Monocyte/macrophage chemokine receptor CCR2 mediates diabetic renal injury

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Submitted 16 June 2011; accepted in final form 29 August 2011

Monocyte/macrophage chemokine receptor CCR2 mediates diabetic renal injury. Am J Physiol Renal Physiol 301: F1358–F1366, 2011. First published August 31, 2011; doi:10.1152/ajprenal.00332.2011.—Monocyte/macrophage recruitment correlates strongly with progression of renal impairment in diabetic nephropathy (DN). C-C chemokine receptor (CCR)2 regulates monocyte/macrophage migration into injured tissues. However, the direct role of CCR2-mediated monocyte/macrophage recruitment in diabetic kidney disease remains unclear. We report that pharmacological blockade or genetic deficiency of CCR2 confers kidney protection in Ins2Akita and streptozotocin (STZ)-induced diabetic kidney disease. Blocking CCR2 using the selective CCR2 antagonist RS504393 for 12 wk in Ins2Akita mice significantly attenuated albuminuria, the increase in blood urea nitrogen and plasma creatinine, histological changes, and glomerular macrophage recruitment compared with vehicle. Furthermore, mice lacking CCR2 (CCR2−/−) mimicked CCR2 blockade by reducing albuminuria and displaying less fibronectin mRNA expression and inflammatory cytokine production compared with CCR2+/+ mice, despite comparable blood glucose levels. Bone marrow-derived monocytes from CCR2+/+ or CCR2−/− mice adoptively transferred into CCR2−/− mice reversed the renal tissue-protective effect in diabetic CCR2−/− mice as evaluated by increased urinary albumin excretion and kidney macrophage recruitment, indicating that CCR2 is not required for monocyte migration from the circulation into diabetic kidneys. These findings provide evidence that CCR2 is necessary for monocyte/macrophage-induced diabetic renal injury and suggest that blocking CCR2 could be a novel therapeutic approach in the treatment of DN.

albuminur; fibrosis; macrophages; CCR2 antagonist

IMMUNOLOGICAL AND INFLAMMATORY mechanisms play a significant role in the development and progression of diabetic nephropathy (DN) (21, 30). Monocytes and/or macrophages and their adherence to endothelial cells, and exoexpression of proinflammatory cytokines and chemokines, contribute to the pathogenesis of DN (10–12, 31). Deletion or blockade of C-C chemokine ligand-2 (CCL2/MCP-1) results in diminished macrophage infiltration and reduced renal injury in both type 1 and type 2 diabetes in mice (11, 12, 32). Furthermore in humans, macrophage accumulation occurs in DN (24, 31) and correlates strongly with the progression of renal impairment (31). These data and our observation of a direct correlation between CCL2/MCP-1 and urinary albumin excretion (UAE) in streptozotocin (STZ)-induced diabetes (1) suggest a critical role for macrophages in DN. Infiltrating macrophages release lysosomal enzymes, nitric oxide, reactive oxygen species, transforming growth factor-β, vascular endothelial growth factor, and cytokines such as TNF-α, interleukin-1, and IFN-γ (38), which could play a pivotal role in the development and progression of DN.

Monocyte recruitment to sites of inflammation is regulated by members of the C-C chemokine ligands, also known as monocyte chemoattractant proteins (MCPs). CCL2/MCP-1 attracts macrophages by signaling through the CCR2 chemokine receptor (16). CCL8/MCP-2, CCL7/MCP-3, and CCL12/MCP-5 are also ligands for this receptor (22). Recently, it was found that both CCL2/MCP-1 and CCL7/MCP-3 provided parallel contributions to CCR2-mediated inflammatory monocyte recruitment and that both chemokines were required for an optimal immune response (20). A previous report showed increased intrarenal CCL7/MCP-3 transcripts in CCL2/ MCP-1−/− mice and that CCL7/MCP-3 may compensate for the lack of glomerular CCL2/MCP-1 signaling through CCR2 (39). Thus results obtained with CCR2−/− mice would not necessarily be the same as those obtained with CCL2/MCP-1−/− mice. CCR2 is expressed mainly on monocyte subsets (36) that are associated with the pathogenesis of inflammatory disorders and contribute to progressive fibrosis. CCR2 receptors are required for monocyte emigration from the bone marrow (BM) such that CCR2−/− mice have fewer circulating monocytes (36). CCR2−/− mice have been shown to be protected from monocyte/macrophage-induced injury in atherosclerosis (14), experimental autoimmune encephalitis (19), bronchiolitis obliterans syndrome (5), hepatotoxicity (13), and ureteral obstruction (25). Furthermore, recent studies demonstrate that CCR2 inhibitors can block the development of DN in association with a reduction in kidney macrophage infiltration in type 2 diabetic mice (23, 34). Whether inhibition of CCR2 can also reverse DN is not clear. Accordingly, the current studies tested the hypothesis that CCR2-mediated monocyte/macrophage infiltration is a critical determinant of diabetic kidney disease. We found that diabetic mice treated with a CCR2 antagonist or lacking CCR2 are protected from albuminuria and display reduced histological changes associated with DN, macrophage recruitment, and production of inflammatory cytokines in Ins2Akita and STZ-induced diabetic kidney disease. These protective effects were reversed by adoptive transfer of CCR2+/+ or CCR2−/− monocytes into diabetic CCR2−/− mice, as evaluated by increased UAE and kidney macrophage recruitment, indicating that CCR2 is not required for monocyte mobilization from the circulation into diabetic kidneys. These results demonstrate that targeting CCR2 may be a novel therapeutic intervention in the treatment of DN.

