Role of FTY720 on M1 and M2 macrophages, lymphocytes, and chemokines in 5/6 nephrectomized rats

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Schaier M, Vorwalder S, Sommerer C, Dikow R, Hug F, Gross M, Waldherr R, Zeier M. Role of FTY720 on M1 and M2 macrophages, lymphocytes, and chemokines in 5/6 nephrectomized rats. Am J Physiol Renal Physiol 297: F769–F780, 2009. First published June 17, 2009; doi:10.1152/ajprenal.90530.2008.—Renal injury is accompanied by the presence of infiltrating inflammatory cells in the glomerulus and tubulointerstitium. FTY720 modifies lymphocyte migration into injured tissues by lymphocyte sequestration to secondary lymphoid organs. The purpose of this study was to examine the potential of FTY720 to influence the inflammatory response in a nonimmunological model of renal failure. Sham-operated and 5/6 nephrectomized (SNX) rats received two different doses of FTY720 or vehicle orally for 14 wk. Treatment with FTY720 reduced glomerular and tubulointerstitial damage in SNX rats but failed to stabilize creatinine clearance. The increase in gene expression of chemokine receptors CCR1, CCR2, and CCR5 in kidneys of vehicle-treated SNX rats was significantly attenuated by high-dose FTY720. Treatment with high-dose FTY720 tended to normalize RANTES and MCP-1 renal gene expression. FTY720 affected not only glomerular and tubulointerstitial lymphocytes, but M1 and M2 phenotype macrophages were also reduced. FTY720 significantly reduced key mediators of renal inflammation and fibrosis. FTY720 also decreased immunoregulation of M2 macrophages, which are beneficial for tissue remodeling and repair.

INFLAMMATORY CELL INFILTRATION of the renal parenchyma is a common finding in various progressive renal diseases (32). Infiltrating cells initiate and maintain renal scarring with a consequent loss of renal function. Extracellular matrix components are secreted and thereby contribute to matrix accumulation (23). Mononuclear cells are relevant sources of profibrotic molecules such as transforming growth factor (TGF)-β1 (28).

Chemokines are small cytokines that mediate cell chemotaxis and activation (17). They are also leukocyte chemotactants that interact with profibrotic cytokines in the development of fibrosis by recruiting myofibroblasts, macrophages, and other key effector cells to sites of tissue injury (52, 60). Chemokine expression secondary to stimulation with proinflammatory cytokines has been reported in various types of intrinsic renal cells in vitro and in vivo, including tubular epithelial, interstitial, endothelial, and mesangial cells (51).

The β-chemokines MCP-1/CCL2 and RANTES/CCL5 have been shown to play important roles in the pathology of allograft nephropathy (12) and are key chemokines in the progression of tubulointerstitial injury. The expression of chemokines and chemokine receptors may be influenced by a new generation of immunosuppressive medication, among others S1P receptor modulators.

The S1P receptor agonist fingolimod; 2-amino-2[2-(4-octylphenyl)ethyl]-1,3-propanediol (FTY720) represents the prototype of a new generation of S1P receptor modulators (9). The drug may act as a functional “antagonist” or “agonist,” depending on the S1P receptor subtype and the targeted cell type/tissue. FTY720 effectively inhibits the egress of T cells (19, 34) and B cells (25) from lymph nodes, thereby reducing the number of antigen-primed/restimulated cells that recirculate to peripheral inflammatory tissues (9). FTY720 reduces plasma concentrations of the proinflammatory cytokines IFNγ, TNF-α, IL-6, IL-12, and RANTES (53).

Previous works have shown that in experimental neural inflammation, the recruitment of lymphocytes is significantly reduced and progressive scarring is prevented (5, 62). FTY720 demonstrated promising results in phase II trials and recently entered phase III in patients with relapsing multiple sclerosis (11). The S1P receptor modulator reduces ischemia-reperfusion injury in the kidney (14), however, although a more detailed insight into the potential pathomechanisms is of interest.

In the present study, we examined the hypothesis of whether FTY720 retards the progression of chronic renal disease by reducing peripheral lymphocytes. To achieve this aim, we administered FTY720 to rats with 5/6 renal ablation (SNX), a model of progressive renal disease due to reduction of nephron mass and without immunological mechanisms.

MATERIALS AND METHODS

Animals and Experimental Protocol

Male Sprague-Dawley rats (Charles River, Sulzfeld, Germany), with an initial weight of 240–260 g, were housed in pairs in cages at constant room temperature (22°C) and humidity (55%) under a 12:12-h light-dark cycle.

