2-month-old NZB/W mice (Figure 3).

As shown in Table 2, at 8 months, NZB/W mice given vehicle revealed glomerular changes consisting of intracapillary hypercellularity associated with focal extracapillary proliferation. Immune-type deposits were detected in the mesangium and in the glomerular capillary walls with subendothelial distribution. Tubular damage, interstitial inflammation, and fibrosis were present. Treatment with imatinib limited glomerular hypercellularity, deposits, and tubulointerstitial damage ($P < 0.05$ vs vehicle) (Table 2).

At 8 months, in vehicle-treated mice, there were diffuse granular deposits of IgG and C3 in the mesangium and in the glomerular capillary wall. In mice treated with imatinib, less IgG and C3 deposits were detected than that of mice treated with vehicle, but a statistical significance was not reached (score, IgG: 1.80 ± 0.16 vs 2.27 ± 0.13; C3: 1.60 ± 0.19 vs 2.21 ± 0.21).

Imatinib limits renal inflammation and myofibroblast accumulation

A marked accumulation of F4/80-positive monocytes/macrophages was present in the renal interstitium of NZB/W mice given vehicle (85 ± 6 vs CD-1 control mice: 4 ± 1 cells/high-power field (HPF), $P < 0.01$). Treatment with imatinib significantly ($P < 0.01$) reduced peritubular F4/80-positive cell accumulation (45 ± 8 cells/HPF) (Figure 4). Infiltrates of monocytes/macrophages were also observed at periglomerular level in vehicle-mice (13 ± 2 cells/HPF vs controls, 0.9 ± 0.2 cells/HPF, $P < 0.01$), and were limited by the drug (6 ± 2 cells/HPF, $P < 0.05$ vs vehicle) (Figure 4).

In control kidneys, interstitial immunostaining for α-smooth muscle actin (α-SMA) was present only in sparse cells (score, 0.35 ± 0.12). In the kidneys of lupus mice given vehicle, the interstitial expression of α-SMA was markedly increased (score, 2.06 ± 0.05). This increase was significantly ($P < 0.05$) prevented by imatinib (score, 1.37 ± 0.23) (Figure 5).

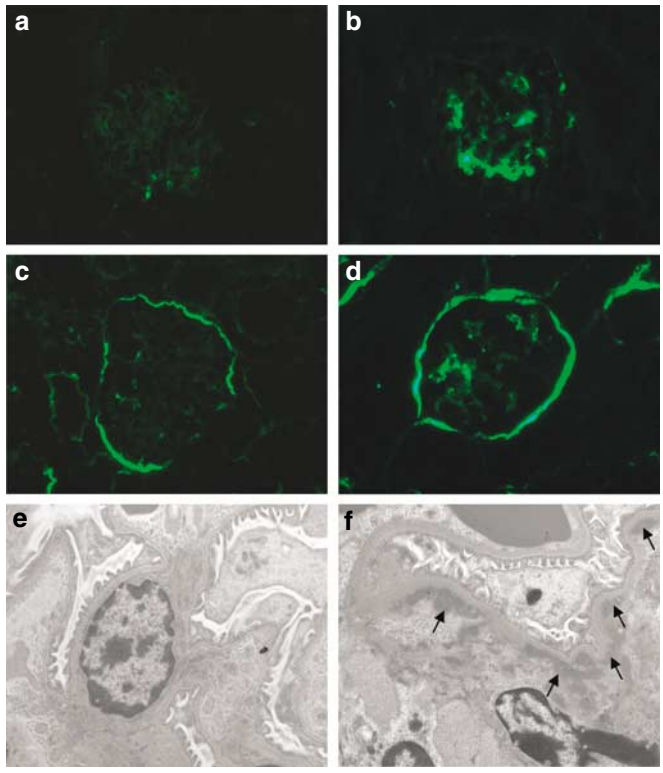

Figure 3 | Evidence of established immune complex deposition in glomeruli at the time at which imatinib treatment is started. Representative sections of glomeruli of (left panels) 2-month-old NZB/W mice, and (right panels) 5-month-old NZB/W mice studied at the time at which the treatment with imatinib started, stained by immunofluorescence for (**a**, **b**) IgG and (**c**, **d**) C3, or (**e**, **f**) analyzed by electron microscopy. Arrows in (**f**) indicate electron dense immune deposits.