MATERIALS AND METHODS

Diabetic mouse models. Experiments were conducted in male Ins2Akita and their wild-type littersmates (DBA background; Jackson Laboratories, Bar Harbor, ME) starting at 6 wk of age until 18 wk of
age and were approved by the Penn State University College of Medicine Institutional Animal Care and Use Committee. Ins2\textsuperscript{Akita} mice, recommended by the Animal Models of Diabetes Complications Consortium (AMDCC) as an optimal model of DN (7, 8), develop hyperglycemia by 3–4 wk of age. Additional experiments were conducted in male 7- to 8-wk-old CCR2\textsuperscript{−/−} and CCR2\textsuperscript{−/+} mice (C57Bl/6 background; Jackson Laboratories) using STZ (Sigma, St. Louis, MO) and were approved by the University of Virginia Animal Research Committee as described previously (3).

Drug delivery. RS504393, a selective CCR2 antagonist (2 \text{ mg·kg}^{-1}·day\textsuperscript{−1}, Tocris Bioscience, Ellisville, MO), or vehicle was administered by continuous subcutaneous infusion for 12 wk via a mini-osmotic pump (Alzet; Durect, Palo Alto, CA). The osmotic pump was incubated in PBS for 60 h at 37°C before implantation and was implanted dorsally between shoulders. The condition of mice and body weight were monitored daily following the pump implantation.

Blood pressure measurement. In the Ins2\textsuperscript{Akita} mice experiment, systolic blood pressure (SBP) was measured using a Coda blood pressure system (Kent Scientific, Torrington, CT) (15). SBP in the CCR2-deficient experiment was measured via the tail-cuff method as described previously (1) (ITC model 179, I/TCH/Life Science Instruments, Woodland Hills, CA) (41). Mice were allowed to rest quietly for 10 min at 26°C. All measurements were performed at the same time for all groups to prevent any diurnal variations.

Renal histochemistry and immunohistochemistry. Kidneys from mice were fixed in 4% paraformaldehyde, embedded in paraffin, and 4-μm sections were cut. Sections were stained with periodic acid-Schiff (PAS) stain, and all glomeruli were examined at ×25, graded to ×40 (W. K. Bolton) in a masked fashion. All images were obtained with an Olympus BX51 microscope and DP71 digital camera using MicroSuite Basic 2.6 imaging software. Images were taken with a ×100 (oil) objective with a total magnification of ×1,000. Semiquantitative scores (0−4+) were assigned based on the masked reading, as previously described (42). Briefly, each glomerulus on a single section was graded from 0 to 4+, where 0 represents no lesions, and 1, 2, 3, and 4+ represent mesangial matrix expansion or sclerosis, involving ≤25, 25–50, 50–75, or >75% of the glomerular tuft area, respectively.

Immunohistochemistry for macrophages was performed in mice using rat anti-mouse Mac-2 monoclonal antibody; clone M3/38 (Cedarlane, Burlington, NC) on paraffin sections. Sections were incubated with primary antibody (50 μg/ml) followed by a biotinylated goat IgG anti-rat (Vector Laboratories, Burlingame, CA) secondary antibody according to the manufacturer’s protocol. Sections were viewed using an Olympus BX51 microscope and DP71 digital camera using MicroSuite Basic 2.6 imaging software. Images were taken with a ×100 (oil) objective with a total magnification of ×1,000. The number of glomerular macrophages was counted in 20 glomeruli/section (number of macrophages in glomeruli divided by the number of glomeruli) in a blinded fashion under ×40 magnification and averaged.

