The lymphocyte migration inhibitor FTY720 attenuates experimental hypertensive nephropathy

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HYPERTENSIVE NEPHROPATHY has become the second major cause of chronic kidney disease in western societies and is considered to be a major driving force in its number one cause, which is type 2 diabetic nephropathy (8, 12). The steadily increasing incidence of hypertensive and hypertensive-diabetic end-stage renal disease has become a major challenge for the health care systems, and there is a great need for more effective and preventive treatment strategies here. Similar to renal disorders of immune origin, hypertensive, nonimmune kidney damage shows significant infiltration with mononuclear white blood cells such as macrophages and lymphocytes (14, 22). Although the function of macrophages in nonimmune renal disease has been unraveled in greater detail in recent years, the potential role of lymphocytes in this setting is far less clear.

In a model of antibody-induced, chronic-progressive renal fibrosis, i.e., anti-thy1-induced chronic glomerulosclerosis, we have previously reported that selective inhibition of lymphocyte migration into the diseased kidney by FTY720 significantly limited the progressive course of the disease toward glomerulosclerosis and tubulointerstitial fibrosis (21). FTY720 [fingolimod; 2-amino-2-[4-octylphenyl(methyl)-1,3-propanediol hydrochloride] is a novel and potent lymphocyte-specific immunomodulator and has currently been tested in phase III studies for the treatment of multiple sclerosis (3, 10). From a cellular point of view, fingolimod inhibits the egress of T and B cells from secondary lymphoid organs and thereby reduces selectively the number of circulating lymphocytes by up to 85–90% (3, 9). From a molecular point of view, FTY720 is a produg that, after endogenous phosphorylation, mainly by sphingosine kinase 2, acts as a structural analog of the biological mediator sphingosine 1-phosphate (SIP) on four out of five G protein-coupled SIP receptors (SIP1, SIP3, SIP4, and SIP5, but not SIP2). The potent inhibitory action of FTY720 on lymphocyte lymph nodes egress has been related to two molecular pathways leading to peripheral lymphocyte depletion. In the first one, FTY720 is sought to act primarily as a functional SIP1 receptor antagonist on lymphocytes. After receptor binding and internalization would follow a prolonged downregulation of the SIP1 receptor. This would deprive lymphocytes from the obligatory SIP1-mediated signal to migrate from lymphoid organs into the circulation. In the second one, it is hypothesized that FTY720 acts primarily as a SIP1 receptor agonist on lymphatic endothelium and thereby increases endothel barrier function. The consequence would be reduced lymphocyte transmigration in the efferent lymph compartment and subsequently into the peripheral blood (3, 9).

The present study was designed to characterize the role of infiltrating lymphocytes in hypertensive nephropathy. Rats subjected to surgical nephrectomy served as a model of hypertensive renal injury, and FTY720 served as a pharmacological tool to deplete circulating lymphocytes from the blood and thus prevent their infiltration in the diseased kidney. Treatment with FTY720 was started after surgery and was continued for 6 wk. During treatment, changes in proteinuria were monitored. After 6 wk, effects of FTY720 on white blood cell count, renal matrix protein accumulation, and underlying fibrosis-related pathways were analyzed.

METHODS

Materials. Unless otherwise indicated, all materials and chemicals were purchased from Sigma Chemical-Aldrich (Taufkirchen, Germany).

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Animals. Male Wistar rats (250 ± 15 g) were obtained from Charles River (Sulzfeld, Germany) and fed a high-protein diet (40% protein; Altromin, Lage, Germany) for at least 3 days before the start of the experiment to enable equilibration. Animals were housed in a constant temperature room with a 12:12-h light-dark cycle. Body weight was determined at the beginning and at the end of the experiment. Food and water intake were monitored. Animal care and treatment were in conformity with the guidelines of the American Physiology Society and approved as well by local authorities.

Surgical procedure of 5/6 nephrectomy. Subtotal nephrectomy was achieved by surgically removing the complete right kidney and truncating two-thirds of the left kidney under isoflurane anesthesia. Renal intersection hemostasis was achieved by using spongostane films (Johnson & Johnson, Norderstedt, Germany). Sham operation consisted of ventral laparotomy and manipulation of renal tissue without injuring structures.