Induction of Chronic Renal Disease by 5/6 Nephrectomy

Chronic renal failure was initiated by a two-step subtotal nephrectomy (SNX). The right-side nephrectomy was performed through a dorsolateral incision with decapsulation and ligation of the renal hilus. After 1 wk, a weight-controlled 5/6 left-side nephrectomy was performed (according to the weight of the left kidney). Sham operation was done by laparotomy and bilateral decapsulation of the kidneys. General anesthesia consisted of an intramuscular injection of ketamine 100 mg/kg body wt (Ketanest, Essex Pharma, München, Germany) and xylazine 5 mg/kg body wt (Rompun, Bayer Vital, Leverkusen, Germany).

Animals were pair-fed to ascertain comparable caloric and FTY720 intakes in SNX animals and sham-operated controls. The dose of FTY720 was adjusted by offering an amount of pellets calculated to deliver the respective dose. The rats had free access to tap water. The
experiments were performed according to the German law on animal protection.

Experimental Groups and Intervention

Forty-eight animals were randomly assigned to the following experimental groups:

- Sham-operated + vehicle (control/vehicle, \( n = 8 \)).
- Sham-operated + low-dose FTY720 (control/low-dose, \( n = 8 \)).
- Sham-operated + high-dose (control/high-dose, \( n = 8 \)).
- Subtotally nephrectomized + vehicle (SNX/vehicle, \( n = 8 \)).
- Subtotally nephrectomized + low-dose (SNX/low-dose, \( n = 8 \)).
- Subtotally nephrectomized + high-dose (SNX/high-dose, \( n = 8 \)).

FTY720 was kindly provided by Novartis Pharma (Basel, Switzerland). For the administration via food, FTY720 was encapsulated in a lactose-gelatin granular carrier substance and subsequently processed into a standard rat chow (Alt. 1324, Altromin, Lage, Germany) (45). The administration of FTY720 was started 1 wk after the first operation (right-side nephrectomy). We did not start our treatment with FTY720 at the time of nephrectomy, since we preferred a strategy that resembles human disease. Furthermore, inflammation starts as early as 2 wk after ablation (15). The animals were pair-fed with a low-dose of 0.3 mg/kg body wt and a high-dose of 3 mg/kg body wt. Low-dose treatment of 0.3 mg/kg body wt is an adequate dose to reduce lymphocytes; higher doses have shown some additive effects such as direct effects on endothelial barrier (44). According to pharmacological studies, a peak concentration is reached after 4 h, with lymphocyte depletion which happens within 12 h. The experiment was terminated 14 wk after the induction of chronic renal disease.

Systolic Blood Pressure

Systolic blood pressure was measured on day 0 and weeks 5, 10, and 14 after the subtotal nephrectomy in conscious animals by tail-cuff plethysmography and calculated as an average of three separate measurements at each session.

Blood Analysis, Albuminuria, and Creatinine Clearance

At the time of death, blood was sampled for routine chemistry and blood count. Albuminuria was determined in 24-h urine specimens from animals housed in metabolic cages 7, 11 and 14 wk after the subtotal nephrectomy. The urine was frozen at −20°C until measure-

![Graphs showing effects of FTY720 treatment on body weight, systolic blood pressure, and albuminuria.](http://ajprenal.physiology.org/)
FTY720 MODULATES CHEMOKINE GENE EXPRESSION

Table 1. Effects of FTY720 on blood count

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Vehicle</th>
<th>Low-dose</th>
<th>High-dose</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hemoglobin, mg/dl</td>
<td>13.8±0.38</td>
<td>13.4±0.29</td>
<td>14.1±0.25</td>
</tr>
<tr>
<td>Leukocytes/μl</td>
<td>7,473±794</td>
<td>1,768±110*</td>
<td>2,412±254*</td>
</tr>
<tr>
<td>Lymphocytes/μl</td>
<td>5,900±360</td>
<td>587±70*</td>
<td>636±118*</td>
</tr>
<tr>
<td>SNX</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hemoglobin, mg/dl</td>
<td>12.3±0.33</td>
<td>12.9±0.19</td>
<td>12.6±0.25</td>
</tr>
<tr>
<td>Leukocytes/μl</td>
<td>7,958±488</td>
<td>2,339±298†</td>
<td>2372±199†</td>
</tr>
<tr>
<td>Lymphocytes/μl</td>
<td>6,200±370</td>
<td>730±120†</td>
<td>530±65†</td>
</tr>
</tbody>
</table>

Values are means ± SE. SNX, % nephrectomy. *P < 0.001 vs. controls/vehicle. †P < 0.001 vs. SNX/vehicle (analysis of variance and Bonferroni’s multiple comparison test).

ment. Albuminuria was measured using the microplate technique described by Magnotti et al. (31) but modified by using a peroxidase-conjugated anti-rat-albumin antibody (ICN Biomedical, Eschwege, Germany). Measurements were performed in quadruplicate (31).

