Obstructive Nephropathy in the Mouse: Progressive Fibrosis Correlates with Tubulointerstitial Chemokine Expression and Accumulation of CC Chemokine Receptor 2- and 5-Positive Leukocytes

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Abstract. The infiltration of leukocytes plays a major role in mediating tubulointerstitial inflammation and fibrosis in chronic renal disease. CC chemokines participate in leukocyte migration and infiltration into inflamed renal tissue. Because CC chemokine-directed leukocyte migration is mediated by target cell expression of a group of CC chemokine receptors, this study examined the expression of CC chemokines and their receptors during initiation of tubulointerstitial fibrosis after unilateral ureteral obstruction in C57BL/6 mice. Obstructed kidneys developed hydronephrosis, tubular cell damage, interstitial inflammation, and fibrosis. From days 2 to 10, a progressive interstitial influx of F4/80+ macrophages and CD3+ lymphocytes occurred (macrophages, 4-fold; lymphocytes, 20-fold at day 10, compared with contralateral control kidneys). In parallel, the number of activated fibroblast-specific protein 1+ fibroblasts and interstitial collagen IV accumulation increased from days 2 to 10. The mRNA expression of CC chemokines (predominantly monocyte chemoattractant protein-1 [MCP-1]/CCL2, RANTES/CCL5) and their receptors CCR1, CCR2, CCR5 increased progressively from days 2 to 10. By in situ hybridization, a prominent interstitial mRNA expression of MCP-1 and RANTES and their receptors CCR2 and CCR5 localized to interstitial mononuclear cell infiltrates. MCP-1 and RANTES expression was also seen in tubular epithelial cells. Fluorescence-activated cell sorter analysis of single-cell suspensions from obstructed kidneys revealed a prominent expression of CCR2 and CCR5 by infiltrating macrophages, whereas most lymphocytes expressed CCR5 only. These data demonstrate an increased expression of MCP-1/CCL2 and RANTES/CCL5 at sites of tubulointerstitial damage and progressive fibrosis during unilateral ureteral obstruction that correlates with simultaneous accumulation of interstitial macrophages and T lymphocytes expressing the respective surface receptors CCR2 and CCR5. The chemokine receptor–mediated leukocyte influx into the tubulointerstitium could offer a new potential target for therapeutic intervention in progressive renal tubulointerstitial fibrosis.
membrane-spanning G-protein–linked receptors, which are specific for individual chemokine subgroups. Within one subgroup, multiple chemokines can bind to a single receptor, which leads to a certain degree of redundancy (8,9). The pivotal role for chemokines in the pathogenesis of renal inflammation has been verified by blocking chemokine activity with neutralizing antibodies, chemokine receptor antagonists, and targeted chemokine gene disruption in various animal models (8,10). The CC chemokines monocyte chemoattractant protein-1 (MCP-1)/CCL2, RANTES/CCL5, and macrophage inflammatory protein-1α (MIP-1α)/CCL3 emerged as important mediators of renal leukocyte attraction and inflammation by these neutralization studies (11–20). In two animal models of glomerulonephritis, administration of anti–MCP-1 antiserum or the CC chemokine receptor antagonist AOP-RANTES decreased not only glomerular leukocyte infiltrates but also glomerular and interstitial collagen deposition (13,15,17). Thus, CC chemokines and their receptors might also play a role in mediating tubulointerstitial leukocyte recruitment, local inflammation, and subsequent tubulointerstitial fibrosis. However, the expression pattern and role of CC chemokines and particularly their specific receptors in tubulointerstitial inflammation and progressive fibrosis have not been investigated. We used unilateral ureteral obstruction (UUO) in C57Bl/6 mice as a model, which is characterized by an infiltration of macrophages and T cells into the interstitium and concomitant renal fibrosis (6,21). In the present investigation, we demonstrate a progressively increasing tubulointerstitial expression of MCP-1 and RANTES during the development of obstructive nephropathy, which correlates with a simultaneous interstitial accumulation of macrophages and lymphocytes expressing the respective surface receptors CCR2 and CCR5 and a progressive interstitial fibrosis. Blockade of the CC chemokine receptor–mediated leukocyte influx by chemokine receptor antagonists therefore may offer a new therapeutic approach for progressive interstitial fibrosis in chronic renal disease.

Materials and Methods

Experimental Model of Obstructive Nephropathy

Female inbred C57Bl/6 mice weighing 20 to 26 g were obtained from Charles River (Sulzfeld, Germany) and kept in macrolone type III cages under a 12-h light/dark cycle. Water and standard chow (Sniff, Soest, Germany) were available ad libitum. All experimental protocols were approved by the Animal Care and Ethics Committee of the Bavarian government.

Under general ether anesthesia, unilateral ureteral ligation resulting in UUO was performed by ligating the left distal ureter with a 2/0 Mersilene suture through a low midline abdominal incision. Unobstructed contralateral kidneys served as controls. Groups of mice were killed at 2, 6, and 10 d after UUO by cervical dislocation under general anesthesia with inhaled ether.