Experimental groups and FTY720 administration. Following surgery, animals were divided into the following three groups: 1) sham surgery (n = 4); 2) 5/6 nephrectomy, no treatment (n = 5); and 3) 5/6 nephrectomy with FTY720 administration (n = 9). Treatment with FTY720 was started 2 days after complete surgery and was continued until the end of the experiment in week 6. The compound FTY720 was kindly provided by Novartis Pharma (Basel, Switzerland). The drug was administered by the food in a dose of 0.3 mg/kg body wt/−1 day/−1. This dose was chosen on the basis of previous reports that, using this amount of FTY720, indicated an ~80–90% reduction in the number of circulating lymphocytes (17, 21). The food containing FTY720 was produced in our laboratory using the rat chow (40% protein content, A1314; Altromin) that was given to the untreated animals. The drug was mixed in an appropriate amount into the dry flour, water was added, and then pellets were formed. After the pellets were air-dried, these were then fed to the animals (21).

Experimental sequence and death. After surgery (2, 4, and 6 wk), the animals were housed in metabolic cages for urine collection. On week 6, the animals were killed using 0.1 mg ketanest/0.01 mg xylazine/100 g body wt/−10% Ketamin (WDT, Garbsen, Germany); 2% Rompun (Bayer Vital, Leverkusen, Germany)). Following a midline abdominal incision, 5–10 ml of heparinized blood was drawn from the abdominal aorta, and kidneys were subsequently perfused with 30 ml of ice-cold PBS (14). Materials and tissues were subsequently processed as described in the following sections. All analyses are based on results assessed in individual animals.

Measurement of proteinuria and systolic blood pressure. Urinary protein was measured using a pyrogallol red microtiter plate technique as described previously (14). Proteinuria is expressed as milligrams protein per 24 h. Systolic blood pressure was assessed 1 or 2 days as described previously (14). Proteinuria is expressed as milligrams protein per 24 h. Systolic blood pressure was assessed 1 or 2 days after surgery (2, 4, and 6 wk).

Blood analysis. White blood cell counts were determined in an automated cell counter (XE 2100; Sysmex, Norderstedt, Germany) using a standard fluorescent flow cytometry technology (17). Plasma and urine creatinine and plasma urea were measured spectrophotometrically in enzyme-based assays (13). The glomerular filtration rate was calculated on the basis of both serum and urinary creatinine concentrations and the corresponding urine volume, and this factor is expressed as milliliters per minute per 100 grams body weight (15).

Light and immunohistochemistry microscopy. For histological examination, cortical tissue was fixed in Carnoy’s solution. All histological studies were performed in a blinded fashion as previously described by a computer-based morphometric analysis (15). Briefly, renal sections were examined on a Zeiss Axiostar imaging system equipped with a digital Axiostar camera and the Axiosvision 4.6 image analysis system (all from Carl Zeiss Vision, Munich, Germany) using a 10 X 10 orthographic grid overlaid on digital images. Tubulointerstitial and glomerular sections were analyzed separately.

Tubulointerstitial and glomerular fibrosis. Paraffin sections (3 μm) were stained by use of the periodic acid-Schiff reaction. The relative degree of tubulointerstitial fibrotic lesions, i.e., matrix deposition, tubular atrophy, cell infiltration, and dilation, was calculated in 15 randomly selected tubulointerstitial areas/animal observed at ×200 magnification graded from 0 to 100% of the tubulointerstitial area (15). The relative degree of glomerular matrix expansion was evaluated by rating the mesangial matrix occupying the area of 15 glomeruli/animal using a scoring system from 0 to 100% (15).

For the analysis of renal collagen I, III, and IV, laminin, and fibronectin protein expression, paraffin-embedded tissues were incubated with primary goat anti-collagen type I, III, and IV antibodies (all Southern Biotech, distributed by Biozol, Eching, Germany) or laminin and fibronectin antibodies (both DakoCytomation, Glostrup, Denmark) (15). A horseradish peroxidase (HRP)-conjugated rabbit-anti-goat antibody was used in the second step and then visualized with AEC reagent (both from DakoCytomation). Tubulointerstitial collagen I- and III- and laminin- and fibronectin-positive areas were analyzed by randomly selecting at least 15 cortical sections/animal using ×200 magnification and glomerular collagen I-, III-, and IV- and laminin- and fibronectin-positive areas in at least 15 glomerular areas/animal respectively. Matrix protein expression is indicated by the percentage of the positive-staining area per tubulointerstitial or glomerular section (15).