For light microscopy, paraffin-embedded tissue slides of 4 μm were stained with the periodic acid-Schiff (PAS) reagent.

Glomerular sclerosis index. Glomerulosclerosis was assessed by PAS-stained sections using a semiquantitative scoring system according to the method of Raij et al. (40). The extent of glomerulosclerosis was evaluated by examining 30 randomly selected glomeruli at a magnification of ×400 and applying a score system to each glomerulus according to the percentage of sclerosed glomerular area. This score was graded from 0 to 4: (e.g., 0 = 0%; 1 = 1–25% affected glomerular area; 2 = 26–50% affected glomerular area; 3 = 51–75% affected glomerular area; 4 = 76–100% affected glomerular area). The resulting index in each animal was expressed as a mean of all scores obtained.

Total glomerular cell count. The total glomerular cell count was determined in PAS-stained sections in 30 glomeruli/kidney with a diameter of at least 100 μm.

Tubulointerstitial lesion score. Twenty nonoverlapping cortical areas were assessed in PAS-stained paraffin sections at a magnification of ×100 using a scoring system introduced by Veniant et al. (56).

Renal Morphology

For light microscopy, paraffin-embedded tissue slides of 4 μm were stained with the periodic acid-Schiff (PAS) reagent.

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Interstitial space and cells. For the quantitative evaluation of interstitial space and number of interstitial cells, PAS-stained sections were analyzed using a grid containing 121 fields. The proportion of interstitial space of the evaluated field was quantified, and the interstitial cells per grid were counted in 20 nonoverlapping cortical areas.

Glomerular area. The glomerular area was quantitated by superimposing the 121-point grid (Leica, Wetzlar, Germany) and counting the points overlying the glomerular area. Afterward, the proportion of the glomerular area of the evaluated field was quantitated.

Immunohistochemistry

Renal tissue was fixed in 10% buffered formalin (CD3, CD4, CD8, CD20, CD163, CCR7, TGFB1, fibronectin) or methyl Carnoy’s solution (ED-1), paraffin-embedded, and cut into 4-μm slices. The primary antibodies were a polyclonal rabbit anti-rat CD3 antibody (DakoCytomation, Hamburg, Germany), a monoclonal mouse anti-rat CD4 (Serotec, Oxford, UK), a monoclonal mouse anti-rat CD8 (Serotec), a monoclonal mouse anti-rat CD163 (Serotec), a polyclonal...
Table 2. Effects of FTY720 on glomerular morphology

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Controls</th>
<th>SNX</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Vehicle</td>
<td>Low-dose</td>
</tr>
<tr>
<td>Glomerulosclerosis index, glomerular score</td>
<td>1.1±0.14</td>
<td>1.3±0.03</td>
</tr>
<tr>
<td>Glomerular cell count, cells/glomerulus</td>
<td>66±1.7</td>
<td>66±2</td>
</tr>
<tr>
<td>Glomerular area, %/grid</td>
<td>16.8±0.29</td>
<td>17.4±0.43</td>
</tr>
</tbody>
</table>

Values are means ± SE. *P < 0.05, †P < 0.01 vs. SNX/vehicle (analysis of variance and Bonferroni’s multiple comparison test).

Table 3. Effects of FTY720 on tubulointerstitial and glomerular staining of macrophage subtypes

<table>
<thead>
<tr>
<th>Group</th>
<th>Tubulointerstitial</th>
<th>Glomerular</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CCR7/M1</td>
<td>CD163/M2</td>
</tr>
<tr>
<td>Control/vehicle</td>
<td>0.22±0.06</td>
<td>0.48±0.11</td>
</tr>
<tr>
<td>Control/low-dose</td>
<td>0.23±0.04</td>
<td>0.09±0.12</td>
</tr>
<tr>
<td>Control/high-dose</td>
<td>0.27±0.04</td>
<td>0.80±0.11</td>
</tr>
<tr>
<td>SNX/vehicle</td>
<td>1.70±0.34</td>
<td>2.11±0.37</td>
</tr>
<tr>
<td>SNX/low-dose</td>
<td>0.48±0.085</td>
<td>0.46±0.07‡</td>
</tr>
<tr>
<td>SNX/high-dose</td>
<td>0.69±0.11†</td>
<td>0.83±0.15‡</td>
</tr>
</tbody>
</table>

Values are means ± SE. *P < 0.05, †P < 0.01, ‡P < 0.001 vs. SNX/vehicle (analysis of variance and Bonferroni’s multiple comparison test).