Table 2 | Effect of imatinib treatment on renal histology in NZB/W mice

	Glomeruli		Tubulo-interstitial damage	
	Intracapillary hypercellularity (score)	Extracapillary hypercellularity (score)	Deposits (score)	(score)
Vehicle	2.13 ± 0.13	2.5 ± 0.3	2.1 ± 0.2	1.30 ± 0.1
Imatinib	1.33 ± 0.22*	1.5 ± 0.3*	1.2 ± 0.3*	0.75 ± 0.1*

NZB/W, New Zealand Black/White.
Data are mean ± s.e. *$P < 0.05$ *vs* vehicle.

As to the mechanism underlying imatinib's effect on the accumulation of α-SMA-positive cells, we have analyzed the expression of transforming growth factor-β (TGF-β), a strong stimulus for the differentiation of fibroblasts and other cell types to α-SMA-positive myofibroblasts. Real-time polymerase chain reaction (PCR) analysis showed that TGF-β mRNA levels were increased in the kidneys of NZB/W mice given vehicle (fourfold increase over CD-1 control mice). Cytokine upregulation was significantly ($P < 0.05$) reduced by

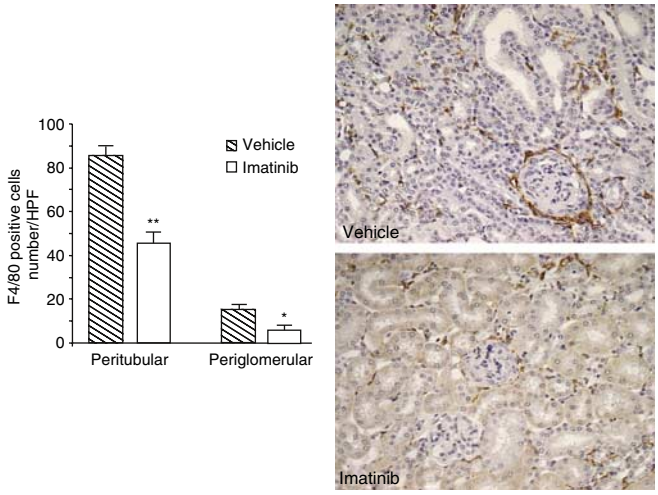

Figure 4 | F4/80-positive monocytes/macrophages infiltrating the kidney of NZB/W mice and the effect of imatinib. Data are mean ± s.e. HPF, high-power field. $^{**}P<0.01$ vs vehicle. Original magnification × 400.

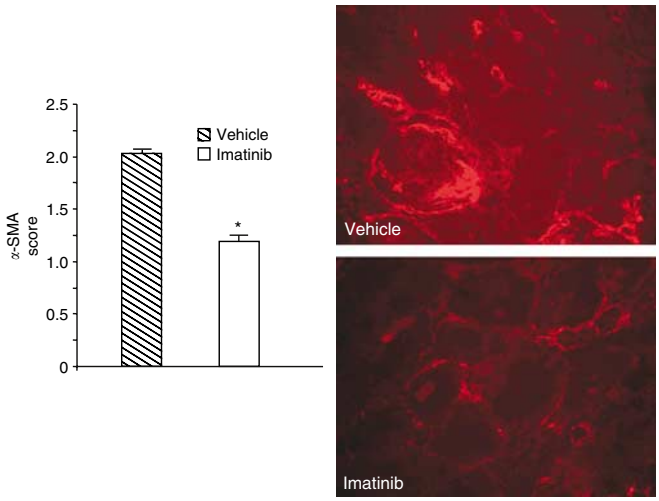

Figure 5 | Accumulation of α-smooth muscle actin (SMA)-positive cells in the renal interstitium of NZB/W mice is reduced by imatinib. Data are mean ± s.e. $^{*}P<0.05$ vs vehicle. Original magnification × 400.

imatinib, to the extent that in treated mice, renal TGF-β expression increased by only twofold over controls.