Tubulointerstitial and glomerular lymphocyte and macrophage infiltration. For analysis of renal cell infiltration, tissues were immunohistochemically stained for ED1 (macrophages)- and CD3 (lymphocytes)-positive cells (14, 15). Paraffin-embedded tissues were incubated with primary mouse anti-CD3 or anti-ED1 antibodies (both Serotec, Oxford, UK). In the case of CD3 staining, a secondary goat HRP-conjugated anti-mouse IgM antibody was used and visualized with alkaline phosphatase (AP) antialkaline phosphatase, and, in the case of ED1 detection, an AP-conjugated secondary goat anti-mouse antibody was used in conjunction with Fast Red as substrate (all from DakoCytomation). The numbers of CD3- or ED1-positive cells were evaluated by counting infiltrating cells in at least 15 randomly selected tubulointerstitial and in at least 15 glomerular sections (14, 15).

Tubulointerstitial and glomerular cell proliferation. Renal cell proliferation was detected by using a primary mouse anti-proliferating cell nuclear antigen (PCNA), a secondary AP-conjugated secondary goat anti-mouse antibody, and Fast Red as substrate (all from DakoCytomation) (14, 15). The numbers of CD3- or ED1-positive cells were evaluated by counting infiltrating cells in at least 15 randomly selected tubulointerstitial and in at least 15 glomerular sections.

Western blot analysis. For Western blot analysis, cortical tissue was snap-frozen in liquid nitrogen. For preparation, tissue was homogenized and then transferred to the following buffer (10% wt/vol): 50 mM NaCl, 50 mM Tris·HCl (pH 7.5), 1 mM EDTA, 2 mM EGTA, 1 mM vanadate, 1 mM glycerol, 1% Triton X-100, and 1% protease inhibitor cocktail (Sigma-Aldrich, Taufkirchen, Germany) on ice. Tissue extracts were treated in an ultrathorax for 20 s and centrifuged at ×10,000 for 5 min at 4°C. The protein concentration of the supernatant was determined by use of the Pierce BCA-protein kit (Pierce, Rockford, IL). Samples (30 μg) were fractionated by electrophoresis on a 10% Tris-glycine gel (Invitrogen, Carlsbad, CA) at 125 volts for ~90 min. After electrophoresis, the proteins were transferred to an Immobilon P membrane (Sera, Heidelberg, Germany), at 20 volts for 90 min using the X Cell II blot module (Invitrogen) with Tris-glycine buffer, pH 8.3. Broad-range prestained molecular weight markers (Santa Cruz Biotechnology, Heidelberg, Germany) were used to assure proper protein transfer. The membranes were then blocked for 1 h at room temperature in TBS containing 0.2% Tween 20 (TBST) and 6% nonfat dry milk. After being washed in TBST, polyclonal rabbit anti-rat transforming growth factor (TGF)-β1 antibody (Santa Cruz Biotechnology) was diluted to 1:1,000, and incubation took place overnight at 4°C. Next, incubation with goat-anti-rabbit HRP-bound antibody (DakoCytomation) diluted 1:2,000 for 1.5 h followed. After each step, washes of 30 min were undertaken in TBST, which was changed every 10 min. Finally, the
membrane was analyzed for the amount of antibody binding by chemiluminescence using enhanced chemiluminescence detection reagents (Amersham Pharmacia, Piscataway, NJ) and subjected to autoradiography for 20 s. The films were scanned, and relative optical density was assessed using Image J 1.35q (NIH, Bethesda, MD). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Dako-Cytomation) served as a loading control.

**Statistical analysis.**

The results are expressed as means ± SE. Statistical analysis between groups was performed by using the Kruskal-Wallis test and subsequently Mann-Whitney U-testing. A P value of <0.05 was considered significant.

**RESULTS**

**Basic data.** In FTY720-treated animals, the drug intake actually achieved was 0.32 ± 0.07, 0.29 ± 0.01, and 0.31 ± 0.05 mg/kg body wt in weeks 2, 4, and 6, respectively, and thus close to the targeted dose of 0.30 mg/kg body wt throughout the experiment. Food intakes did not differ between the groups throughout the experiment [exemplary results from week 4: Sham 26 ± 2; hypertensive nephropathy (HN) 25 ± 1, and HN + FTY720 25 ± 2 g/day, respectively]. Water consumption was higher in both HN groups, indicating renal disease with partly impaired urinary concentration capacity (Sham 51 ± 2, HN 70 ± 3, and HN + FTY720 71 ± 3 ml/day, respectively).