Renal Morphology and Immunohistochemistry

From each mouse cranial kidney halves were used for histologic assessment. Ligated and contralateral kidney tissue was fixed for 24 h at room temperature in 4% neutral buffered formalin and then embedded in paraffin. For quantitative analysis, 4-μm horizontal sections were cut. Every fifth of 15 subsequent sections, chosen by systematic uniformly random sampling, was used for analysis (22). Slides were stained with periodic acid-Schiff (PAS) reagent for routine histology and morphometric analysis. For immunohistologic studies, sections were deparaffinized, rehydrated, transferred into citrate buffer, and either autoclaved or microwave treated. Sections were blocked with 3% peroxidase, avidin, and biotin (Vector Blocking Kit; Vector Laboratories, Burlingame, CA) for 20 min each. After slides were washed in phosphate-buffered saline, they were incubated with the primary antibody for 1 h at room temperature. The following monoclonal rat antibodies were used: anti-mCD45 against leukocytes (Pharmingen, San Diego, CA; 1:200), anti-F4/80 against macrophages (Serotec, Oxford, UK; 1:50), anti-mCD3 against human lymphocytes (Serotec; 1:100), and anti-type IV collagen (Research Diagnostics, Flanders, NY; 1:100). Activated fibroblasts were stained with a polyclonal antiseraum (1:1000) against recombinant murine fibroblast-specific protein 1 (FSP1) generated in a New Zealand white rabbit as described elsewhere (23). For mRANTES/CCL5, a polyclonal rabbit anti-mouse antibody (PeproTech, Rocky Hill, NY; 1:50) was used. For detection of mMCP-1/CCL2, a custom antipeptide antiserum against murine MCP-1 was raised in rabbits as described previously (24). Signals of all primary antibodies were detected with a commercial mouse link and label kit following the instructions of the supplier (SuperSensitive; BioGenex, San Ramon, CA). 3-amino-9-ethylcarbazole substrate was used for signal development. All sections were counterstained with hemaluma. For quantitation of the interstitial cell infiltrate, 20 cortical high-power fields (hpf, 400×) per kidney were selected randomly by a blinded observer and the mean cell count in each kidney was expressed as cells per hpf.

Morphometric Analysis of Tubular Damage, Interstitial Volume, and Interstitial Type IV Collagen Deposition

Markers of tubular damage and interstitial fibrosis were assessed morphometrically (25). In brief, a grid containing 117 (13 × 9) sampling points was superimposed on images of cortical hpf (400×) from sections stained with PAS reagent or type IV collagen antibody. The number of grid points overlying the tubular lumen (tubular dilation index), atrophic or necrotic tubular cells (index of tubular cell damage), interstitial space (interstitial volume index), and interstitial type IV collagen deposits (interstitial collagen IV index) was counted and expressed as a percentage of all sampling points. For each kidney, 10 randomly selected, nonoverlapping fields were analyzed by a blinded observer.

In Situ Hybridization

Single-stranded radiolabeled riboprobes for mMCP-1/CCL2, mRANTES/CCL5, mCCR2, and mCCR5 were prepared by in vitro transcription of subcloned cDNA fragments. The 161-bp MCP-1 probe corresponds to nucleotides 299 to 459 (GenBank accession number, J04467) and the 321-bp mRANTES probe corresponds to nucleotides 124 to 444 (GenBank accession number, S37648) of the respective cDNA sequence. For mCCR2 and mCCR5 probes, cDNA fragments in the untranslated 5' and 3' end, respectively, with low sequence homology were subcloned. The 153-bp mCCR2 probe represents nucleotides 1736 to 1888 (GenBank accession number, U47035), and the 220-bp mCCR5 probe represents nucleotides 1384 to 1604 (GenBank accession number, D83648). Antisense and sense RNA transcripts were labeled with α32P-UTP (1250 Ci/mmol; NEN, Cologne, Germany) to a specific activity of 8 × 106 cpm/μg and served as hybridization probe and control, respectively. In situ hybridization was performed as described previously following modified
standard protocols (24). Briefly, 4-μm paraffin sections were digested with 5 μg/ml proteinase K and acetylated. Prehybridization was performed in 50% formamide, 0.3 M NaCl, 10 mM sodium phosphate buffer, 10 mM Tris-HCl, 5 mM ethylenediaminetetraacetic acid (EDTA; pH 7.4), 10% dextrose, 25 mM DTT, 1× Denhardt’s, and 1.25 mg/ml RNA at 52°C for 2 h. Sections were hybridized at 52°C overnight with 40 μl of prehybridization solution containing labeled RNA probe with an activity of 0.8 × 10^6 cpm. After hybridization, four washes in 4 × SSC were followed by a washing procedure with 50% formamide, 1 × SSC at 50°C for 60 min. Sections then were incubated in a mixture of RNase A (40 μg/ml) and RNase T1 (50 units/ml) at 37°C for 30 min, followed by two high-stringency washes in 0.1 × SSC (37°C, 15 min). Slides were dehydrated, dipped in Ilford K2 nuclear research emulsion (Ilford, Mobberly, UK) diluted 1:1 with distilled water at 42°C, and exposed for 2 to 4 wk at 4°C in a dry chamber. After development, sections were counterstained with Harris hematoxylin and eosin. In situ hybridization for chemokine receptors and immunostaining for CD3+ cells was combined on some sections. After in situ hybridization, immunohistochemistry was performed as described above without autoclaving or microwave pretreatment. After developing the 3-amin-9-ethylcarbazole substrate, slides were dipped, exposed, developed, and counterstained with hematoxylin.