DISCUSSION

In the present study, we have investigated the tyrosine kinase inhibitor imatinib as a novel therapeutic option for the cure of lupus nephritis. The drug retarded the evolution of renal disease of NZB/W lupus mice and ameliorated survival as compared to untreated animals. Specifically, imatinib orally administered after the onset of immune complex deposition delayed the development of proteinuria to a significant extent, prevented renal function impairment, and limited glomerular hypercellularity, immune type deposits, tubular damage, and interstitial inflammation and fibrosis. These effects were associated with lower levels of circulating anti-DNA antibodies with respect to NZB/W mice given vehicle.

Imatinib has been originally developed as a therapeutic agent for chronic myeloid leukemia.[25,26] By inhibiting BCR-ABL tyrosine kinase, it targets the molecular event implicated as the direct cause of the disease. Although highly specific, imatinib occupies the adenosine triphosphate-binding site of several tyrosine kinase molecules such as ABL, ABL-related gene product, the PDGF receptor, and the stem cell factor receptor c-kit, impeding the ensuing signal transduction.[26] Clinical trials in chronic myeloid leukemia showed that the drug produced an unprecedented rate of hematologic and cytogenetic remission.[26] Moreover, imatinib yielded encouraging results in patients with gastrointestinal stroma tumors caused by c-kit mutations and myeloproliferative disorders with rearrangements of PDGF receptor, diseases where the activation of an imatinib-sensitive kinase is central to the pathogenesis.[29–31]

The inhibitory effect of imatinib on PDGF receptor activation suggested its potential application to nonmalignant diseases in which PDGF has been strongly implicated, such as glomerulonephritis. Imatinib ameliorated experimental mesangial proliferative glomerulonephritis causing significant reductions in mesangial cell proliferation, numbers of activated/α-SMA-positive mesangial cells and extracellular matrix accumulation.[27] Renoprotective effects of imatinib have been recently reported against the diabetic nephropathy of apolipoprotein E-knockout mice, a model of accelerated renal disease associated with increased renal expression of PDGF-B.[28] The drug administered starting immediately after diabetes induction for 20 weeks reduced albuminuria and limited glomerular changes. Tubulointerstitial inflammation and fibrosis were also attenuated by the treatment. Protective effects achieved in NZB/W lupus mice by interrupting PDGF signal transduction with imatinib, while emphasizing the role of PDGF in the manifestation of the disease, suggest the PDGF pathway as a therapeutic target in lupus nephritis. Experimental studies showed progressively increasing PDGF mRNA expression in the renal cortex of lupus mice during the evolution of the disease.[19,21] Moreover, in renal tissue from patients with diffuse proliferative lupus nephritis, gene and protein expression of both PDGF-B chain and PDGF-β receptor increased in correlation with the histologic grade. Sites of localization were the mesangium, cells of the capillary walls, crescents, and mononuclear cells infiltrating the interstitium.[22] In the current study, PDGF-receptor tyrosine kinase inhibition was associated with reduced accumulation of monocytes/macrophages in the kidneys, suggesting that PDGF may contribute, together with other cytokines,[32] to inflammatory cell influx into the kidney in lupus nephritis. A chemotactic activity of PDGF on monocytes as well as granulocytes and fibroblasts has been documented *in vitro*.[33] Another significant finding here was the reduction after imatinib of α-SMA-positive myofibroblasts in the renal interstitium. This could be either the result of a direct effect on PDGF, known to induce

