Targeting renal macrophage accumulation via c-fms kinase reduces tubular apoptosis but fails to modify progressive fibrosis in the obstructed rat kidney

Frank Y. Ma,1 Jian Liu,1 A. Richard Kitching,1 Carl L. Manthey,2 and David J. Nikolic-Paterson1

1Department of Nephrology and Monash University Department of Medicine, Monash Medical Centre, Clayton, Victoria, Australia; and 2Johnson and Johnson Pharmaceutical Research and Development, Spring House, Pennsylvania

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Targeting renal macrophage accumulation via c-fms kinase reduces tubular apoptosis but fails to modify progressive fibrosis in the obstructed rat kidney. Am J Physiol Renal Physiol 296: F177–F185, 2009. First published November 15, 2008; doi:10.1152/ajprenal.90498.2008.—The role of macrophages in promoting interstitial fibrosis in the obstructed kidney is controversial. Macrophage depletion studies in the unilateral ureter obstruction (UUO) model have produced opposing results, presumably reflecting the subtleties of the individual depletion methods used. To address this question, we targeted the macrophage colony-stimulating factor receptor, c-fms, which is uniquely expressed by cells of the monocyte/macrophage lineage. Administration of 5, 12.5, or 30 mg/kg (bid) of a selective inhibitor of c-fms kinase activity (fms-I) resulted in a dose-dependent inhibition of renal macrophage accumulation in the rat UUO model. This was due to inhibition of local macrophage proliferation in the obstructed kidney and, at higher doses, to depletion of circulating blood monocytes. To determine the contribution of macrophages to renal pathology in the obstructed kidney, groups of animals were treated with 30 mg/kg fms-I and killed 3, 7, or 14 days later. Complete inhibition of renal macrophage accumulation prevented upregulation of the macrophage-associated proinflammatory mediators, tumor necrosis factor (TNF)-α and matrix metalloproteinase-12, and significantly reduced tubular apoptosis. Macrophage depletion caused a minor reduction of interstitial myofibroblast accumulation and deposition of interstitial collagen IV at day 7, but no difference was seen in renal fibrosis on day 7 or 14. Similarly, the upregulation of collagen IV, fibronectin, transforming growth factor-β1 and connective tissue growth factor mRNAs levels on day 7 and 14 in the obstructed kidney was unaffected by macrophage depletion. In conclusion, c-fms blockade was shown to selectively prevent interstitial macrophage accumulation and to reduce tubular apoptosis in the obstructed kidney, but it had no significant impact on the development of interstitial fibrosis.

Connective tissue growth factor; monocyte; myofibroblast; proliferation; transforming growth factor-β1

Interstitial fibrosis is a common feature of progressive kidney disease leading to end-stage renal failure. Clinical and experimental studies have identified a key role for α-smooth muscle actin (α-SMA)+ myofibroblasts and growth factors such as transforming growth factor (TGF)-β1 and connective tissue growth factor (CTGF) in the development of interstitial fibrosis (3). Macrophage accumulation is a prominent feature during the development of interstitial fibrosis in virtually all situations (3), but the role of these cells in promoting the fibrotic process is uncertain.

Defining the role of macrophages in interstitial fibrosis is a complex question since macrophages can induce renal inflammation and damage, events that promote the fibrotic response. Thus it is often difficult to distinguish between a direct profibrotic role for macrophages and an indirect role in promoting fibrosis secondary to tissue damage. Another complexity is the potential role of macrophages in promoting tissue repair (6). One reason for this complexity is that the local microenvironment encountered by monocyte/macrophages within a tissue has a major influence in determining the type of response that these macrophages make (17).

The most commonly used model to examine mechanisms of interstitial fibrosis is unilateral ureteric obstruction (UUO). Fibrosis in this model is driven by the same factors implicated in promoting fibrosis in human kidney disease and in other models of renal fibrosis, including α-SMA + myofibroblasts, TGF-β1, CTGF and ANG II (20, 30). Development of interstitial fibrosis in the obstructed kidney is very rapid, enabling functional blockade studies. Another important strength of this model is that injury to the tubulointerstitium is caused by an irreversible surgical insult. Thus the UUO model provides an opportunity to examine the direct role of macrophages in promoting interstitial fibrosis.

Different strategies have been employed to delete macrophages from the obstructed kidney. These studies have, in general, produced agreement that macrophages promote tubular apoptosis in the obstructed kidney (13, 16). However, conflicting results have been found in regard to whether macrophages do (1, 9, 14) or do not (22–24, 31) promote interstitial fibrosis in the obstructed kidney. These apparent discrepancies may be because of subtleties of the individual depletion methods used, making it difficult to interpret the role of macrophages in the development of interstitial fibrosis in the obstructed kidney.