RNA Preparation and Renal Chemokine and Chemokine Receptor Expression

The lower half of each kidney was snap-frozen in liquid nitrogen and stored at −80°C. Total RNA was prepared with the use of the method of Chomczynski and Sacchi (26). Chemokine and chemokine receptor expression was analyzed with the use of ribonuclease protection assays (RPA). RiboQuant multiprobe template sets for murine chemokines (mCK-5) and murine CC chemokine receptors (mCR-5) were obtained from Pharmingen. Twenty μg (chemokines) or 50 μg (receptors) of total RNA were used for each determination. Transcription of antisense riboprobes with α-32P-UTP (3000 Ci/mmol; NEN), hybridization, and RNase treatment was performed according to the manufacturer’s instructions. Efficacy of the RNase step was ensured by a yeast t-RNA sample in every assay as a negative control. RNase protected hybridization products were separated on a 6% denaturing polyacrylamide gel. The intensity of the specific bands was quantitated with the use of a Storm 840 PhosphorImager (Molecular Dynamics, Sunnyvale, CA) and a standard software program (ImageQuant; Molecular Dynamics) and normalized to glyceraldehyde phosphate dehydrogenase gene expression.

Isolation of Renal Cells

A preparation of isolated renal cells, including inflammatory leukocytes, was obtained from obstructed kidneys and contralateral control kidneys 10 d after UUO, following a method adapted from Cook et al. (27). In a first enzyme step, mechanically disaggregated tissue was incubated for 20 min in 5 ml of Hanks’ balanced salt solution (1 × HBSS) containing 1 mg/ml collagenase type I and 0.1 mg/ml deoxyribonuclease type III (both from Sigma, Deisenhofen, Germany) at 37°C. Trypsin was omitted to avoid degradation of cell surface antigens, especially CD4 and CD8. After incubation in 5 ml of 2 mM EDTA, 1 × HBSS (without calcium and magnesium) for 20 min at 37°C, the supernatant containing isolated cells was removed and kept on ice. In a second enzyme step, the remaining pellet was incubated in 5 ml of 1 mg/ml collagenase I in 1 × HBSS for 20 min at 37°C. The resulting supernatant containing free cells was pooled with the first supernatant from the EDTA incubation, washed twice in 1 × HBSS, and resuspended in 1 ml of the same buffer. After staining with 0.4% trypan-blue, cells were counted in a Neubauer chamber. Typically, 7 to 8 × 10^5 cells/ml could be isolated from two pooled kidneys. Before kidneys were removed for cell isolation, blood samples were taken from the anesthetized mice by retrobulbar puncture, collected in 4 mM EDTA and stored on ice until labeling for flow cytometry.

Flow Cytometry

Rat monoclonal antibodies to detect murine CCR2 and CCR5 expression on isolated leukocytes were generated as described elsewhere (28). Renal cell suspensions and antiaggregated full blood samples were incubated with 5 μg/ml of the monoclonal antibodies MC-21 or MC-68 for 60 min on ice. The antibody MC-21 binds specifically to murine CCR2, and the antibody MC-68 binds to murine CCR5. As isotype control, samples also were stained with rat IgG2b (PharMingen). After 3 washing steps, cells were incubated for 60 min on ice with a biotin-labeled anti-rat polyclonal antibody followed by phycoerythrin-labeled streptavidin (both from Dako, Hamburg, Germany). For the identification of leukocyte subsets, samples were finally incubated with a combination of the following directly conjugated cell-specific antibodies: CD11b fluorescein-isothiocyanate (clone M1/70), CD4 allophycocyanin, and CD8 cy-chrome (all from Pharmingen). After lysis of erythrocytes with fluorescence-activated cell sorter (FACS)-lysing solution (Becton-Dickinson, Franklin-Lakes, NJ), stained cells were analyzed on a flow cytometer (FACSCalibur, Becton-Dickinson). Monocytes/macrophages were identified by their light scatter properties and expression of CD11b; T lymphocytes were identified by expression of CD4 and CD8. The cutoff to define chemokine receptor–positive cells was set according to the staining with the isotype control antibody. For CCR2 and CCR5 expression on macrophages, shifts of the mean fluorescence compared with isotype control are given, as some unspecific binding of the IgG2b antibody on activated renal macrophages occurred. Approximately 100,000 gated events were collected in each analysis.

Statistical Analyses

Values are expressed as mean ± SD. Statistical analysis was performed with the use of the t test for unpaired data (RPA) or paired data (morphometric analysis). Statistical significance was defined as P < 0.05.

Results

Induction of Interstitial Renal Disease and Fibrosis

After left-sided UUO mice developed hydronephrosis with progressive dilation of the renal pelvis and thinning of the renal parenchyma. Body weight, serum creatinine, and blood urea nitrogen levels remained unchanged to preobstruction values for mice with UUO at all time points (data not shown).

Histopathologic Changes

Renal tubular damage after UUO increased progressively from days 2 to 10, involving the cortex and the medulla. Tubular damage was characterized by dilation with flattened epithelium, atrophy, and necrosis (Figure 1, A and B; Figure 2A; Table 1). In parallel, a progressive expansion of the interstitial space and an increased extracellular matrix deposition occurred in the cortex (Table 1), as revealed by PAS staining and immunohistochemical detection of type IV collagen deposits (Figure 1, E and F; Figure 2A). In addition, fibrotic
Figure 1. Tubular damage, interstitial fibrosis, and interstitial accumulation of macrophages and lymphocytes in obstructed kidneys 10 d after unilateral ureteral obstruction (UUO). (A) A marked tubular dilation with flattened tubular epithelial cells, atrophy, and necrosis was present in the cortex of obstructed kidneys, which was paralleled by expansion of the interstitial space, accumulation of interstitial cells, and extracellular matrix deposition. (B) Unobstructed contralateral kidneys showed no histomorphologic changes. (C) In obstructed kidneys,
changes were characterized by the progressive accumulation of interstitial FSP1+ fibroblasts from day 2 (8.8 ± 1.1 \textit{versus} 5.0 ± 0.8 cells/hpf in the contralateral kidneys) to day 10 (20.0 ± 2.8 \textit{versus} 4.7 ± 1.5 cells/hpf) (Figure 1, C and D; Figure 2A; Table 1. Results are mean ± SD from five mice per time point). FSP1 recently was characterized as a marker for activated fibroblasts contributing to tissue fibrogenesis. It is a S100A4 protein expressed constitutively in the cytoplasm of resident tissue fibroblasts (23). No gross glomerular pathology was evident in mice with UUO at any time point.