transdifferentiation of renal fibroblasts into α-SMA-positive myofibroblasts,[34] or involve other profibrotic factors such as TGF-β, activated through PDGF receptor-dependent pathways.[28,35] Our data showing that the overexpression of TGF-β mRNA in the kidneys of lupus mice was decreased by imatinib are in line with findings of reduced expression of TGF-β in the glomeruli and the tubulointerstitium after treatment with the drug in diabetic mice.[28] On the other hand, the antifibrotic effect afforded by imatinib could be related to the fact that TGF-β may directly signal through the c-ABL tyrosine kinase, a target of imatinib. Thus, in cultured fibroblasts, TGF-β directly stimulated c-ABL activity, independently of Smad 2/3 phosphorylation or PDGF receptor activation, and the inhibition of c-ABL by imatinib prevented TGF-β-induced morphologic transformation and extracellular matrix gene expression.[36] Finally, in a mouse model of bleomycin-induced pulmonary fibrosis, treatment with imatinib was found to prevent cytokine-induced lung fibrosis.[36]

The finding that imatinib treatment slightly retarded anti-DNA antibody formation as compared to vehicle reflects the immunoregulatory potential of the drug in NZB/W mice. Consistent with this possibility, imatinib delayed to some extent immune complex deposition as suggested by less glomerular IgG and C3 staining, thereby resulting in the attenuation of the downstream disease. *In vitro* studies have shown that imatinib, at concentrations achieved *in vivo*, inhibited the proliferation[37] and activation[38] of T cells via inhibition of early signaling mediated by the T-cell receptor.[39] Consistently with inhibition on T-cell activation, it was found that imatinib reduced IL-2 production by activated T cells.[39] Moreover, that imatinib may affect the T-cell response *in vivo* rests on the observation that imatinib inhibited delayed-type hypersensitivity in mice.[37] Mechanisms by which imatinib might modulate T- and B-cell-mediated pathogenic pathways in lupus nephritis are beyond the scope of the present paper and merit to be investigated further.

In conclusion, this study documents the effect of tyrosine kinase inhibition with imatinib in attenuating renal manifestations and prolonging survival in NZB/W lupus mice with established disease. These results imply the need to assess whether imatinib may function as steroid-sparing drug in human lupus nephritis.

MATERIALS AND METHODS

Experimental design

NZB/W F1 female mice, 2 months of age, were obtained from Harlan Italy s.r.l., Milano, Italy. Animal care and treatment were conducted in conformity with the institutional guidelines that are in compliance with national (Decreto Legislativo n.116, Gazzetta Ufficiale suppl 40, 18 febbraio 1992, Circolare n.8, Gazzetta Ufficiale 14 luglio 1994) and international laws and policies (EEC Council Directive 86/609, OJL358-1, December 1987; *Guide for the Care and Use of Laboratory Animals,* US National Research Council, 1996). Animals were housed in a constant temperature room with a 12-h dark/12-h light cycle and fed a standard diet. Lupus mice were allocated to the following groups. Group 1 ($n=30$) was given the drug vehicle (water) by gavage; Group 2 ($n=30$) was given imatinib mesylate/Glivec (Novartis Pharma AG, Basel, Switzerland) by gavage at the dose of 50 mg/kg b.i.d./day. Treatment started at 5 months of age. Fifteen animals for each group were used for survival studies. The others were killed at 8 months. Five NZB/W mice were killed at 2 months of age. Five normal CD-1 mice (Charles River Italia, Calco, Italy) were killed at 8 months and used as controls. Five additional NZB/W mice were killed at 5 months of age to confirm that in the present cohort of mice, immune complex deposition occurs at the time at which imatinib treatment is started. Immunofluorescence and electron microscopy studies were performed in comparison with the kidneys taken from 2-month-old NZB/W mice.

Urinary protein excretion was measured at 5 months (baseline) and every 2 weeks. Levels >4 mg/day were considered abnormal. Serum BUN was evaluated monthly. At the time of killing, blood was collected for measurements of serum anti-DNA antibodies and transaminase (aspartate aminotransferase, alanine aminotransferase). The kidneys were processed for histological analysis by light microscopy. Renal TGF-β mRNA expression was evaluated by real-time PCR.