Macrophage colony-stimulating factor (M-CSF, also known as CSF-1) is the principal growth factor regulating the proliferation, differentiation, and survival of monocyte/macrophages (26). M-CSF acts on monocyte/macrophages via the receptor tyrosine kinase c-fms (26). Expression of c-fms is restricted to cells of the monocyte/macrophage lineage, even in inflammatory conditions such as in the obstructed kidney (15). Many tissues, including the kidney, express M-CSF. Renal M-CSF expression is upregulated in a wide variety of diseases, which is correlated with macrophage accumulation and local macrophage proliferation (12). Furthermore, we have previously shown that administration of a neutralizing anti-c-fms antibody selectively reduced interstitial macrophage accumulation in the obstructed kidney, an effect that was attributed to inhibition of
local macrophage proliferation within the kidney (15). Thus blockade of c-fms is a strategy that can be used to selectively deplete macrophages from the obstructed kidney.

The aim of the current study was to use a specific inhibitor of the tyrosine kinase activity of c-fms to prevent macrophage accumulation in the obstructed kidney and thereby determine the functional role of macrophages in promoting interstitial fibrosis and tubular apoptosis in this model.

MATERIALS AND METHODS

Antibodies. Antibodies used in this study were as follows: OX-42, mouse anti-rat CD11b/c recognizing monocye/macrophages (27); OX-6, mouse anti-rat major histocompatibility complex (MHC) class II (18); ED1, mouse anti-rat CD68 recognizing monocye/macrophages (Serotec, Oxford, UK); R73, mouse anti-rat T cell receptor (Seropec); goat anti-collagen IV (Santa Cruz Biotechnology, Santa Cruz, CA); mouse anti-a-SMA (Sigma-Aldrich, St. Louis, MO); RP-1, mouse anti-rat neutrophils (Becton-Dickinson, San Diego, CA); and mouse antibodies against vimentin and Ki67 (both from Dako, Glostrup, Denmark). Secondary antibodies included biotinylated rabbit anti-goat IgG, biotinylated goat anti-mouse IgG, and streptavidin-conjugated horseradish peroxidase (all from Zymed-Inviron, Carlsbad, CA). Horseradish peroxidase-conjugated goat anti-mouse IgG, mouse peroxidase-conjugated anti-peroxidase complexes (PAP), and alkaline phosphatase-conjugated goat anti-mouse IgG were all from Dako (Glostrup, Denmark).

c-fms kinase inhibitor and compound characterization. fms-I (4-cyano-1H-imidazole-2-carboxylic acid [2-cyclohex-1-enyl-4-[1-(2-methanesulfonyl-ethyl)-piperidin-4-yl]-phenyl]-amide) is a selective inhibitor of the tyrosine kinase activity of c-fms synthesized by Johnson & Johnson Pharmaceutical Research and Development (11). To assess selectivity, the Invitrogen SelectScreen Kinase Profiling Service was employed to evaluate fms-I at 1 μM for activity against 32 serine/threonine kinases and 29 tyrosine kinases in the presence of 100 μM ATP. Of these, c-fms, c-kit, Flt-3, and Trk-A were inhibited >50%. IC50 values for these four kinases were determined using biochemical and cellular assays (Table 1). Kinase assays were as described previously (10). Functional impact on cellular c-fms activity was determined by measuring compound inhibition of CSF-1-driven proliferation of mouse macrophages as previously described (25). Cell proliferation dependent on Flt-3, c-kit, and Trk-A was assessed using MV-4–11 (ATCC no.: CRL-9591), M-07e (DSMZ no.: ACC 104), and TF-1 (ATCC no.: CRL-2003) cells, respectively. MV-4–11 cells grew independent of growth factor because of the expression of a constitutive active FLT3 mutation, and M-07e and TF-1 cells were driven to proliferate by 25 ng/ml stem cell factor and 1.4 ng/ml nerve growth factor, respectively. Following a culture period of 72 h, relative cell numbers were determined using CellTiterGlo reagent (Promega, Madison, WI).

Animal experiments. Male Sprague Dawley rats (180–220 g) were obtained from Monash Animal Services (Clayton, Australia). All animal experimentation was approved by the Monash Medical Centre Animal Ethics Committee. UUO was performed on rats under ketamine/xylazine anesthesia in which a midline incision was made, and the left ureter was exposed and tied off at two points and then cut between these ties. An initial study was performed to assess the ability of fms-I to inhibit macrophage accumulation in the obstructed kidney. Groups of four animals underwent UUO surgery and were treated with 5, 12.5, or 30 mg fms-I/kg body wt by two times daily oral gavage, beginning 3 h before surgery and continuing until animals were killed on day 7. Control groups of UUO rats had either no treatment or vehicle treatment (20% hydroxylpropyl-β-cyclodextrin in H2O).