Table 4. Effects of FTY720 on tubulointerstitial and glomerular staining of T cell subtypes

<table>
<thead>
<tr>
<th>Group</th>
<th>Tubulointerstitial</th>
<th>Glomerular</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CD4</td>
<td>CD8</td>
</tr>
<tr>
<td>Control/vehicle</td>
<td>0.58±0.09</td>
<td>0.51±0.19</td>
</tr>
<tr>
<td>Control/low-dose</td>
<td>0.18±0.05</td>
<td>0.13±0.09</td>
</tr>
<tr>
<td>Control/high-dose</td>
<td>0.12±0.07</td>
<td>0.19±0.04</td>
</tr>
<tr>
<td>SNX/vehicle</td>
<td>3.41±0.6</td>
<td>2.42±0.5</td>
</tr>
<tr>
<td>SNX/low-dose</td>
<td>1.14±0.2‡</td>
<td>0.82±0.1‡</td>
</tr>
<tr>
<td>SNX/high-dose</td>
<td>1.10±0.2‡</td>
<td>1.12±0.2*</td>
</tr>
</tbody>
</table>

Values are means ± SE. *P < 0.05, †P < 0.01, ‡P < 0.001 vs. SNX/vehicle (analysis of variance and Bonferroni’s multiple comparison test).
compared with sham-operated controls (6.9 ± 0.5 ml/min/kg body wt). Treatment with FTY720 at neither dose affected the creatinine clearance (SNX low-dose 2.4 ± 0.5 ml·min⁻¹·kg body wt⁻¹; SNX high-dose 2.1 ± 0.1 ml·min⁻¹·kg body wt⁻¹).

The urinary albuminuria excretion was elevated in SNX rats compared with sham-operated rats. Treatment with FTY720 reduced albuminuria but the reduction failed to reach statistical significance (Fig. 1C).

**Effects of FTY720 on Peripheral Blood Cells**

Peripheral lymphocytes and leukocytes were significantly reduced in animals treated with high- and low-dose FTY720. Low-dose was as effective as high-dose treatment concerning the reduction of white blood cells (Table 1).

**Effects of FTY720 on Glomerular Damage**

Figure 2 depicts typical PAS stains of glomeruli of vehicle-treated, sham-operated, and SNX rats that were treated with vehicle or low- and high-dose FTY720. FTY720-treated rats showed an obvious reduction in glomerular and tubulointerstitial damage. The glomerulosclerosis index was significantly higher in vehicle-treated SNX rats than in sham-operated controls. Low- and high-dose FTY720 had no effects on sham-operated controls but reduced significantly the glomerulosclerosis index in SNX rats treated with FTY720 (Table 2).

The total glomerular cell number was not affected by FTY720 treatment in sham-operated controls compared with the vehicle group. In vehicle-treated SNX rats, the glomerular cell number was significantly higher and was lowered in SNX rats treated with either low- or high-dose FTY720 (Table 2).

The glomerular area was increased in vehicle-treated SNX rats compared with vehicle-treated sham-operated controls. Treatment with FTY720 had no effects on the glomerular area (Table 2).

**Effects of FTY720 on Glomerular Inflammation**

The number of monocytes/macrophages per glomerular cross section was higher in vehicle-treated SNX rats than in sham-operated controls (see Fig. 3 and Tables 3 and 4). In rats treated with FTY720, the number of glomerular monocytes/macrophages was significantly reduced. Glomerular mononuclear cells with M1 (CCR7+) and M2 (CD163+) phenotypes were increased in vehicle-treated SNX rats. The numbers of M1 and M2 macrophages were significantly lower in SNX rats treated with FTY720 (Table 3).

Glomerular staining for T lymphocytes (CD3+) and the subtypes T helper cells (CD4+) and cytotoxic T cells (CD8+) showed significantly more glomerular positive cells in vehicle-treated SNX rats than in sham-operated controls. CD3+, CD4+, and CD8+ T cells were significantly less in SNX rats treated with either low- or high-dose FTY720 compared with the vehicle-treated group (Table 4).