**Effects of FTY720 on peripheral white blood cell number.** In rats following 5⁄6 nephrectomy, the number of circulating leucocytes was similar to that in the animals having had sham surgery (Sham 6,064 ± 170/μl; HN 5,898 ± 310/μl) (Fig. 1A). The same was found for the circulating lymphocyte number (Sham 4,312 ± 37/μl; HN 3,613 ± 661/μl) (Fig. 1B). As anticipated, treatment with FTY720 significantly reduced the blood lymphocyte number by ~85% (HN + FTY720 644 ± 146/μl, P < 0.001) (Fig. 1A) and thereby the total blood’s white blood cell count by 75% (HN + FTY720 1,477 ± 258/μl, P < 0.001) (Fig. 1A and B). The number of circulating eosinophils, neutrophils, and monocytes was not altered after administration of FTY720.

**Effects of FTY720 on proteinuria and blood pressure.** As shown in Fig. 2, urinary protein excretion before surgery (week 0) did not differ between the groups (Sham 34 ± 2, HT 28 ±
2, and HT + FTY720 29 ± 3 mg/day). In the untreated HN rats, proteinuria continuously increased from 154 ± 13 mg/day in week 2, over 238 ± 20 mg/day in week 4, to a maximum of 382 ± 47 mg/day in week 6. Treatment with FTY720 significantly reduced proteinuria throughout the experiment. The relative reductions found in the second week were 33%, in the sixth week 28% (all relative reductions found in the second week were 33%, in the sixth week 28% (all P < 0.05 vs. HN). Systemic blood pressure was significantly increased in the untreated animals with % nephrectomy in week 6 (HN 161 ± 5 vs. Sham 122 ± 2 mmHg, P < 0.001). Treatment with FTY720 did not significantly alter blood pressure in the subtotally nephrectomized rats (HN + FTY720 160 ± 5 mmHg, P = NS vs. HN).

Effects of FTY720 on renal matrix accumulation. In week 6, untreated % nephrectomy was characterized by marked tubulointerstitial fibrosis and glomerulosclerosis. Characteristic histological photographs are shown in Fig. 3. Compared with the control animals, untreated subtotal nephrectomy rats showed a tubulointerstitial matrix protein accumulation that had a highly significant increase (matrix score: HN 54.7 ± 3.8% vs. Sham 2.9 ± 1.1%). In conformance with histological fibrosis at the molecular level, this went along with the markedly increased tubulointerstitial protein deposition of collagen I (HN 7.3 ± 0.6% vs. Sham 1.2 ± 0.4%; Fig. 4), collagen III (HN 5.1 ± 0.3% vs. Sham 0.9 ± 0.1%), laminin (HN 4.4 ± 0.4% vs. Sham 0.8 ± 0.2%; Fig. 5A), and fibronectin (HN 15.1 ± 2.1% vs. Sham 6.7 ± 0.9%; Fig. 5C). Treatment with FTY720 for 6 wk significantly limited renal disease severity as shown by the presence of reduced tubulointerstitial histological matrix protein expansion (matrix score: 40.3 ± 3.2%, P < 0.001; Fig. 3) and protein expression of collagen I (2.5 ± 0.4%, P < 0.001; Fig. 4), collagen III (2.1 ± 0.2%, P < 0.001), laminin (1.8 ± 0.4%, P < 0.001; Fig. 5A), and fibronectin (8.2 ± 0.8%, P < 0.01; Fig. 5C).

At the glomerular level, disease in % nephrectomy was characterized by marked glomerular matrix protein expansion (HN 52.4 ± 3.4% vs. Sham 22.6 ± 0.8%; Fig. 3) and protein expression of collagen I (HN 4.0 ± 0.6% vs. Sham 0.4 ± 0.2%; Fig. 4), collagen IV (HN 1.2 ± 0.2% vs. Sham 0.1 ± 0.01%), laminin (HN 6.9 ± 1.3% vs. Sham 0.7 ± 0.5%; Fig. 5B), and fibronectin (HN 7.8 ± 0.3% vs. Sham 1.4 ± 0.3%; Fig. 5D) (all P < 0.01 vs. Sham). Treatment with FTY720 limited glomerular fibrosis as well, but less prominently than in the interstitial compartment. Glomerular matrix score was reduced by 20% (P < 0.001 vs. HN), collagen I by 61% (P < 0.01 vs. HN), collagen IV by 52% (P < 0.01 vs. HN), laminin protein by 72% (P < 0.001 vs. HN), and fibronectin by 44% (P < 0.001 vs. HN). Histological collagen III expression was very low in all groups, with a small increase in the untreated % nephrectomy group (HN 0.011 ± 0.001% vs. Sham 0.003 ± 0.001%) and a nonsignificant change in the FTY720-treated group (0.018 ± 0.01%).