In the second study, designed to achieve complete depletion of macrophages in the obstructed kidney, groups of rats underwent UUO surgery and were administered 30 mg fms-I/kg bid, no treatment, or vehicle treatment beginning 3 h before UUO surgery and continued until being killed on day 3 (n = 6), day 7 (n = 8), or day 14 (n = 8). Animals were killed by cardiac puncture under anesthesia, and heparinized blood was collected.

White blood cell counts and flow cytometry. White blood cell (WBC) counts were performed on heparinized blood using a Cell Dyn 3500 Cell Counter (Abbott Laboratories, Abbott Park, IL). To analyze blood monocyte numbers, mononuclear leukocytes were isolated from heparinized peripheral blood by centrifugation on Ficoll (GE Healthcare, Uppsala, Sweden). After being washed in PBS, cells were resuspended in 2% paraformaldehyde-lysolese-periodate for 20 min at 4°C. These fixed cells were then incubated with either ED1 or OX42 antibodies in PBS containing 10% normal sheep serum and 10% normal rat serum for 30 min at 4°C. For ED1 detection, 0.1% saponin was also included in the antibody buffer. After being washed three times, cells were then incubated with fluorescein isothiocyanate-conjugated sheep anti-mouse IgG in the same antibody buffers for 30 min at 4°C. After further washing, the cells were analyzed on a MoFlo flow cytometer connected to a data acquisition system (Becton Dickinson Cytomation, Fort Collins, CO). A fluorescence histogram of ~30,000 cells was obtained for each sample.

Immunocytochemistry. Immunoperoxidase staining for ED1, collagen IV, vimentin, and Ki67 was performed on paraffin sections of the obstructed kidney tissue. Immunoperoxidase staining for α-SMA was performed on formalin-fixed tissue sections using antigen retrieval (microwave oven heating in 0.1 M sodium citrate for 10 min) followed by a three-layer PAP staining method. Immunostaining for α-SMA was performed on formalin-fixed tissue sections without antigen retrieval and using a three-layer avidin-biotin peroxidase complex (ABC) staining method. Immunostaining for OX42, OX6, R73, and RPL1 was performed on frozen tissue sections fixed in 2% paraformaldehyde-lysolese-periodate with a three-layer PAP method. Two-color immunostaining was performed using formalin sections in which Ki67+ cells were first stained using the PAP peroxidase-based method. After microwave treatment to prevent antibody cross-reactivity, the sections were then stained for ED1+ macrophages using an alkaline phosphatase-conjugated ABC method with NBT/BCIP substrate development. ED1+ macrophages in the renal cortex were quantified by image analysis. Medium-power digital images of ED1 staining covering at least 90% of the cortex (×250) were analyzed using Image-Pro software (Media Cybernetics) to determine the area of ED1 staining as a percentage of the cortex. The interstitial area of α-SMA and collagen IV immunostaining was quantified in ×250 power fields that covered at least 90% of the cortex by image analysis using Image-Pro software. Large blood vessels and glomeruli were excluded from the analysis. The percentage of cortical tubules exhibiting one or more vimentin-positive cells was scored in ×250 fields of the entire cortex. To assess macrophage proliferation, the number of ED1+ Ki67+ and ED1+ Ki67− macrophages was counted under high power (×400) in the entire cortex. All scoring was performed on blinded slides.

Detection of apoptotic cells. Apoptotic cells in kidneys were identified using the ApopTag Peroxidase In Situ Apoptosis Detection Kit (Millipore-Chemincon, Temecula, CA). Briefly, 4 μm formalin-fixed paraffin sections were treated with freshly diluted 20 μg/ml

Table 1. Specificity of fms-I

<table>
<thead>
<tr>
<th>IC50 for fms-I μM</th>
<th>Kinase-Dependent Cell Proliferation μM</th>
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<tr>
<td>c-fms</td>
<td>0.0015</td>
</tr>
<tr>
<td>c-kit</td>
<td>0.012</td>
</tr>
<tr>
<td>Flt-3</td>
<td>0.058</td>
</tr>
<tr>
<td>Trk-A</td>
<td>0.030</td>
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</table>

Values represent the means of at least three determinations. *32 additional serine/threonine kinases and 25 additional tyrosine kinases were inhibited <50% by 1 μM fms-I.
proteinase K (Roche, Mannheim, Germany) for 10 min at room temperature. Endogenous peroxidase activity was blocked by incubating the sections in 3% H₂O₂ in PBS for 10 min. After treatment with the kit equilibration buffer, working-strength TdT enzyme was added for 60 min at 37°C and then incubated with anti-digoxigenin antibody conjugated to a peroxidase reporter molecule for 30 min at room temperature. Sections were then washed, developed in 3,3'-diaminobenzidine hydrochloride, and counterstained with a light hematoxylin. The numbers of the tubular and interstitial TdT-dUTP nick end labeling (TUNEL)-positive apoptotic cells in the entire cortex was scored on coded slides.