In sham-operated rats, B lymphocytes (CD20+) were almost undetectable irrespective of FTY720 treatment. In vehicle-treated SNX rats, B lymphocytes (CD20+) were almost undetectable irrespective of FTY720 treatment.
Fig. 4. Effects of FTY720 on tubulointerstitial damage. A: tubulointerstitial lesion score. There was a significant reduction of the tubulointerstitial lesion score by treatment with both high- and low-dose FTY720. Values are means ± SE.

B: tubulointerstitial area. The tubulointerstitial area was significantly expanded in vehicle-treated SNX rats compared with sham-operated controls, but was significantly smaller in FTY720-treated animals.

C: tubulointerstitial cell count. The interstitial cell count was reduced significantly in rats treated with low- and high-dose FTY720.

Fig. 5. Effects of FTY720 on tubulointerstitial cell infiltration. A: macrophages/monocytes in the interstitium. Both low-dose and high-dose FTY720 treatment reduced the interstitial infiltration significantly in SNX rats. B: T lymphocytes (CD3-positive staining) in the interstitium. The number of interstitial CD3-positive cells was markedly higher in vehicle-treated SNX rats compared with controls. FTY720 limited the infiltration of CD3-positive cells in SNX rats. Values are means ± SE.

C: B lymphocytes (CD20-positive staining) in the interstitium. Treatment reduced B lymphocytes in SNX rats.
cle-treated rats with SNX, glomerular infiltration with B lymphocytes was detectable compared with controls (Fig. 3). In contrast, the infiltration with B lymphocytes was significantly lower in SNX rats with low- or high-dose FTY720.

Effects of FTY720 on Tubulointerstitial Damage

The tubulointerstitial lesions score was significantly higher in vehicle-treated SNX rats compared with sham-operated controls (Fig. 4). Treatment with FTY720 significantly reduced the tubulointerstitial lesions in SNX rats.

The tubulointerstitial area was larger in SNX rats treated with vehicle than in sham-operated controls. The tubulointerstitial area was significantly smaller in rats treated with FTY720 than in vehicle-treated rats.

Similarly, the interstitial cell count was reduced in rats treated with low- and high-dose FTY720 compared with vehicle-treated SNX rats.

Effects of FTY720 on Tubulointerstitial Inflammation

The number of interstitial monocytes/macrophages (ED-1/CD68-positive) and their different phenotypes, M1 (CCR7+ and M2 (CD163+), were significantly higher in SNX rats compared with sham-operated controls (Fig. 5). Either dose of FTY720 lowered interstitial positive cell counts of monocytes/macrophages and their different phenotypes M1 and M2 in SNX rats (Table 3).

In sham-operated rats, T lymphocytes (CD3-positive) and the subtypes T helper cells (CD4+) and cytotoxic T cells (CD8+) were almost undetectable irrespective of FTY720 treatment (Fig. 6). However, in vehicle-treated rats with SNX, a marked interstitial infiltration by different T cells was detected compared with controls. The interstitial infiltration by different T lymphocyte subtypes were significantly lower in SNX rats treated with low- or high-dose FTY720 (Table 4).

Interstitial B lymphocytes (CD20-positive) were elevated in vehicle-treated SNX rats compared with vehicle-treated sham-operated rats. Treatment with FTY720 significantly reduced interstitial B lymphocytes in SNX rats.

Effects of FTY720 on Chemokines

The renal gene expression of chemokine receptor-1 (CCR1) was significantly increased in SNX rats compared with sham-operated control rats (Fig. 7). High-dose FTY720 treatment significantly reduced the renal CCR1 expression in SNX rats; low-dose FTY720 failed to be effective.

Similarly, renal cortical chemokine receptor-2 (CCR2) was higher in the renal cortex of SNX rats treated with vehicle than in sham-operated controls. CCR2 gene expression was lower in SNX rats treated with high-dose FTY720. Low-dose FTY720 treatment had no effects on renal cortical CCR2 gene expression.

Renal cortical chemokine receptor-5 (CCR5) gene expression also was significantly higher in vehicle-treated SNX rats compared with sham-operated controls. It was significantly lower in SNX rats treated with high-dose FTY720. Low-dose FTY720 treatment did not influence the CCR5 gene expression.

Renal monocyte chemoattractant protein-1 (MCP-1) gene expression was significantly greater in vehicle-treated SNX rats than in sham-operated controls. High-dose FTY720 treatment markedly lowered cortical renal MCP-1 gene expression.