These results document that FTY720 limits tubulointerstitial fibrosis and glomerulosclerosis in an animal model of hypertensive kidney injury. To further explore and document the potentially operating mechanisms, we next analyzed renal lymphocyte infiltration as a primary mode of action for FTY720 and potential pathways secondarily involved in me-
diating renal fibrosis such as TGF-β expression, macrophage infiltration, and cell proliferation.

**Effects of FTY720 on renal lymphocyte number.** Remnant renal tissue after 5⁄6 nephrectomy showed a highly significant increase in the number of tubulointerstitial (HN 4.1 ± 4 vs. Sham 2.1 ± 1 CD3-positive cells/tubulointerstitial section) (Fig. 6A) and glomerular (HN 3.7 ± 0.8 vs. Sham 0.2 ± 0.1 CD3-positive cells/glomerular section) (Fig. 6B) lymphocytes. Treatment with FTY720 significantly prevented tubulointerstitial and glomerular lymphocyte influx both by −63% (14.9 ± 2.6 CD3-positive cells/tubulointerstitial section; \( P < 0.001 \); 1.4 ± 0.2 CD3-positive cells/glomerular section; \( P < 0.001 \)). Together with the markedly lower number of circulating lymphocytes, these results confirm FTY720’s primarily mode of pharmacological action as inhibitor of lymphocyte migration in diseased tissue.

**Effects of FTY720 on renal TGF-β expression, macrophage infiltration, and cell proliferation.** Compared with the Sham group, tubulointerstitial fibrosis and glomerulosclerosis in subtotal nephrectomy was paralleled by markedly increased renal protein expression of the key fibrosis mediator TGF-β (Western blot of cortical tissue: HN 39.7 ± 4.7% vs. Sham 13.4 ± 3.4% relative to GAPDH) (Fig. 7), infiltration with macrophages (tubulointerstitial HN 17.8 ± 1.4 vs. Sham 1.5 ± 0.3 and glomerular HN 4.0 ± 0.3 vs. Sham 0.4 ± 0.02 ED1-positive cells/section) (Fig. 8), and proliferation of cells (tubulointerstitial HN 26.2 ± 6.5 vs. Sham 0.3 ± 0.1 and glomerular HN 2.3 ± 0.2 vs. Sham 0.4 ± 0.1 PCNA-positive cells/section) (Fig. 9). Inhibition of renal lymphocyte infiltration by FTY720 went along with significant reductions in cortical TGF-β1 protein expression: to 28.6 ± 3.4% relative to GAPDH, \( P < 0.05 \); tubulointerstitial and glomerular macrophage infiltration, to 7.7 ± 0.8 ED1-positive cells/tubulointerstitial section, \( P < 0.001 \); to 1.9 ± 0.2 ED1-positive cells/glomerular section, \( P < 0.01 \); as well as tubulointerstitial and glomerular cell proliferation to 10.3 ± 2.9 PCNA-positive cells/tubulointerstitial section, \( P < 0.05 \); and 1.0 ± 0.1 PCNA-positive cells/glomerular section, \( P < 0.001 \) (Figs. 7–9).

**Effects of FTY720 on plasma markers of renal function.** To see whether the anti-fibrotic actions of FTY720 in 5⁄6 nephrectomy can lead to any improved renal function, this was analyzed as well. Table 1 shows that, in this experiment, 5⁄6 nephrectomy was characterized by increases in plasma creatinine and urea levels and a reduced glomerular filtration rate. The anti-fibrotic effects of FTY720 went along with moderate improvement of renal function as shown by plasma creatinine and urea levels and glomerular filtration rate, although this did reach not any statistical significance.