Analysis of kidney macrophage content by fluorescence-activated cell sorter. We used flow cytometry to analyze kidney macrophage content (CD11b+ FITC) at the end of the experiments as described previously (2, 4). In brief, kidneys were extracted, minced, digested, and then passed through a filter and a cotton wool column. Fresh kidney suspensions were incubated with anti-mouse CD45-FITC (30-F11) for 30 min on ice. Kidney macrophages were then identified using allophycocyanin (APC)-labeled rat anti-mouse F4/80 (BM8) and phycoerythrin (PE)-labeled rat anti-mouse CD11b (M1/70). All samples were treated with anti-mouse CD16/CD32 (2.4G2) to block the nonspecific FcR binding and 7-AAD to eliminate dead cells (Invitrogen, Carlsbad, CA). Counting beads (Caltag, Carlsbad, CA) were used to determine the total number of CD45\textsuperscript{+} cells/g of kidney tissue. Subsequent flow cytometry data acquisition was performed on FASCalibur or LSR2 (Becton Dickinson, San Jose, CA). Data were analyzed by Flowjo software 6.4 (Tree Star, Ashland, OR). All the antibodies were purchased from eBioscience (San Diego, CA). BM-derived monocyte isolation and adoptive transfer. BM cells were isolated from the tibia and femur of CCR2\textsuperscript{−/−} or CCR2\textsuperscript{−/+} mice under sterile conditions. Bones were flushed with RPMI 1640 ( invitrogen Life Technologies) plus 10% FCS. The marrow cells were passed sequentially through a 22-gauge needle followed by three passages through a 25-gauge needle to obtain single-cell suspensions of BM cells. Monocytes were isolated from bone marrow cells using a STEMCELL (Vancouver, BC) mouse monocyte enrichment negative selection kit according to the manufacturer’s protocols. The enriched population comprised over 90% monocytes. Enriched monocytes (5 × 10\textsuperscript{6}) were injected intravenously into CCR2\textsuperscript{−/−} mice at days 3 and 10 after STZ induction of diabetes.

Quantitative real-time PCR. Total RNA was extracted from mouse kidneys using an RNeasy Mini Kit (Qiagen, Hilden, Germany). Single-strand cDNA was synthesized using iScript cDNA Synthesis Kits (Bio-Rad, Hercules, CA) for two-step real-time RT-PCR. Gene-specific primers for fibronectin and CCR2 were designed using Beacon Designer Probe/Primer Design Software (Premier Biosoft International, Palo Alto, CA). The sense primers are AGGCAATG-GACGCATCAC and GTGATTGCAAGCATTGAGAC, and the antisense primers are TTCTCCGTTGCTTCTTGTG and ACTC-GATCTGCTGTCCTC; respectively (Integrated DNA Technologies, Coralville, IA). Specificity of the PCR products was verified by melting curve analysis. Quantitative real-time PCR was performed using the ABI 7900HT sequence detection system (Applied Biosystems, Foster City, CA). Reactions were performed in duplicate, and threshold cycle numbers were averaged. Samples were calculated with normalization to GAPDH (1).

Analytic methods. UAE was measured by ELISA using Albuwell M (Exocell, Philadelphia, PA) as described previously (1). Urine TNF-α was measured by ELISA (eBioscience) (1). Blood urea nitrogen (BUN) was measured using VITROS DT601 chemistry slides (Ortho-Clinical Diagnostics, Rochester, NY). Plasma creatinine was measured using (DZ072B, Diazyme Labs, Poway, CA). Body composition was determined using LF90 Minispec Time Domain Nuclear Magnetic Resonance Spectrometer (Bruker Optics, Billerica, MA).

Statistical analysis. Comparisons between groups were examined by using SPSS version 17.0 software for Windows (SPSS, Chicago, IL). Data are expressed as means ± SE. One-way ANOVA was used.
when more than two groups were compared, and the significance of observed differences among the groups was evaluated with a least significant difference post hoc test. Statistical significance was identified at $P < 0.05$.