Proteinuria and renal function

Urinary protein concentration was determined by the Coomassie blue G dye-binding assay with bovine serum albumin as standard. Renal function was assessed as BUN in heparinized blood by the Reflotron test (Roche Diagnostics Corporation, Indianapolis, IN, USA).

Anti-DNA antibodies

Serum levels of anti-dsDNA autoantibodies were evaluated by enzyme immunoassay (Diastat anti-ds DNA kit, Bouty Laboratory, Milano, Italy) as described before.[13]

Serum transaminase

Serum levels of aspartate aminotransferase and alanine aminotransferase were measured using the Reflotron test (Roche Diagnostics Corporation).

Renal morphology

Light microscopy. Fragments of renal cortex were fixed in Duboscq-Brazil, dehydrated in alcohol, and embedded in paraffin. Sections (3 μm) were stained with hematoxylin and eosin, Masson's trichrome, and periodic acid-Schiff's reagent stain. Glomerular intracapillary hypercellularity was given a score ranging from 0 to 3+ (0 = no hypercellularity; 1+ = mild; 2+ = moderate; 3+ = severe). Extracapillary proliferation was graded from 0 to 3+ (0 = no hypercellularity; 1+ = less than 25% of glomeruli involved; 2+ = 25–50% of glomeruli involved; 3+ = more than 50% of glomeruli involved). Deposits were graded from 0 to 3+ (0 = no deposits; 1+ = less than 25% of glomeruli involved; 2+ = 25–50% of glomeruli involved; 3+ = more than 50% of glomeruli involved). At least 100 glomeruli were examined for each biopsy. Tubular (atrophy, casts, and dilatation) and interstitial changes (fibrosis and inflammation) were graded from 0 to 3+ (0 = no changes; 1+ = changes affecting less than 25% of the sample; 2+ = changes affecting 25–50% of the sample; 3+ = changes affecting more than 50% of the sample).[32] At least 10 fields per sample were examined at low magnification ($\times 10$) for scoring of the interstitium. Biopsies were analyzed by the same pathologist, in a single-blind fashion.

Immunofluorescence. Studies were performed on frozen tissue embedded in 22-oxacalcitriol. Sections of 3 μm were cut, fixed in acetone, washed with phosphate buffer saline pH 7.4 for 10 min, blocked with phosphate-buffered saline/1% bovine serum albumin for 15 min, and stained with fluorescein isothiocyanate-conjugated antibodies to mouse IgG (Sigma-Aldrich, St Louis, MO, USA) or C3 (NL Cappel Laboratories Inc., West Chester, PA, USA). Glomerular staining intensity was graded from 0 to 3+ (0 = no staining; 1+ = faint staining; 2+ = intense staining; 3+ = very intense staining).

Electron microscopy. Fragments of cortical kidney tissue from 2-month-old and 5-month-old NZB/W mice were fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.4, overnight at 4°C, and washed repeatedly in cacodylate buffer. After post-fixation in 1% OsO_4 for 1 h, specimens were dehydrated through ascending grades of alcohol and embedded in Epon resin. Ultrathin sections were stained with uranyl acetate for examination using a Philips Morgagni transmission electron microscope.

Immunohistochemical analysis

F4/80-positive cells. Paraffin sections (3 μm) were dewaxed and taken through graded alcohols. Rat monoclonal antibody against a cytoplasmic antigen of mouse monocytes and macrophages (F4/80, 2.5 μg/ml, Caltag Laboratories, Burlingame, CA, USA) was used for detection of infiltrating cells. Sections were incubated for 30 min with 0.3% H_2O_2 in methanol to quench endogenous peroxidase. The tissue was permeabilized in 0.1% Triton X-100 in phosphate-buffered saline 0.01 mol/l, pH 7.2, for 30 min and then incubated with 2% normal goat serum (Vector Laboratories, Burlingame, CA, USA) for 30 min. Primary antibody was incubated overnight at 4°C, followed by the secondary antibody (biotinylated goat anti-rat IgG, Vector Laboratories) and avidin–biotin peroxidase complex solution, and finally development with diaminobenzidine. The sections were counterstained with Harris hematoxylin. Negative controls were obtained by omitting the primary antibody. F4/80-labeled cells were counted in 20 randomly selected high-power microscopic fields (× 400) per each animal.