Taken together, these data indicate that FTY720 significantly limits histological and molecular fibrosis in the model of 5⁄6 nephrectomy without affecting the degree of systemic hypertension. FTY720 markedly prevented renal lymphocyte infiltration as anticipated. This was paralleled by significant renoprotective actions on key pathways of renal disease progression such as TGF-β overexpression, macrophage infiltration, and renal cell proliferation.
DISCUSSION

Hypertensive tissue injury to both the kidney and large arteries, in advanced stages known as nephrosclerosis and atherosclerosis, share a number of cellular and molecular similarities. One of these characteristics is that the two chronic diseases both exhibit prominent infiltration by monocytes and lymphocytes, although they are not of primary immune origin. In recent years, the pathogenesis of atherosclerosis has been more and more understood as a chronic inflammatory disorder (24). The cellular and molecular contribution of adhesion molecules, proinflammatory cytokines, and infiltrating leukocytes has been well characterized in the pathophysiological cascade leading to sclerosis of the arterial wall (26). Of importance to this study, the lymphocyte migration inhibitor FTY720 has recently been shown to limit atherosclerosis in two rodent models, i.e., in apolipoprotein E knockout (11) or low-density lipoprotein receptor knockout mice (19).

The goal of the present study was to characterize the contribution of infiltrating lymphocytes to the degree and severity of hypertensive nephrosclerosis. With the use of FTY720 as a tool to prevent renal lymphocyte infiltration and 5/6 nephrectomy as a model of hypertensive tissue injury, the present investigations document the following.

1) As anticipated, FTY720 administration markedly reduced the number of circulating lymphocytes by 85% and renal infiltration by 63%.

2) With this lymphocyte inhibition having been achieved, there was a markedly lower tubulointerstitial and glomerular collagen, laminin, and fibronectin protein deposition and overall renal matrix protein accumulation in the matrix score.

3) The anti-fibrotic action of FTY720 went along with significant reduction in renal TGF-β protein overexpression, macrophage infiltration, and cell proliferation. The sum of these findings suggests that lymphocytes actively contribute to renal disease progression in a model of hypertensive nonimmune kidney tissue injury. The relevance and importance of these findings will be discussed in the following paragraphs.

FTY720’s key mode of action is lymphocyte depletion in the circulation and subsequently of diseased tissues via S1P1 receptor modulation (3, 9). This was likely the primary mechanism of how the drug mediated anti-fibrotic effects in the rats following 5/6 nephrectomy of this study. The contribution of infiltrating lymphocytes has already been the focus of previous studies in comparable models of renal mass reduction employing the transplant drug mycophenolate mofetil (MMF; see Refs. 23 and 28). MMF is an inhibitor of the inosine monophosphate dehydrogenase and acts as potent inhibitor of lymphocyte proliferation and action. However, the mode of action with MMF is far less lymphocyte-specific than that initially assumed. In addition to lymphocytes, MMF turned out to be a potent proliferation inhibitor of a number of renal cells, including mesangial cells, interstitial fibroblasts, and epithelial cells and thereby may directly act on the renal disease (14, 22, 31). Thus, although MMF was clearly protective in models of renal mass reduction form a therapeutic point of view, the mechanisms involved were likely beyond the state of selective lymphocyte inhibition. More selective and conclusive results for an

Fig. 5. Tubulointerstitial and glomerular laminin and fibronectin deposition 6 wk after induction of HN. Graphs depict the percentages of laminin- and fibronectin-positive staining of at least 15 tubulointerstitial (A and C) and glomerular (B and D) sections, visualized with a HRP-conjugated staining system and applying computer-based histomorphometry. Treatment with FTY720 was started 2 days after complete 5/6 nephrectomy (HN + FTY720; n = 13–15). Untreated animals with 5/6 nephrectomy (n = 13–17) or sham surgery (Sham; n = 4) served as control. **P < 0.01 and ***P < 0.001 vs. HN.
FTY720 IN 5⁄6 NEPHRECTOMY

Downstream of any less renal lymphocyte infiltration by FTY720, we found that its nephroprotective actions went along in a profibrotic manner as well by stimulation of the matrix protein production of resident renal cells. Furthermore, significantly less renal cell proliferation was observed in the FTY720-treated 5⁄6 nephrectomy animals. Although FTY720, similar to its endogenous analog S1P, has shown some anti-proliferative actions in cell culture (3, 9), it is more likely that this effect was secondary to less renal lymphocyte infiltration than a direct FTY720 action. In this study, we used FTY720 in a very low dose of 0.3 mg·kg body wt⁻¹·day⁻¹ that yields FTY720 blood concentration in the nanomolar range (4, 16). In cell culture experiments, micromolar concentrations of FTY720 were required to demonstrate a growth inhibitory effect of the drug (3, 9).