 Accumulation of Interstitial Macrophages and Lymphocytes in Obstructed Kidneys

Increasing numbers of infiltrating CD45+ leukocytes were identified in the cortical interstitium at 2, 6, and 10 d after UUO by immunohistochemical staining (Table 1). At 2 and 6 d, more F4/80+ macrophages than CD3+ lymphocytes could be detected in the mononuclear cell infiltrate, whereas at 10 d after UUO approximately 60% of infiltrating cells were lymphocytes (Figure 1, G through J; Figure 2B; Table 1. Results are mean ± SD from five mice per time point). Maximal numbers of interstitial macrophages were observed at day 6 (10.9 ± 1.5 \textit{versus} 1.5 ± 0.4 cells/hpf in the control kidneys), with a decline at day 10 (8.7 ± 1.4 \textit{versus} 2.1 ± 0.4 cells/hpf). In contrast, the interstitial influx of lymphocytes increased progressively from day 2 (0.3 ± 0.1 \textit{versus} 0.1 ± 0.0 cells/hpf) to day 10 (12.4 ± 1.4 \textit{versus} 0.6 ± 0.1 cells/hpf). In glomeruli of obstructed kidneys, no leukocyte infiltrates (CD45+, F4/80+, CD3+ cells) could be detected.

Increased Chemokine mRNA Expression in Obstructed Kidneys

Expression of chemokines involved in the recruitment of macrophages and lymphocytes to sites of interstitial inflammation was determined by RPA. Whole-organ mRNA samples from obstructed and contralateral kidneys were obtained from 4 mice at each time point. The mRNA levels of lymphotactin/CXCL1, RANTES/CCL5, eotaxin/CCL11, MIP-1α/CCL3, MIP-1β/CCL4, MIP-2, γ interferon-inducible protein-10 (IP-10)/CXCL10, MCP-1/CCL2, and TCA-3/CCL1 were determined relative to the internal standards L32 and glyceraldehyde phosphate dehydrogenase. At 2 d after UUO, no significant increase of tested chemokine mRNA levels was evident in obstructed kidneys compared with contralateral controls, with the exception of a slightly elevated TCA-3 expression (Figure 3). At day 6, obstructed kidneys contained significantly more mRNA coding for MCP-1/CCL2, RANTES/CCL5, MIP-2, and IP-10/CXCL10 relative to contralateral control kidneys (Figure 3). There was also a slight but significant increase of lymphotactin/XCL1, eotaxin/CCL11, MIP-1α/CCL3, and MIP-1β/CCL4 mRNA expression. Ten d after UUO, a further induction of MCP-1/CCL2 (60-fold compared with contralateral controls), MIP-2 (55-fold), RANTES/CCL5 (21-fold), and IP-10/CXCL10 (11-fold) mRNA could be detected (Figure 3). In addition, a further upregulation of lymphotactin/XCL1 (8.7-fold), MIP-1α/CCL3 (7.8-fold), MIP-1β/CCL4 (7.5-fold), and eotaxin/CCL11 (4.7-fold) mRNA expression occurred. Increased TCA-3 mRNA levels were present at all time points.

Tubulointerstitial Localization of MCP-1 and RANTES mRNA and Protein

To localize the sites of chemokine mRNA expression, we performed \textit{in situ} hybridization on paraffin-embedded sections with antisense probes for MCP-1/CCL2 and RANTES/CCL5, two of the four major upregulated chemokines. For both MCP-1 and RANTES, a strong positive staining was found in the cortex of obstructed kidneys at days 6 and 10 (Figure 4, A and E). No signals were detected with the use of sense sequence templates as a negative control. Strong expression of MCP-1 and RANTES mRNA was seen in the expanded interstitial compartment of the cortex, co-localizing to interstitial mononuclear cell infiltrates (Figure 4, C and G). In addition, MCP-1 expression by cortical tubular epithelial cells frequently could be detected, whereas only a few tubular cells were positive for RANTES mRNA (Figure 4, B and F). No MCP-1 or RANTES signals could be detected in glomeruli. Immunohistochemical staining with antisense peptide antiserum against murine MCP-1 revealed a strong MCP-1 protein expression in interstitial cells and positive staining in cortical tubules (Figure 5A). Similar results were obtained for RANTES protein expression with the use of the polyclonal rabbit anti-mouse antibody (Figure 5B). Compared with detected mRNA transcripts by \textit{in situ} hybridization, more tubular cells showed a positive immunostaining for MCP-1 and RANTES protein. As chemokines bind to the cellular glyocalyx and to extracellular matrix, the distribution detected by immunohistochemistry could be broader than that by \textit{in situ} hybridization. As for MCP-1 and RANTES mRNA transcripts, no protein could be detected in glomeruli by immunostaining.
Figure 2. Morphometric analysis was performed as described in the Materials and Methods section. (A) Results demonstrate a progressive increase of tubular cell damage, interstitial accumulation of FSP1+ fibroblasts, and type IV collagen deposition in obstructed kidneys from mild changes at day 2 to severe renal fibrosis at day 10 (see also Table 1). (B) A progressive interstitial accumulation of CD45+ leukocytes, F4/80+ macrophages, and CD3+ lymphocytes in obstructed kidneys occurred from day 2 to day 10. Differences between obstructed and contralateral control kidneys were significant for all three cell types at days 6 and 10. Values are given as mean ± SD from five obstructed and five contralateral kidneys at each time point. *, $P < 0.01$.