Real-time RT-PCR. Rat kidney samples used in PCR analysis were snap-frozen in liquid nitrogen and stored at −80°C until RNA was extracted using a RiboPure RNA isolation kit (Ambion, Austin, TX). cDNA was prepared from total RNA by reverse transcription using random hexamer primers and Superscript II (Invitrogen, Carlsbad, CA). Real-time PCR of prepared cDNA were examined over 50 cycles on a Rotor-Gene 3000 system (Corbett Research, Sydney, NSW, Australia). Each PCR involved thermal cycling conditions of 37°C for 10 min, 95°C for 5 min, followed by 50 cycles of 95°C for 15 s, 60°C for 20 s, and 68°C for 20 s. The primer pairs and probes used were as follows: collagen IV (forward: GGC GTG GCA CAG TCA GAC CAT; reverse: GGA ATA GCC AAT CCA CAG TGA; probe: CAG TGC CCC AAC GGT); fibronectin (forward: CTG TGC GCT ATT ACA GAA TC; reverse: AGT GAC AGC ATA CAG GGT GA; probe: GGA GAG ACA GGA GGA A); CTGF (forward: CAA ACT CCA AAC ACC A; reverse: CGT TTG TGC CTA TTG TTC TTG T; probe: GAT CCA TTG CTT TAC CGT CTA C); TGF-β1

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Fig. 1. Macrophage accumulation in the obstructed kidney. Immunostaining for ED1+ macrophages (A–C), OX-42+ macrophages (D–F), and major histocompatibility complex (MHC) class II (G–I) is shown for normal rat kidney (A, D, G) and day 7 unilateral ureter obstruction (UUO) kidneys treated with vehicle (B, E, H) or 30 mg/kg fms-I (C, F, I) (magnification ×250). J: graph showing quantification of ED1+ macrophages in the renal cortex in the dose-response study of fms-I treatment on day 7 of UUO. K: graph showing quantification of ED1+ macrophages on days 3 and 14 UUO in vehicle and 30 mg/kg fms-I-treated groups. *+ + + P < 0.001 vs. normal; * P < 0.05, ** P < 0.01, and *** P < 0.001 vs. no treatment and vehicle groups at the same time point. Results are shown as means ± 1 SD and analyzed by ANOVA with Bonferroni’s posttest for multiple comparisons.
Macrophages Induce Apoptosis but Not Fibrosis in Rat UUO

The obstructed kidney also features minor T cell and neutrophil infiltrates, and these were not affected by fms-I treatment (data not shown).

Effect of fms-I treatment on blood leukocyte populations. WBC counts were elevated in the vehicle and no treatment UUO groups compared with normal rats, but this did not reach statistical significance (Table 2). Treatment with 5 or 12.5 mg fms-I/kg had no effect on total WBC counts, whereas high-dose fms-I treatment resulted in a significant reduction in WBC counts compared with control UUO groups, but this was not different from WBC counts in normal rats (Table 2). Because rats have low levels of blood monocytes, this population was further analyzed by flow cytometry. This showed a dose-dependent reduction in blood monocyte numbers, with the two higher doses of fms-I producing a significant reduction. Further analysis showed that fms-I had no effect on blood neutrophils, lymphocytes, or platelets at 5 or 12.5 mg/kg. However, a significant reduction in blood neutrophils was seen with 30 mg fms-I/kg compared with control UUO groups, but this was not different from blood neutrophil levels in normal rats (Table 2).

A very similar picture of reduced blood monocytes and a minor reduction in blood neutrophil numbers was evident after 3 or 14 days treatment with 30 mg fms-I/kg in the UUO model (data not shown). Animal body weight was not affected by any dose of fms-I treatment (data not shown).

### RESULTS

**fms-I treatment inhibits macrophage accumulation in the obstructed kidney.** Immunostaining with the ED1 antibody showed a marked accumulation of interstitial CD68+ macrophages on day 7 in the obstructed kidney (Fig. 1). Administration of fms-I resulted in a dose-dependent reduction of interstitial CD68+ macrophage accumulation (Fig. 1). To confirm that c-fms inhibition had not simply downregulated CD68 antigen expression, we examined the expression of two other macrophage antigens (CD11b/c and MHC class II). Both antigens were expressed on the interstitial macrophage infiltrate seen in the vehicle and no treatment UUO groups, but interstitial staining for both macrophage antigens was abrogated in the high-dose fms-I-treated group (Fig. 1). The number of ED1+ cells in the contralateral kidney was also reduced by high-dose fms-I treatment (data not shown), indicating an effect on the resident macrophage population.