Fig. 6. Representative examples of immunostainings in glomerular and tubulointerstitial areas. A–D: typical stains for CD3-positive lymphocytes in vehicle-treated controls (A) or vehicle-treated SNX rats (B) or low-dose FTY720-treated SNX rats (C) or high-dose FTY720-treated SNX rats (D). E–H: CD20-positive B lymphocytes in vehicle-treated controls (E) or vehicle-treated SNX rats (F) or low-dose FTY720-treated SNX animals (G) or high-dose FTY720-treated SNX animals (H). I–L: ED-1-positive monocytes/macrophages in vehicle-treated controls (I) or vehicle-treated SNX rats (J) or low-dose FTY720-treated SNX rats (K) or high-dose FTY720-treated SNX animals (L).
in SNX rats. Low-dose treatment was not sufficient in reducing MCP-1 gene expression. RANTES gene expression was not different in vehicle-treated SNX rats than in sham-operated controls at the endpoint of the experiment. Met-RANTES gene expression was increased in vehicle-treated SNX rats compared with vehicle-treated sham-operated controls. Treatment with FTY720 did normalize RANTES gene expression in SNX rats.

**Effects of FTY720 on Renal Gene Chemokine Expression**

The expression of chemokine receptor-1 (CCR1), CCR2, CCR5, and RANTES was studied in vehicle-treated SNX rats compared with sham-operated controls. Treatment with high-dose FTY720 reduced the renal gene expression of all chemokines significantly; low-dose treatment with FTY720 did not influence gene expression. Values are means ± SE.

**Effects of FTY720 on Fibronectin and TGF-β1 Immunostaining**

The immunostaining for fibronectin in the tubulointerstitial space was significantly enhanced in vehicle-treated SNX rats compared with sham-operated controls (Table 5). It was significantly reduced by either low- or high-dose FTY720 in SNX rats. The tubulointerstitial staining for TGF-β1 was markedly higher in SNX rats than in sham-operated controls. Significantly less staining was found in the tubulointerstitial space of SNX rats after treatment with FTY720.

**DISCUSSION**

Renal scarring is a hallmark of almost all kidney diseases with progressive renal failure (30). Previous work has shown that the antiproliferative substance mycophenolic acid is capable of reducing glomerular and interstitial damage in the 5/6 nephrectomy model (43).
We chose the nonimmunological model of 5/6 nephrectomy to study the effect of FTY720 on glomerular and tubulointerstitial damage (42). Previous work shows that recruitment/activation of inflammatory cells is notable in the remnant kidney (47). There are different mechanisms which are important for the inflammation in the 5/6 nephrectomy model, as, for example, activated tubular epithelial cells with a rich pool of cytokines, chemokines, and other mediators that promote leukocyte recruitment, cytotoxicity and fibrogenesis (21).

FTY720 acts through a SIP signaling pathway, sequestering lymphocytes into secondary lymphatic tissues and keeping them away from inflammatory lesions and graft sites (8). In addition, the reduction of monocytes and macrophages could not be explained. Similar to our study, fewer monocytes and macrophages were detected just as well as in other inflammatory models where FTY720 (33, 39, 61, 62) was applied.

In the present study, we tried to clarify the role of FTY720 on lymphocytes, subtypes of macrophages, and chemokines in a 5/6 nephrectomy model of renal damage. The effect on albuminuria was notably reduced compared with sham-operated animals. This reduction was not only a simple blood pressure effect, since FTY720-treated and untreated SNX rats had no significantly different blood pressure levels during the study period. Statistical significance was not reached because of high interindividual variability of vehicle-treated SNX rats (33). In contrast, there was a marked reduction in the tubulointerstitial damage, with less scarring, reduced interstitial fibronectin, and reduced TGF-β expression in the tubulointerstitium. In this remnant kidney model, the effects of FTY720 were more pronounced in the tubulointerstitial compound rather than in the glomerulus.

The lower glomerulosclerosis index probably reflects the direct effects of FTY720 on glomerular cell proliferation and immigration of inflammatory cells; however, FTY720 reduced glomerulosclerosis, but not completely. SNX rats developed an early surge of renal cell proliferation, with a peak level in the first week, which then gradually decreased, although never descending to control levels (16). Tubular cells show most of the proliferative activity, which might reflect a feasible participation of tubular cells in the pathogenesis of interstitial fibrosis (27). This view is paralleled by the observation that, in addition to lymphocytes, large numbers of monocytes/macrophages accumulated progressively in the interstitial area. Treatment with FTY720 not only lowered lymphocytes in the peripheral blood, it also reduced significantly the infiltration of glomerular and tubulointerstitial monocytes/macrophages and lymphocytes. The concomitant reduction of renal inflammation had positive effects on markers of renal damage, in particular tubulointerstitial lesions. These beneficial effects of FTY720 on renal structure are even more remarkable, since the renal damages attending this model are not attributed to a primary immunological disorder.