Table 1. Morphometric analysis of tubulointerstitial damage, interstitial fibrosis, and interstitial cell accumulation in mice after UUO a

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Day 2</th>
<th>Day 6</th>
<th>Day 10</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>UUO CLK</td>
<td>UUO CLK</td>
<td>UUO CLK</td>
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<tr>
<td>Indices of tubulointerstitial damage</td>
<td></td>
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</tr>
<tr>
<td>$I_{TD}$</td>
<td>1.6 ± 0.5</td>
<td>1.1 ± 0.3</td>
<td>3.5 ± 0.7 b</td>
</tr>
<tr>
<td>$I_{TCD}$</td>
<td>0.9 ± 0.4</td>
<td>0.1 ± 0.1</td>
<td>2.1 ± 0.3 b</td>
</tr>
<tr>
<td>$I_{IVol}$</td>
<td>6.6 ± 1.1</td>
<td>4.8 ± 1.2</td>
<td>11.6 ± 0.8 b</td>
</tr>
<tr>
<td>$I_{Col IV}$</td>
<td>3.1 ± 1.2</td>
<td>2.3 ± 0.4</td>
<td>5.7 ± 1.0 b</td>
</tr>
<tr>
<td>$I_{Cells}$</td>
<td>3.6 ± 1.2</td>
<td>2.5 ± 0.5</td>
<td>5.9 ± 0.7 b</td>
</tr>
<tr>
<td>Interstitial cells</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FSP1+ cells</td>
<td>8.8 ± 1.1 b</td>
<td>5.0 ± 0.8</td>
<td>14.8 ± 2.9 b</td>
</tr>
<tr>
<td>CD45+ cells</td>
<td>2.1 ± 1.3</td>
<td>2.4 ± 0.5</td>
<td>19.6 ± 2.3 b</td>
</tr>
<tr>
<td>F4/80+ cells</td>
<td>1.3 ± 0.5</td>
<td>1.2 ± 0.2</td>
<td>10.9 ± 1.5 b</td>
</tr>
<tr>
<td>CD3+ cells</td>
<td>0.3 ± 0.1</td>
<td>0.1 ± 0.0</td>
<td>6.8 ± 1.6 b</td>
</tr>
</tbody>
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a Morphometric analysis was performed as described in the Materials and Methods section. UUO, obstructed kidney; CLK, unobstructed contralateral kidney; $I_{TD}$, tubular dilation index; $I_{TCD}$, index of tubular cell damage; $I_{IVol}$, interstitial volume index; $I_{Col IV}$, interstitial type IV collagen index; $I_{Cells}$, interstitial cell index. Data are given as mean ± SD from five obstructed and five contralateral kidneys at each time point.

b $P < 0.01$ versus contralateral control.
Figure 3. Time course of chemokine mRNA expression in obstructed kidneys (+) and contralateral control kidneys (−) 2, 6, and 10 d after UUO. Chemokine mRNA was detected by a multiprobe RNase protection assay (RPA) and quantified by image densitometry after normalization to glyceraldehyde phosphate dehydrogenase (GAPDH) expression, as described in the Materials and Methods section. (A) Representative gel segments from three obstructed and two control kidneys per time point are shown. (B) The line graphs demonstrate a marked increase of mRNA encoding for RANTES, macrophage inflammatory protein-2 (MIP-2), γ-interferon-inducible protein-10 (IP-10), and monocyte chemoattractant protein-1 (MCP-1) in obstructed kidneys compared with control kidneys at 6 and 10 d after UUO. In addition, there was a moderate but significant upregulation of lymphotactin, eotaxin, MIP-1β, and MIP-1α mRNA at days 6 and 10. Average fold induction at day 10 (compared with contralateral controls) were as follows: MCP-1, 60-fold; MIP-2, 55-fold; RANTES, 21-fold; IP-10, 11-fold; Lymphotactin, 8.7-fold; MIP-1α, 7.8-fold; MIP-1β, 7.5-fold; Eotaxin, 4.7-fold. Increased TCA-3 mRNA levels were present at all time points. Results are given as mean ± SD from three to four obstructed kidney or two contralateral control kidney RNA samples at each time point. *, P < 0.05; **, P < 0.01.
Figure 4. Tubulointerstitial expression of MCP-1 and RANTES mRNA in obstructed kidneys 6 d after UUO as demonstrated by *in situ* hybridization. (A) Low-magnification illustration of hybridization with a specific antisense probe demonstrates MCP-1 mRNA expression (black silver grains) in the cortex of obstructed kidneys. (B) Tubular epithelial cells frequently expressed MCP-1 mRNA (arrow). (C) A prominent expression of MCP-1 mRNA localized to interstitial cells at sites of mononuclear cell infiltration. No glomerular expression of MCP-1 mRNA was detected by *in situ* hybridization. (D) In unobstructed contralateral kidneys, occasional MCP-1 mRNA expression was found only on some interstitial cells. (E) A prominent RANTES mRNA expression was present in obstructed kidneys with localization to the renal cortex. Note the extent of tubular dilation in the low-magnification illustration. (F) In contrast to MCP-1, tubular epithelial cells were only...
Increased mRNA Expression of CCR1, CCR2, and CCR5 Chemokine Receptors in Obstructed Kidneys