In contrast to the pathways of renal progression mentioned above, the anti-fibrotic action of FTY720 in hypertensive nephropathy was not only independent of changes in systemic blood pressure but it occurred even in the presence of systemic hypertension. This finding has two major implications. First, FTY720 has been found to modulate blood pressure in general, an effect that depends on the relative distribution of S1P receptors in the endothelium and smooth muscle cell layer of the blood vessel exposed. Some studies have shown a small, transient decrease in mean arterial pressure in animals and humans (20, 25), which is possibly explained by a similar modest and by a transient decrease in heart rate by FTY720 (25). In contrast, increases in mean arterial pressure by S1P active role of infiltrating lymphocytes in the progression of hypertensive are now provided by the present study inasmuch as they employ the selective lymphocyte migration inhibitor FTY720.

Downstream of any less renal lymphocyte infiltration by FTY720, we found that its nephroprotective actions went along with significant reductions in renal TGF-β protein expression, macrophage infiltration, and cell proliferation, three key pathways of chronic kidney disease. The relation between FTY720 and TGF-β in vitro and in vivo appears to be complex and multidimensional. Mesangial cell culture experiments have indicated that phosphorylated FTY720, similar to S1P, can act in a profibrotic manner via induction of connective TGF and an intracellular cross talk with the TGF-β/SMAD signaling cascade (9, 30). On the other hand, infiltrating activated lymphocytes themselves can release important profibrotic mediators such as TGF-β1 and ANG II and thereby directly amplify pathological matrix protein production by resident renal cells. In 5⁄6 nephrectomy, we found a significant reduction in renal TGF-β1 expression with FTY720 administration. This suggests that the contribution of infiltrating lymphocytes to renal tissue TGF-β overexpression is of greater magnitude than the potential direct profibrotic actions of FTY720 on resident renal cells. In line with this interpretation are experiments that documented no signs of either glomerulosclerosis or tubulointerstitial fibrosis in normal rats treated with FTY720 at rather high doses of 5 mg·kg body wt⁻¹·day⁻¹ for 3 wk (29).

Another secondary downstream effect of preventing renal lymphocyte infiltration was the ~50% reduction in kidney macrophage numbers (22). Infiltrating macrophages can act in a profibrotic manner as well by stimulation of the matrix protein production of resident renal cells. Furthermore, significantly less renal cell proliferation was observed in the FTY720-treated 5⁄6 nephrectomy animals. Although FTY720, similar to its endogenous analog S1P, has shown some anti-proliferative actions in cell culture (3, 9), it is more likely that this effect was secondary to less renal lymphocyte infiltration than a direct FTY720 action. In this study, we used FTY720 in a very low dose of 0.3 mg·kg body wt⁻¹·day⁻¹ that yields FTY720 blood concentration in the nanomolar range (4, 16). In cell culture experiments, micromolar concentrations of FTY720 were required to demonstrate a growth inhibitory effect of the drug (3, 9).

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analogs have been reported in conscious rodents during continuous infusion (7). Long-term effects on blood pressure may also involve S1P-induced modulation of renal function. In the present experiment, the net effect of FTY720 on blood pressure was apparently zero.

The second major implication is related to the previous discussion on whether or not renal lymphocyte infiltration may play a direct role in the development of renal hypertension. Previous experiments using rather unspecific modes to inhibit lymphocyte actions such as thymus removal or anti-thymocyte serum have reported significantly lower systemic blood pressure following the intervention in rodent models of hypertensive nephropathy (2, 27). Now with the use of a more selective mode of lymphocyte inhibition, the present investigation implies that lymphocyte infiltration is not contributing to the pathogenesis of renal hypertension and that, in the experiments mentioned above, it was likely other mechanisms that had been operating.

It was the primary intention of this study to find a mechanistic insight in the role of lymphocytes in hypertensive nephropathy. For this, we found a moderate, but significant, contribution of infiltrating lymphocytes to the degree and severity of a number of profibrotic changes in 5/6 nephrectomy. In a line, renal function was only slightly improved. For the interpretation of this finding, it is important to mention that benefits in renal functional parameters are inconsistent results in nephrectomy and have in fact sometimes not been demonstrable using even more potent interventions, including, for instance, MMF (1, 23, 28).

Fig. 8. Tubulointerstitial and glomerular macrophage infiltration 6 wk after induction of HN by 5/6 nephrectomy. Macrophage infiltration was assessed by counting the ED1-positive cells per tubulointerstitial (A) and glomerular (B) section. Analysis was performed using a specific murine primary antibody with a HRP-conjugated detection system and a computer-based histomorphometry. Treatment with FTY720 was started 2 days after complete 5/6 nephrectomy (HN + FTY720; n = 18). Untreated animals with 5/6 nephrectomy (n = 21) or sham surgery (Sham; n = 4) served as controls. ***P < 0.001 vs. HN.