The renal damage in SNX rats was likely associated with several cell abnormalities detected in these rats. Fujihara et al. (16) have observed an early interstitial infiltration by T lymphocytes in 5/6 nephrectomized rats. These findings suggest that these cells may have contributed to the subsequent development of renal injury. The role of lymphocytes in the progression of tubulointerstitial injury is not clearly defined (50). Lymphocytes are rapidly recruited in immune-mediated glomerulonephritis and graft rejection (3, 24). It remains largely unclear why and how lymphocytes influence the rather unspecific inflammation going along with progressive renal diseases (42). Several potential mechanisms are conceivable. Within a cytokine-rich milieu, renal epithelial cells can present a variety of antigens and promote CD4(+)/T cell activation and proliferation, or CD8(+) cell-mediated cytotoxicity (26). Although there is no convincing evidence in nonimmune progressive renal diseases, the lymphocytes may indeed interact with yet undefined renal antigens (39). Lymphocytes interact closely with macrophages. Activated T cells also express proinflammatory cytokines that induce the tubular epithelial cell expression of macrophage-directed chemokines (29). In the adriamycin nephropathy model of nonimmune progressive proteinuric renal disease, CD8+ T cell depletion was associated with reduced macrophage infiltration and tubulointerstitial injury (58). In our model, there was an increase in CD4+ and CD8+ cells in vehicle-treated SNX rats in contrast to sham-operated controls. FTY720 significantly prevented glomerular and tubulointerstitial infiltration with CD4+ and CD8+ cells. Peters et al. (39) have described comparable effects in an immunological model of chronic anti-thy-1-induced nephritis. Previously, the relative percentage of B lymphocytes in the renal tubulointerstitium in chronic kidney disease was considered to be low (22). In our study, we detected only a weak CD-3-positive T cell staining in the glomerulus; however, there was a marked staining for CD20-positive B cells in vehicle-treated SNX rats compared with sham-operated controls. FTY720 treatment significantly lowered not only T lymphocytes in the blood but also reduced the infiltration of CD-20-positive B cells in the tubulointerstitial space in SNX rats.

There is complicated interaction between lymphocytes and macrophages in a mutual and complex manner (47, 55). Macrophages represent, in conjunction with lymphocytes, an important component of inflammatory cell infiltrates in several forms of experimental and human glomerulonephritis and are thought to contribute directly to renal injury (49). As a consequence of primary injury, proteinuria, chronic hypoxia, and glomerular-derived cytokines may all differentially modulate the expression of factors that promote macrophage recruitment.

A series of cytokines/chemokines secreted by infiltrating mononuclear cells has been shown to influence fibroblast proliferation and thus contribute to the progression of renal disease (49). MCP-1 is involved in tubulointerstitial damage in chronic kidney failure associated with glomerular hypertension (38). It is a secreted protein which specifically attracts blood

<table>
<thead>
<tr>
<th>Group</th>
<th>Tubulointerstitial</th>
<th>Glomerular</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>TGF-β</td>
<td>Fibronectin</td>
</tr>
<tr>
<td>Control/vehicle</td>
<td>0.83 ± 0.10</td>
<td>0.49 ± 0.07</td>
</tr>
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<td>0.55 ± 0.08</td>
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<tr>
<td>Control/high-dose</td>
<td>0.351 ± 0.02</td>
<td>0.50 ± 0.08</td>
</tr>
<tr>
<td>SNX/vehicle</td>
<td>2.86 ± 0.21</td>
<td>3.26 ± 0.23</td>
</tr>
<tr>
<td>SNX/low-dose</td>
<td>2.16 ± 0.31*</td>
<td>2.20 ± 0.28f</td>
</tr>
<tr>
<td>SNX/high-dose</td>
<td>1.55 ± 0.11†</td>
<td>2.07 ± 0.11t</td>
</tr>
</tbody>
</table>

Values are means ± SE. TGF-β1, transforming growth-factor-β1. Glomerular and tubulointerstitial staining intensity was scored using a 4-grade scale.

*P < 0.05, †P < 0.01, ‡P < 0.001 vs. SNX/vehicle (analysis of variance and Bonferroni’s multiple comparison test).
monocytes and tissue macrophages to its source via interaction with its cell surface receptor, CCR2 (7). MCP-1/CCL2 gene expression correlates with interstitial macrophage infiltration and fibrosis (10). In our study, MCP-1/CCL2 gene expression was significantly higher in SNX rats than in sham-operated controls. The expression of MCP-1/CCL2 decreased significantly after high-dose treatment with FTY720. Different authors have provided indirect evidence for this effect by demonstrating that renoprotective strategies are associated with lowered tubulointerstitial MCP-1/CCL2 expression and disease progression in SNX rats (13, 54).