RPA revealed minimal expression of CC chemokine receptors CCR1 (binding RANTES/CCL5 and MIP-1α/CCL3), CCR2 (binding MCP-1/CCL2), and CCR5 (binding RANTES/CCL5 and MIP-1α/CCL3) in contralateral kidneys 2, 6, and 10 d after UUO (Figure 6). In obstructed kidneys, CCR1, CCR2, and CCR5 mRNA expression increased progressively from days 2 to 10. At 2 d, no significantly increased expression was noted compared with control kidneys. Six and 10 d after UUO, the induction of mRNA expression was 3.6-fold and 8.4-fold for CCR1, 3.6-fold and 12.7-fold for CCR2, and 4.4-fold and 12.8-fold for CCR5, respectively, compared with transcript levels in contralateral kidneys. No mRNA expression of CCR1b, CCR3, or CCR4 was detected in obstructed or contralateral kidneys at any time point (Figure 6).

Interstitial Localization of Murine CCR2 and CCR5 mRNA

In situ hybridization with murine CCR2 and CCR5 antisense probes revealed an exclusive interstitial mRNA expression of the two chemokine receptors in the renal cortex of obstructed kidneys 6 and 10 d after UUO (Figure 7, A and B). No specific hybridization signals could be detected in contralateral kidneys, which showed a background signal comparable to the results after hybridization with the sense probes (not shown). In obstructed kidneys, interstitial mRNA expression of CCR2 and CCR5 was restricted to infiltrating mononuclear cells. Tubular epithelial cells at sites with tubular damage, atrophy, and interstitial mononuclear cell infiltrates or in areas with absence of severe interstitial inflammation showed no detectable CCR2 or CCR5 expression (Figure 7, A and B). CCR2 and CCR5 expression was absent in endothelial cells and smooth muscle cells of the vascular compartment. No glomerular CCR2 or CCR5 mRNA transcripts could be detected besides occasional positive cells, which most likely reflect circulating leukocytes in glomerular capillaries. When in situ hybridization was combined with immunostaining for CD3 on the same section, the majority of CD3+ lymphocytes expressed CCR5 mRNA, whereas no clear co-localization of CD3+ cells with CCR2 mRNA transcripts could be found (Figure 7, C and D). Thus, infiltrating lymphocytes in the interstitial compartment seemed to express predominantly CCR5 but not CCR2.

CCR2 and CCR5 Expression on Infiltrating Leukocytes

We were unsuccessful in obtaining specific immunohistology for CCR2 and CCR5 with our antibodies on tissue sections. The antibodies are, however, suitable for FACS analysis. Therefore, to characterize further the expression of CCR2 and CCR5 chemokine receptors on the interstitial mononuclear cell infiltrate, we performed a four-color flow cytometry analysis. Renal cells including inflammatory leukocytes were isolated from obstructed kidneys and contralateral control kidneys 10 d after UUO. CCR2 and CCR5 expression was detected on CD11b+ macrophages from obstructed kidneys (Figure 8). Although incubation with an irrelevant IgG2b antibody as isotype control gave a relative high background fluorescence on the activated macrophages, a clear shift of the mean fluorescence could be seen after staining with the specific antibodies directed against murine CCR2 (mean fluorescence, 1267 arbitrary units versus isotype control 520) and CCR5 (1075 versus 520). The few macrophages isolated from contralateral control kidneys showed a similar expression of CCR2 and CCR5 receptors (Figure 8). In contrast, peripheral blood monocytes of the same mice expressed high levels of CCR2 (mean fluorescence, 680 versus isotype control 222) but only moderate amounts of CCR5 (348 versus 222) (Figure 8). Thus, infiltrating renal macrophages express CCR2 and CCR5 chemokine receptors after UUO. Analysis of CD4+ and CD8+ lymphocytes isolated from obstructed kidneys revealed a high expression of CCR5 on both lymphocyte subsets: 43.1% of CD4+ and 93.1% of CD8+ cells stained positive for CCR5 (Figure 8B), confirming the results obtained by combined CD3 immunohistology and in situ hybridization. In contrast, only a weak CCR2 expression could be detected on infiltrating CD4+ or CD8+ lymphocytes (10.6% of CD4+ cells and 6.1% of CD8+ cells showed a positive staining; Figure 8A). A similar pattern of CCR2 and CCR5 expression was detectable on the few CD4+ and CD8+ lymphocytes isolated from contralateral control kidneys. However, a lower percentage of cells expressed CCR5, and slightly more CD4+ lymphocytes from contralateral kidneys were positive for CCR2 (Figure 8). In the peripheral blood only 5.4% of CD4+ lymphocytes expressed CCR2, and only 5.9% expressed CCR5. Nineteen and 16.2% of CD8+ lymphocytes were positive for CCR2 and CCR5, respectively (Figure 8). These results indicate that CCR5-positive lymphocytes of both the CD4+ and CD8+ subtype accumulate preferentially in the inflamed renal interstitium after 10 d of UUO compared with their frequency in the peripheral blood, whereas only a small number of infiltrating lymphocytes express CCR2.