In a further series of studies, the 30 mg fms-I/kg dose was administered to groups of rats that were killed on day 3 or day 14 after UUO surgery. Interstitial macrophage accumulation in the obstructed kidney was abrogated at both early and late time points (Fig. 1).

### Table 2. White blood cell counts on day 7 of UUO

<table>
<thead>
<tr>
<th>Cell Population*</th>
<th>Normal</th>
<th>No Tx UUO</th>
<th>Vehicle UUO</th>
<th>5 mg/kg fms-I</th>
<th>12.5 mg/kg fms-I</th>
<th>30 mg/kg fms-I</th>
</tr>
</thead>
<tbody>
<tr>
<td>ED1</td>
<td>0.31±0.16</td>
<td>0.45±0.21</td>
<td>0.52±0.16</td>
<td>0.25±0.12</td>
<td>0.16±0.05†</td>
<td>0.03±0.03‡</td>
</tr>
<tr>
<td>OX42</td>
<td>0.37±0.17</td>
<td>0.73±0.31</td>
<td>0.65±0.27</td>
<td>0.36±0.08</td>
<td>0.19±0.07†</td>
<td>0.08±0.04§</td>
</tr>
<tr>
<td>Total WBC</td>
<td>6.79±2.29</td>
<td>9.25±2.13</td>
<td>9.86±2.17</td>
<td>7.10±1.64</td>
<td>6.95±1.62</td>
<td>5.74±1.49§</td>
</tr>
<tr>
<td>Monocyte</td>
<td>0.39±0.27</td>
<td>0.45±0.19</td>
<td>0.50±0.16</td>
<td>0.22±0.10</td>
<td>0.19±0.09</td>
<td>0.07±0.03*</td>
</tr>
<tr>
<td>Neutrophil</td>
<td>1.49±1.19</td>
<td>2.98±1.23</td>
<td>3.17±1.06</td>
<td>2.13±0.90</td>
<td>1.27±0.47</td>
<td>1.12±0.53§</td>
</tr>
<tr>
<td>Lymphocyte</td>
<td>4.49±0.80</td>
<td>5.54±0.62</td>
<td>5.45±2.06</td>
<td>4.47±0.90</td>
<td>4.70±1.85</td>
<td>4.42±1.15</td>
</tr>
<tr>
<td>Platelet</td>
<td>8.15±1.82</td>
<td>9.67±1.08</td>
<td>10.02±1.56</td>
<td>10.66±1.31</td>
<td>8.54±2.58</td>
<td>8.83±0.89</td>
</tr>
</tbody>
</table>

Data are means±SD. *ED1- and OX-42- positive blood monocytes were analyzed by flow cytometry. White blood cell (WBC) counts were performed on a Cell Dyn 3500 Cell Counter. No Tx, no treatment; UUO, unilateral ureteric obstruction. WBC: ×10⁹/L; platelets: ×10¹²/L. †P < 0.01, ‡P < 0.001, and §P < 0.05 vs. vehicle group.

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treatment prevented the increase in TNF-α and MMP-12 mRNA levels at all time points (Fig. 3). Low-dose fms-I treatment had no impact on the upregulation of TNF-α or MMP-12, whereas the 12.5 mg/kg dose of fms-I suppressed upregulation of TNF-α but not MMP-12. These data are consistent with macrophages being responsible for upregulation of these factors in the obstructed kidney.

Effect of fms-I treatment on renal fibrosis in the obstructed kidney. The development of interstitial fibrosis in the obstructed kidney is characterized by prominent accumulation of α-SMA+ myofibroblasts and deposition of collagen IV. Interstitial accumulation of α-SMA+ myofibroblasts is first seen on day 3 of the UUO model with the development of marked fibrosis on days 7 and 14. We examined whether macrophage depletion using high-dose fms-I treatment affected the development of renal fibrosis in the obstructed kidney. The minor accumulation of α-SMA+ myofibroblasts and collagen IV deposition seen at day 3 in untreated UUO was partially reduced by macrophage depletion (Fig. 4, B and C). However, the prominent accumulation of α-SMA+ myofibroblasts and deposition of collagen IV seen on days 7 and 14 were not affected by fms-I treatment (Fig. 4). Similarly, the UUO animals given 5 or 12.5 mg fms-I/kg for 7 days exhibited no change in myofibroblast accumulation or collagen IV deposition (Fig. 4).

Consistent with the histologic findings, high-dose fms-I treatment caused a significant reduction in TGF-β1 mRNA levels on day 3 UUO with a nonsignificant trend for a reduction in collagen IV mRNA levels (Fig. 5). However, fms-I treatment had no impact on the significant increase in renal mRNA levels for matrix proteins collagen IV and fibronectin and profibrotic growth factors TGF-β1 and CTGF, on days 7 or 14 in the obstructed kidney (Fig. 5).