α-SMA-positive cells. Tissue specimens were embedded in 22-oxacalcitriol medium and frozen in liquid nitrogen. Tissue sections were cut at 3 μm using a Mikrom 500 O cryostat (Mikrom, Walldorf, Germany) and either stained immediately or stored at −80°C. Non-specific binding of antibodies was blocked with phosphate-buffered saline/1% bovine serum albumin for 15 min (room temperature). Sections were incubated for direct immunofluorescence using a Cy3-conjugated monoclonal anti-α-SMA antibody (1A4, purified mouse IgG, 1:200, Sigma Co., St Louis, MO, USA) for 1 h at room temperature. For the evaluation of immunofluorescence staining, randomly selected, consecutive fields of view (at least 20 in each section, × 400) were examined by an investigator without the knowledge of the experimental group. The staining for α-SMA was assessed semiquantitatively assigning a score for each field as follows: 0, no staining; 1+, mild (up to 25% of interstitial area); 2+, moderate (>25–50%); 3+, marked accumulation of α-SMA cells (>50% of the interstitial area). The mean values were calculated for each specimen and used for statistical analysis.

Renal expression of TGF-β mRNA

Total RNA was isolated from whole kidney tissue by using TRIzol Reagent (Invitrogen, San Giuliano milanese, Italy). Contaminating genomic DNA was removed by RNase-free DNase (Promega, Ingelheim, Germany) for 1 h at 37°C. Purified RNA (2 μg) was reverse transcribed using random examers (50 ng) and 200 U of SuperScript II RT (Life Technologies, San Giuliano Milanese, Italy) for 1 h at 42°C. No enzyme was added for reverse transcriptase-negative controls.

Real-time PCR was performed with ABI PRISM 5700 Sequence Detection System (PE Biosystems, Warrington, UK) using SYBR GREEN PCR Master Mix (PE biosystems). After an initial hold of 2 min at 50°C and 10 min at 95°C, the samples were cycled 40 times at 95°C for 15 s and 60°C for 60 s. Fluorescent detection, defined as threshold cycle (C_t), is picked in exponential phase of PCR and used for relative quantification of target gene. The comparative C_t method normalizes the number of target gene copies to an endogenous control gene as 18S (ΔC_t). Gene expression was then evaluated by a quantification of a cDNA corresponding with the target gene relative to a calibrator serving as physiologic reference (e.g. control group, $\Delta\Delta C_t$). On the basis of exponential amplification of target gene as well as calibrator, the amount of amplified molecules at the threshold cycle is given by: $2^{(-\Delta\Delta C_t)}$.

The following specific primers were used: mouse TGF-β1 (300 nM): forward 5′-GCTGAACCAAGGAGACGGAAT-3′, reverse 5′-AAGAGCAGTGAGCGCTGAATC-3′; 18S (50 nM): forward 5′-ACGGCTACCACATCCAAGGA-3′, reverse 5′-CGGGAGTGGGTAATTTGCG-3′.

Statistical analysis

Data are expressed as mean ± s.e. The time occurrence of death was assessed by Kaplan–Meier cumulative survival functions. Time-to-event (death) was calculated in months. Survival distribution between the two groups (i.e. vehicle vs imatinib) was compared by log-rank test. Proteinuria data were analyzed by Fisher's exact test. All the other parameters were analyzed by Kruskall Wallis or Mann–Whitney *U*-test as appropriate. Statistical significance was defined as $P < 0.05$.

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NOTE ADDED IN PROOF

During preparation and resubmission of the manuscript, the following article has been published:

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