Discussion

Interstitial leukocyte infiltration is a prominent and early feature of tubulointerstitial inflammation and renal fibrosis. There is increasing experimental evidence that infiltrating leukocytes and predominantly the accumulation of interstitial macrophages propagate the tubulointerstitial inflammatory state to renal fibrosis (5,6,29,30). Chemokines and their spe-
Figure 5. Tubulointerstitial expression of MCP-1 and RANTES protein in obstructed kidneys 10 d after UUO. (A) Immunohistology of sections from obstructed kidneys with anti-murine MCP-1 serum localized MCP-1 protein to interstitial cells and tubular epithelium. (B) Staining of obstructed kidneys with anti-murine RANTES demonstrated predominantly interstitial accumulation of RANTES protein and a less intense signal within the tubular epithelium. Endothelial cells within the tubulointerstitium and intrinsic glomerular cells showed no significant staining for MCP-1 and RANTES protein. Hematoxylin counterstain. Magnification, ×400.

Figure 6. Time course of CC chemokine receptor mRNA expression in obstructed kidneys (+) and contralateral control kidneys (−) 2, 6, and 10 d after UUO. Chemokine receptor expression was detected by a multiprobe RPA and quantified by image densitometry after normalization to GAPDH expression. (A) Representative gel segments from two obstructed and one control kidney per time point showed a progressive upregulation of mRNA encoding for CCR1, CCR2, and CCR5 during the course of UUO. Unprotected templates are shown on the left, and the protected fragments are indicated on the right. (B) Ten d after UUO, the average fold induction was 8.4-fold for CCR1, 12.7-fold for CCR2, and 12.8-fold for CCR5 compared with contralateral control kidneys. Results are given as mean ± SD from four obstructed kidney or two contralateral control kidney RNA samples at each time point. *, P < 0.05.
specific receptors play a central role in directing peripheral blood leukocytes into inflamed renal tissue (8). We used the progressive course of obstructive nephropathy with interstitial accumulation of leukocytes and concomitant fibrosis to investigate chemokine and chemokine receptor expression in interstitial nephritis.

In obstructed kidneys, we found a progressive increase of mRNA coding for the CC chemokines MCP-1/CCL2, RANTES/CCL5, and for the CXC chemokines MIP-2 and IP-10/CXCL10 during the course of UUO. To a lesser extent, increased expression of the CC chemokines eotaxin/CCL11, MIP-1α/CCL3, and MIP-1β/CCL4 and of the C chemokine lymphotactin/XCL1 was noted. By in situ hybridization and immunohistochemistry, we demonstrated expression of the two major upregulated CC chemokines, MCP-1 and RANTES, in tubular epithelial cells (predominantly MCP-1) and a very strong expression of both CC chemokines in the interstitial compartment, co-localizing to interstitial mononuclear cell infiltrates. Either resident interstitial cells and/or infiltrating leukocytes may produce MCP-1 and RANTES (31–34). MCP-1 is a potent chemoattractant for monocytes/macrophages, and RANTES attracts both lymphocytes and monocytes, which are a prominent histologic feature in the UUO model. Increased expression of MCP-1 and RANTES has been described in various forms of experimental (8,11,12,24,35) and human glomerulonephritis (36–38), as well as in secondary tubulointerstitial disease (13,18,19). MCP-1 and RANTES expression could be demonstrated in tubular epithelial cells and, in some studies, in the interstitial compartment by in situ hybridization or immunohistochemistry. In a rat model of puromycin aminonucleoside–induced tubulointerstitial nephritis, MCP-1 and IP-10 mRNA production was localized to intrinsic tubulointerstitial cells (39). In experimental hydronephrosis, an increase in MCP-1 mRNA and protein expression was demonstrated (40,41), and in a human biopsy study of congenital obstructive nephropathy, Grandalliano et al. (42) located MCP-1 mRNA expression to tubules and infiltrating mononuclear interstitial cells. The present study confirms the expression of MCP-1 and RANTES by tubular epithelial and interstitial cells in obstructive nephropathy. Increasing chemokine mRNA levels correlated with the extent of tubular damage, progressive interstitial infiltration of macrophages and lymphocytes, and concomitant fibrosis. These data suggest that locally secreted MCP-1 and RANTES are important chemoattractant...
Figure 8. Flow cytometry analysis of CCR2 and CCR5 expression on macrophages and lymphocytes isolated from obstructed kidneys, unobstructed contralateral kidneys, and blood in mice 10 d after UUO. (A) CD11b-labeled macrophages of obstructed kidneys, contralateral kidneys, and blood monocytes expressed CCR2 as indicated by a clear shift of the mean fluorescence intensity compared with values for IgG2b as isotype control (obstructed kidneys, 1267 versus 520 arbitrary units; contralateral kidneys, 977 versus 487 units; blood, 680 versus 222 units).
mediators of interstitial leukocyte infiltration during the course of obstructive nephropathy.