In addition, we analyzed the effect of fms-I treatment on MMP-3 and MMP-9. A marked increase in MMP-3 mRNA levels was apparent on day 3 UUO, which were significantly reduced by high-dose fms-I treatment. However, the further increase in MMP-3 mRNA levels seen on days 7 and 14 were not affected by fms-I treatment (Fig. 5). In contrast, MMP-9 mRNA levels were not increased above normal in the UUO model at any time point (data not shown).

Effect of fms-I on tubular damage in the obstructed kidney. Tubular damage is a prominent feature in the obstructed kidney. Previous studies have found that macrophages contribute, in part, to tubular apoptosis, which peaks around day 7 (13, 16). TUNEL staining identified an increase in the number of apoptotic tubular cells in the obstructed kidney, and fms-I

Fig. 2. Macrophage proliferation in the obstructed kidney. A: presence of proliferating macrophages in the obstructed kidney was identified by double immunostaining for Ki67+CD68+ cells (magnification ×250). B: graph quantifying the no. of Ki67+CD68+ cells in the renal cortex on day 7 in no treatment vs. 5 mg/kg fms-I. C: graph quantifying the percentage of CD68+ macrophages exhibiting Ki67 staining on day 7 in no treatment vs. 5 mg/kg fms-I. Results are shown as means ± 1 SD and analyzed by t-test.

Fig. 3. Real time RT-PCR analysis of mRNA levels for tumor necrosis factor (TNF)-α and matrix metalloproteinase (MMP)-12 in normal kidney compared with no treatment, vehicle, and fms-I-treated groups on days 3, 7, and 14 UUO. Results are shown as means ± 1 SD and analyzed using Kruskal-Wallis ANOVA with Dunn’s posttest for multiple comparisons. +P < 0.05 and ++P < 0.01 vs. normal; *P < 0.05, **P < 0.01, and ***P < 0.001 vs. no treatment and vehicle groups.
cytokines by systemic administration of an inhibitor of the tyrosine kinase activity of the M-CSF receptor, c-fms. The drug was well tolerated, and, when administered at 30 mg/kg, completely prevent macrophage accumulation in the obstructed kidney. This was more effective compared with other strategies for macrophage depletion that have achieved a 40–75% reduction in macrophage accumulation in the obstructed kidney, including liposome clodronate (28), cyclophosphamide (23), monocyte chemoattractant protein-1 (MCP-1/CCR2) blockade (14), and diptheria toxin in susceptible mice (2). These findings are also consistent with studies in CSF-1-deficient op/op mice in which macrophage accumulation in the obstructed kidney is significantly reduced (16).

Two mechanisms were identified that likely account for the ability of fms-I treatment to inhibit macrophage accumulation in the obstructed kidney. First, the lowest dose of fms-I significantly reduced local macrophage proliferation and accumulation within the obstructed kidney without affecting blood monocyte levels. This is consistent with our previous studies using anti-c-fms antibody treatment, which substantially reduced local macrophage proliferation and accumulation without depleting blood monocytes (15). Second, the higher doses of fms-I caused depletion of circulating blood monocytes, thereby preventing monocyte recruitment in the obstructed kidney. Presumably, this reflects increasing levels of fms-I in the bone marrow compartment.

fms-I showed selectivity for inhibition of macrophage accumulation in the obstructed kidney. The reduction in macrophage accumulation was confirmed using three different macrophage antigens, demonstrating that c-fms inhibition had not simply altered monocyte differentiation or modified expression of a single antigen. In addition, the complete downregulation of TNF-α and MMP-12 levels within the obstructed kidney is another indicator that fms-I treatment was effective in preventing macrophage accumulation. In addition, fms-I treatment did not affect WBCs other than monocytes, except for a small reduction in neutrophil counts (although this was still in the normal range), and fms-I had no effect on the T cell or neutrophil infiltrate in the obstructed kidney.

The current study identified a role for macrophages in promoting tubular cell apoptosis and a minor role for macrophages in promoting tubular dedifferentiation resulting in increased vimentin expression. This is consistent with previous reports in which macrophages have been shown to induce tubular cell apoptosis in vitro and in the obstructed kidney (13, 16). Upregulation of the proapoptotic factor TNF-α has been implicated in causing apoptosis of tubular cells in the obstructed kidney (19). Thus suppression of renal TNF-α expression may be one mechanism by which fms-I treatment inhibited tubular apoptosis in this model. A role for macrophage nitric oxide production in causing tubular cell apoptosis in vitro and in vivo has been described (13), although other studies have suggested that nitric oxide can be protective against tubular cell apoptosis in the obstructed kidney (21).