Fig. 9. Tubulointerstitial and glomerular cell proliferation 6 wk after induction of HN by 5/6 nephrectomy. Paraffin-embedded renal sections were stained with a murine primary anti-proliferating cell nuclear antigen (PCNA) antibody and a secondary HRP-conjugated detection system. Tubulointerstitial (A) and glomerular (B) PCNA-positive cells were analyzed by computer-based histomorphometry. Treatment with FTY720 was started 2 days after complete 5/6 nephrectomy (HN + FTY720; n = 18). Untreated animals with 5/6 nephrectomy (n = 19) or sham surgery (Sham; n = 4) served as controls. *P < 0.05 and ***P < 0.001 vs. HN.

Table 1. Parameters of renal function 6 wk after induction of HN by 5/6 nephrectomy

<table>
<thead>
<tr>
<th>Parameter of renal function</th>
<th>Sham</th>
<th>HN</th>
<th>HN + FTY720</th>
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<tbody>
<tr>
<td>Blood creatinine, mg/dl</td>
<td>0.27±0.01</td>
<td>0.95±0.08</td>
<td>0.88±0.05</td>
</tr>
<tr>
<td>Urea, mg/dl</td>
<td>49.1±4.4</td>
<td>236.4±20.6</td>
<td>208.4±19.5</td>
</tr>
<tr>
<td>Glomerular filtration rate, ml/100 g body wt</td>
<td>0.36±0.08</td>
<td>0.25±0.02</td>
<td>0.28±0.02</td>
</tr>
</tbody>
</table>

Values are means ± SE. HN, hypertensive nephropathy. Plasma creatinine, urea, and glomerular filtration rate/100 g body wt are shown. Treatment with FTY720 was started 2 days after complete 5/6 nephrectomy (HN + FTY720; n = 17 animals). Untreated animals with 5/6 nephrectomy (n = 21) or sham surgery (Sham; n = 4) served as controls.
functional and morphological outcome in a rat model of renal ischemia-reperfusion involving lower neutrophil infiltration (6). In addition, the effect of FTY720 was analyzed in both acute and chronic anti-thy1-antibody-induced renal disease of the rat (17, 21). In acute anti-thy1 glomerulonephritis, FTY720 did not affect anti-thy1-antibody-induced mesangial cell lysis at day 1 but significantly lowered subsequent glomerular TGF-β overexpression and matrix accumulation on day 6 (17). In the chronic anti-thy1-model, FTY720 treatment for 19 wk significantly limited the progressive course of the disease toward glomerulosclerosis and tubulo-interstitial fibrosis (21).

Beyond using FTY720 as a tool to deplete lymphocytes from a defined setting such as hypertensive nephropathy, it is worthwhile to speculate on the therapeutic potential for this new class of drugs. FTY720 is the first agent in the class of S1P modulators, and a number of more specific and potent drugs are under development such as SEW2871, KRP-203, VPC23019, JTE-013, BML-241, and W123 (9). FTY720, as regards its use in monotherapy, is currently under investigation in clinical phase III trials for the treatment of multiple sclerosis. In a recently published proof-of-concept study, FTY720 markedly reduced both the number of lesions detected on magnetic resonance imaging and the indexes of clinical disease activity (10). First clinical trials with FTY720 were performed in human kidney transplantation by our group (5). These trials have recently been discontinued since FTY720 in combination therapy with steroids and cyclosporine A have failed to show any efficacy superior to the MMF (18). In addition, FTY720 together with cyclosporine A and steroids was linked to macula edema as a relevant side effect in a subgroup of patients. The most common side effect occurring after FTY720 administration is the induction of a transient bradycardia involving activation of the S1P3 receptor subtype and G protein-gated potassium channel I_KACH in atrial myocytes of the sinus node (3).

In conclusion, the present study shows that FTY720 significantly limits the degree and severity of tissue fibrosis in a model of hypertensive nephropathy without altering hypertensive systemic blood pressure levels. FTY720’s primary and documented action was the prevention of renal lymphocyte abundance. Downstream secondary effects involved a beneficial action on TGF-β overexpression, macrophage infiltration, and renal cell proliferation. These findings suggest that infiltrating lymphocytes play an active, profibrotic role in the progression of hypertensive renal tissue injury.

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