To strengthen this hypothesis, we studied simultaneous expression of the respective CC chemokine receptors during UUO. Indeed, we could demonstrate that leukocytes infiltrating obstructed kidneys differentially expressed MCP-1 and RANTES receptors. Increasing levels of mRNA coding for chemokine receptors CCR1 (binding MIP-1α and possibly murine RANTES), CCR2 (binding MCP-1), and CCR5 (binding RANTES, MIP-1α, MIP-1β) correlate with progressive interstitial mononuclear cell infiltrates, chemokine expression, and fibrotic changes in obstructed kidneys. Expression of CCR2 and CCR5 mRNA transcripts was confined strictly to the interstitial mononuclear cell infiltrate in the cortex, as demonstrated by in situ hybridization. Tubular epithelial cells, endothelial cells, or glomerular cells did not express CCR2 or CCR5, confirming our previous results in mice and in human renal biopsies (35,43). We and others recently found a similar upregulation of CCR1, CCR2, and CCR5 in murine nephrotoxic serum nephritis (35,44) and a glomerular expression of these receptors in experimental immune-complex glomerulonephritis (24), whereas no CCR1b, CCR3, and CCR4 mRNA was detectable. Similarly, receptor induction was associated with the expression of corresponding chemokine mRNA for MCP-1 and RANTES in these and theUUO model. In both immune-complex nephritis and obstructive nephropathy, chemokine receptor expression was localized strictly to mononuclear cell infiltrates at the respective glomerular (immune-complex nephritis) or tubulointerstitial (UUO) sites of renal damage, where the corresponding chemokine production occurred.

Combination of CD3 immunostaining and in situ hybridization revealed expression of CCR5 but not CCR2 on interstitial lymphocytes. FACS analysis of cells from the UUO kidneys confirmed that infiltrating CD4+ and CD8+ lymphocytes expressed mainly CCR5, but only a few T cells were CCR2 positive. In contrast, infiltrating macrophages were positive for both CCR2 and CCR5. Peripheral blood lymphocytes showed a different pattern of CCR2 and CCR5 expression: compared with kidney macrophages, circulating monocytes were positive for CCR2 but expressed only low amounts of CCR5. Circulating CD4+ and CD8+ lymphocytes expressed CCR2 and CCR5 receptors with relative low abundance, which is in striking contrast to the strong CCR5 expression on infiltrating lymphocytes. These data indicate that leukocyte subpopulations infiltrating inflamed kidneys show a distinct expression pattern of CC chemokine receptors. Interestingly, by flow cytometry, the few isolated macrophages and lymphocytes from contralateral unobstructed kidneys showed a similar expression of CCR2 and CCR5 receptors as infiltrating cells in obstructed kidneys, with a higher proportion of CD4+ lymphocytes being positive for CCR2 and a somewhat lower proportion of CD8+ lymphocytes expressing CCR5. However, this pattern clearly is different from the receptor expression on circulating blood leukocytes. These data and the uniform induction of CC chemokine receptors in different forms of nephritis suggest a distinct inflammatory phenotype of infiltrating monocytes/macrophages (expressing CCR2 and CCR5) and T lymphocytes (expressing predominantly CCR5). The differential expression of CCR2 and CCR5 on particular leukocyte subsets may allow their specific accumulation in kidney tissue. Such a tissue-specific homing has been reported for CCR4- and CCR9-positive memory T cells, which accumulate exclusively at cutaneous or intestinal sites of inflammation, respectively (44). Conversely, an accumulation of CCR2- and/or CCR5-positive mononuclear cells has been described in other organs during chronic inflammatory disease, such as arthritis, multiple sclerosis, and inflammatory bowel disease (46–48). Thus, the increased expression of CCR2 and CCR5 by infiltrating leukocytes also may be the result of a local modulation of chemokine receptor expression in chronically inflamed tissue compartments.

During the course of UUO, we found a strong correlation of upregulated chemokines, infiltration of CCR2- and CCR5-positive leukocytes, and progressive interstitial fibrosis, as revealed by the interstitial accumulation of FSP1+ activated fibroblasts and increased type IV collagen deposition. Infiltrating tubulointerstitial leukocytes, especially activated macrophages, are an important source of inflammatory and profibrogenic cytokines such as transforming growth factor-β (TGF-β). An increased tubulointerstitial expression of TGF-β has been reported during UUO (6). Moreover, the release of CC chemokines, particularly MCP-1/CCL2, by infiltrating macrophages and lymphocytes itself may have a direct fibrogenic effect. MCP-1 has been reported to stimulate collagen and TGF-β expression by fibroblasts, the latter resulting in an autocrine upregulation of collagen production (49). Taken together, infiltrating mononuclear cells may contribute significantly to interstitial fibrotic changes in chronic tubulointerstitial nephritis.

In summary, we demonstrated an increased expression of the CC chemokines MCP-1/CCL2 and RANTES/CCL5 at sites of obstructed kidneys differentially expressing CCR1, CCR2, and CCR5. Data shown are from pooled kidneys and blood samples of two animals and are representative of three independent experiments.
progressive tubulointerstitial damage and a simultaneous interstitial accumulation of infiltrating macrophages and T lymphocytes differentially expressing the respective receptors CCR2 and CCR5 in murine obstructive nephropathy. These data suggest that CCR2- and CCR5-positive monocytes as well as CCR5-positive lymphocytes are attracted by locally released MCP-1 and RANTES, resulting in chronic interstitial inflammation. Lymphocytes that bear the CCR5 receptor may be attracted preferentially by RANTES, whereas CCR2- and CCR5-positive monocytes/macrophages could be attracted by both MCP-1 and RANTES. Blocking the ligand/receptor interaction between MCP-1 and CCR2 or RANTES and CCR5 may offer a new therapeutic approach for tubulointerstitial renal disease and progressive fibrosis.

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References


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