We found that macrophages contribute to the very earliest stage in the development of renal fibrosis in the obstructed kidney. Macrophage depletion partially reduced the early myofibroblast infiltrate, increased deposition of collagen IV on day 3 UUO, and inhibited the early increase in TGF-β1 and MMP-3 mRNA levels. However, this effect of fms-I treatment on renal fibrosis was not sustained with time as fibrosis became more marked.

In this study, we were able to achieve dose-dependent inhibition of macrophage accumulation in the obstructed kidney by systemic administration of an inhibitor of the tyrosine kinase activity of the M-CSF receptor, c-fms. The drug was well tolerated, and, when administered at 30 mg/kg, completely prevent macrophage accumulation in the obstructed kidney. This was more effective compared with other strategies for macrophage depletion that have achieved a 40–75% reduction in macrophage accumulation in the obstructed kidney, including liposome clodronate (28), cyclophosphamide (23), monocyte chemoattractant protein-1 (MCP-1/CCR2) blockade (14), and diptheria toxin in susceptible mice (2). These findings are also consistent with studies in CSF-1-deficient op/op mice in which macrophage accumulation in the obstructed kidney is significantly reduced (16).

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In this study, we were able to achieve dose-dependent inhibition of macrophage accumulation in the obstructed kidney by systemic administration of an inhibitor of the tyrosine kinase activity of the M-CSF receptor, c-fms. The drug was well tolerated, and, when administered at 30 mg/kg, completely prevent macrophage accumulation in the obstructed kidney. This was more effective compared with other strategies for macrophage depletion that have achieved a 40–75% reduction in macrophage accumulation in the obstructed kidney, including liposome clodronate (28), cyclophosphamide (23), monocyte chemoattractant protein-1 (MCP-1/CCR2) blockade (14), and diptheria toxin in susceptible mice (2). These findings are also consistent with studies in CSF-1-deficient op/op mice in which macrophage accumulation in the obstructed kidney is significantly reduced (16).

Two mechanisms were identified that likely account for the ability of fms-I treatment to inhibit macrophage accumulation in the obstructed kidney. First, the lowest dose of fms-I significantly reduced local macrophage proliferation and accumulation within the obstructed kidney without affecting blood monocyte levels. This is consistent with our previous studies using anti-c-fms antibody treatment, which substantially reduced local macrophage proliferation and accumulation without depleting blood monocytes (15). Second, the higher doses of fms-I caused depletion of circulating blood monocytes, thereby preventing monocyte recruitment in the obstructed kidney. Presumably, this reflects increasing levels of fms-I in the bone marrow compartment.

fms-I showed selectivity for inhibition of macrophage accumulation in the obstructed kidney. The reduction in macrophage accumulation was confirmed using three different macrophage antigens, demonstrating that c-fms inhibition had not simply altered monocyte differentiation or modified expression of a single antigen. In addition, the complete downregulation of TNF-α and MMP-12 levels within the obstructed kidney is another indicator that fms-I treatment was effective in preventing macrophage accumulation. In addition, fms-I treatment did not affect WBCs other than monocytes, except for a small reduction in neutrophil counts (although this was still in the normal range), and fms-I had no effect on the T cell or neutrophil infiltrate in the obstructed kidney.

The current study identified a role for macrophages in promoting tubular cell apoptosis and a minor role for macrophages in promoting tubular dedifferentiation resulting in increased vimentin expression. This is consistent with previous reports in which macrophages have been shown to induce tubular cell apoptosis in vitro and in the obstructed kidney (13, 16). Upregulation of the proapoptotic factor TNF-α has been implicated in causing apoptosis of tubular cells in the obstructed kidney (19). Thus suppression of renal TNF-α expression may be one mechanism by which fms-I treatment inhibited tubular apoptosis in this model. A role for macrophage nitric oxide production in causing tubular cell apoptosis in vitro and in vivo has been described (13), although other studies have suggested that nitric oxide can be protective against tubular cell apoptosis in the obstructed kidney (21).