Increased RANTES/CCL5 expression is present in acute and chronic renal disease (2, 6). Either resident interstitial cells and/or infiltrating leukocytes may produce MCP-1/CCL2 and RANTES/CCL5 (48). MCP-1 is a potent chemotactant for monocytes/macrophages, and RANTES attracts both lymphocytes and monocytes, which are a prominent histological feature in the SNX model. These data suggest that locally secreted MCP-1 and RANTES are important chemotactant mediators of interstitial leukocyte infiltration during the course of SNX rats.

To confirm this hypothesis, we studied the expression of the respective CC chemokine receptors in SNX rats. We showed that leukocytes infiltrating SNX rats differentially expressed MCP-1 and RANTES receptors. Higher levels of mRNA coding for the chemokine receptors CCR1 (binding MIP-1a and possibly murine RANTES), CCR2 (binding MCP-1), and CCR5 (binding RANTES, MIP-1a, MIP-1b) correlated with progressive interstitial mononuclear cell infiltrates, chemokine expression, and fibrotic changes in SNX rats. Increasing gene expressions of CCR1, CCR2, and CCR5 were also seen in other models of renal damage (1, 57). In these and the SNX model, the receptor induction was similarly associated with the expression of the corresponding chemokine mRNA for MCP-1 and RANTES. Thus the increased expression of CCR2 and CCR5 by infiltrating leukocytes may also be the result of a local modulation of chemokine receptor expression in chronically inflamed tissue compartments. We found that cortical gene expressions of CCR1, CCR2, and CCR5 were significantly elevated in vehicle-treated SNX rats compared with vehicle-treated sham-operated controls. The decrease in cortical gene expression was significantly more pronounced with high-dose than with low-dose FTY720 treatment. This dose effect is difficult to understand; a possible reason may be due to the variability of chemokine expression over the time course of the experiment. CCR1 has been demonstrated to play a major role in the induction of a shear-resistant arrest of monocytes and T lymphocytes (59) and in the recruitment of leukocytes to sites of inflammation in vivo (20). Increasing chemokine mRNA levels correlate with the extent of tubular damage, progressive interstitial infiltration of macrophages and lymphocytes, and concomitant fibrosis (57). High-dose FTY720 treatment reduced the cortical gene expression of Met-RANTES, MCP-1/CCL2, CCR1, CCR2, and CCR5.

Inflammatory monocytes are recruited in response to cytokine cues and undergo differentiation into two broad but distinct subsets of macrophages that are categorized as either M1 or M2 macrophages. There is evidence that macrophages are important in the resolution of injury and promote tissue restoration in both immune- and nonimmune-mediated renal disease (41).

CCR7 is a surface marker indicative of an M1 phenotype, and CD163 is a surface marker representative of an M2 phenotype (4). M1 and M2 macrophage subsets were markedly increased in vehicle-treated SNX rats compared with sham-operated rats. FTY720-treated SNX rats had significantly lower infiltration of glomerular and tubulointerstitial M1 phenotype macrophages. M1 phenotype macrophages are characterized by cells that are associated with classic signs of inflammation, especially chronic inflammation, and are an important source of inflammatory and profibrogenic cytokines (36). An increased tubulointerstitial expression of TGF-β1 has been reported during SNX (37). Moreover, the release of CC chemokines, particularly MCP-1/CCL2, by infiltrating M1 macrophages and lymphocytes may itself have a direct fibrogenic effect. MCP-1 has been reported to stimulate the collagen and TGF-β1 expression by fibroblasts, the latter resulting in an autocrine upregulation of collagen production (18).

FTY720 also significantly reduced M2 phenotype macrophages. The anti-inflammatory macrophage phenotype, signified as M2, promotes immunoregulation, tissue repair, and constructive tissue remodeling.

The reduction of M2 phenotype macrophages interacts with the manifold beneficial effects of FTY720 on lymphocytes, M1 phenotype macrophages, chemokines, and cytokines. The interaction of FTY720 with M2 phenotypes could interfere with tissue repair and remodeling. Although FTY720 demonstrated strong effects on established markers of renal inflammation and fibrosis, treatment with FTY720 could not stabilize creatinine clearance. One possible reason could be the suppressive effects on M2 phenotype macrophages.

REFERENCES

FTTY72 MODULATES CHEMOKINE GENE EXPRESSION