We found that macrophages contribute to the very earliest stage in the development of renal fibrosis in the obstructed kidney. Macrophage depletion partially reduced the early myofibroblast infiltrate, increased deposition of collagen IV on day 3 UUO, and inhibited the early increase in TGF-β1 and MMP-3 mRNA levels. However, this effect of fms-I treatment on renal fibrosis was not sustained with time as fibrosis became more marked.
A major finding in the current study was that abrogation of macrophage accumulation by fms-1 treatment had no detectable impact on progressive interstitial fibrosis in the obstructed kidney. It is, in general, difficult to establish negative findings. The lack of effect of the 5 and 12.5 mg/kg doses of fms-1 treatment on interstitial fibrosis could have been attributed to incomplete macrophage depletion if the remaining macrophage population was sufficient to promote fibrosis. Indeed, this was a sticking point in drawing any conclusions in our previous study using an anti-c-fms antibody that reduced macrophage infiltration by 75% in the obstructed kidney (15) but had no effect on myofibroblast accumulation, collagen synthesis, and deposition or upregulation of TGF-β1 (unpublished results). These data are consistent with a number of studies that argue that macrophages do not promote interstitial fibrosis in the obstructed kidney. Cyclophosphamide-mediated macrophage depletion had no protective effect on interstitial fibrosis in mouse UUO; indeed, this study found a protective, antifibrotic effect of macrophage adoptive transfer in leukocyte-depleted mice in the late stages of the UUO model (23). In another study, wild-type mice reconstituted with bone marrow lacking the ANG II type 1 receptor developed more severe interstitial fibrosis with fewer interstitial macrophages than mice reconstituted with wild-type mice, arguing for a protective role of macrophages in this model (22). Similarly, mice deficient for the urokinase receptor develop more severe interstitial fibrosis and reduced macrophage accumulation in the obstructed kidney (31). Again, blockade of MMP-2 results in more severe interstitial fibrosis in the obstructed kidney with reduced macrophage accumulation (24).

Although the data from the current study are clear-cut, they appear to conflict with a number of published studies. Targeting of MCP-1/CCL2 or its receptor has been shown to cause a partial reduction in both macrophage accumulation and interstitial fibrosis in the obstructed kidney (14), although this could be in part explained by the ability of MCP-1 to induce migration, proliferation, and collagenase production by fibroblast-type cells (7). Reduction of interstitial macrophage in the obstructed kidney through CCR1 blockade has also been shown to reduce interstitial fibrosis (1), providing a strong argument, since CCR1 expression is almost exclusively re-
stricted to leukocyte populations. In a separate study, a two-thirds reduction in the macrophage infiltrate in the UUO model achieved in a transgenic diphtheria-toxin model was highly effective in suppressing interstitial fibrosis (9). An intriguing possibility in this study is whether phagocytosis of the apoptotic macrophages by the remaining macrophage population, or by other neighboring cell types, could have modified a proinflammatory response into an anti-inflammatory and reparative

Fig. 6. Analysis of apoptosis in the obstructed kidney. A: TdT-dUTP nick end labeling (TUNEL) staining identified the presence of apoptotic cells (arrows) in the no treatment group on day 7 UUO (magnification ×250). Graphs show quantification of the no. of TUNEL+ tubular epithelial cells (B), and TUNEL+ interstitial cells (C) in normal rat kidney and in no treatment, vehicle-treated, and 30 mg/kg fms-I-treated groups on day 7 UUO. Results are shown as means ± 1 SD and analyzed using ANOVA with Bonferroni’s posttest for multiple comparisons. +++P < 0.001 vs. normal; *P < 0.05 vs. no treatment and vehicle-treated groups.

Fig. 7. Tubular damage and dedifferentiation. Immunostaining was performed for vimentin in normal rat kidney (A), vehicle-treated day 7 UUO (B), and 30 mg/kg fms-I-treated day 7 UUO (C). Graphs showing quantification of tubular vimentin expression on day 7 UUO (D) and day 14 UUO (E). Results are shown as means ± 1 SD and analyzed using ANOVA with Bonferroni’s posttest for multiple comparisons. +++P < 0.001 vs. normal; *P < 0.05 and ***P < 0.001 vs. no treatment and vehicle-treated groups.
response (4, 8). Finally, gelatin-3-deficient mice are protected from interstitial fibrosis in the UUO model, and this can be restored by macrophage adoptive transfer (9). This clearly demonstrates that macrophages have the potential to induce interstitial fibrosis. However, one limitation of this approach is that the bone marrow-derived macrophages are cultured for 7–9 days in fibroblast-conditioned media before adoptive transfer, thus creating the potential that these macrophages have been programmed toward a profibrotic response (5). Indeed, cytokine-based programming of cultured macrophages before adoptive transfer has been shown to exert quite different effects in a mouse model of adriamycin nephropathy (29).

In summary, the current studies have demonstrated that systemic administration of an inhibitor of the c-fms tyrosine kinase activity is a selective and highly effective method to prevent macrophage accumulation within the obstructed kidney. This inhibition of macrophage accumulation caused a significant reduction in tubular apoptosis. Furthermore, this study has demonstrated that macrophages are not required for progressive myofibroblast accumulation, upregulation of TGF-β1 or CTGF expression, or for increased collagen deposition in the obstructed kidney.

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DISCLOSURES

C. L. Manthey is an employee of Johnson & Johnson. D. J. Nikolic-Paterson acts as a consultant for Johnson & Johnson and sits on a Medical Advisory Board for Johnson & Johnson.

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