**RESULTS**

**Characteristics of Ins$^{2\text{Akita}}$ experiment.** To assess the possible clinical significance of CCR2 in diabetic mice, we continuously infused the CCR2 antagonist RS504393 (2 mg·kg$^{-1}$·day$^{-1}$) or vehicle into Ins$^{2\text{Akita}}$ and their wild-type littermates (DBA background) for 12 wk beginning at 6 wk of age. As shown in Table 1, Ins$^{2\text{Akita}}$ vehicle-treated mice had increased blood glucose (BG) levels, decreased body weight (BW), increased kidney weight, increased kidney weight-to-BW ratio, reduced fat and fluid composition, and increased lean composition compared with control mice. CCR2 antagonist treatment of Ins$^{2\text{Akita}}$ mice significantly reduced kidney weight and kidney weight-to-BW ratio without affecting other measurements. There were no significant changes in SBP between all groups although there was a trend toward increased SBP in the Ins$^{2\text{Akita}}$ groups.

CCR2 antagonist reduces albuminuria, blood urea nitrogen, and plasma creatinine in Ins$^{2\text{Akita}}$ mice. To determine whether CCR2 contributes to diabetic renal injury, we measured 24-h UAE, BUN, and plasma creatinine as indicators of renal injury in Ins$^{2\text{Akita}}$ mice with and without CCR2 antagonist treatment. As shown in Fig. 1, vehicle-treated Ins$^{2\text{Akita}}$ mice had a significant increase in albuminuria compared with controls at 6 and 18 wk of age. Albuminuria was significantly reduced in Ins$^{2\text{Akita}}$ mice treated with RS504393 at 18 wk of age. Similarly, BUN and plasma creatinine were significantly increased in vehicle-treated Ins$^{2\text{Akita}}$ mice compared with other groups (Table 1).

CCR2 antagonist decreases renal histological changes in Ins$^{2\text{Akita}}$ mice. PAS staining of kidney sections (Fig. 2) showed...
Fig. 3. Effect of CCR2 antagonist on macrophage recruitment in Ins2Akita mice. A: immunohistochemical staining for Mac-2-positive macrophages in glomeruli at 18 wk of age in control mice treated with vehicle, control mice treated with CCR2 antagonist, Ins2Akita mice treated with vehicle, and Ins2Akita mice treated with CCR2 antagonist. Images are representative of 20 fields from 5–8 mice/group. B: experimental groups were as described in A. Kidneys were harvested at 18 wk of age and processed for fluorescence-activated cell sorting (FACS) analysis as described in MATERIALS AND METHODS. Macrophages were identified as CD11bhighF4/80low. Graphs show representative contour plots. Values are means ± SE expressed as numbers of CD11bhighF4/80low macrophages/g kidney tissue; n = 5–8 mice/group.
and Ins2Akita (Fig. 2A), respectively. Both CCR2 antagonist-treated control (Fig. 2B) and Ins2Akita (Fig. 2D) mice were similar to vehicle-treated controls and exhibited significantly reduced glomerular changes (scores: 0.7 ± 0.2 and 0.9 ± 0.4, respectively; P < 0.05) compared with vehicle-treated Ins2Akita mice.

**CCR2 antagonist decreases macrophage recruitment in Ins2Akita mice.** To determine whether CCR2 is critical for kidney macrophage infiltration in DN, we show the distribution and quantitation of macrophages in kidneys by immunohistochemistry (Mac-2-positive macrophages) (Fig. 3A). The number of glomerular macrophages in vehicle-treated control mice was low and increased significantly in vehicle-treated Ins2Akita mice (0.3 ± 0.03 and 3.1 ± 0.2 macrophages/glomerulus, respectively; P < 0.001). Both CCR2 antagonist-treated control and Ins2Akita mice displayed significantly reduced glomerular macrophage recruitment (0.6 ± 0.06 and 1.1 ± 0.05 macrophages/glomerulus, respectively; P < 0.001) compared with vehicle-treated Ins2Akita mice at 18 wk of age. Similar results were obtained using flow cytometry (FACS; CD11b+ F4/80low) analysis of total kidney macrophage recruitment (Fig. 3B). Vehicle-treated Ins2Akita mice showed significant kidney macrophage infiltration compared with vehicle-treated control mice (3.1 ± 0.5 vs. 1.8 ± 0.2 macrophages/g kidney tissue; P < 0.05). In contrast, both CCR2 antagonist-treated control and Ins2Akita mice displayed significantly reduced total kidney macrophages (1.8 ± 0.2 and 1.6 ± 0.3 macrophages/g kidney tissue, respectively; P < 0.05) compared with vehicle-treated Ins2Akita mice at 18 wk of age.

**Deficiency of CCR2 does not affect blood glucose levels.** We next assessed whether genetic deficiency of CCR2 mimics the changes observed using CCR2 antagonist treatment in DN. Diabetes was induced in CCR2+/+ and CCR2−/− mice with STZ and mice were observed for an additional 6 wk. Table 2 summarizes data for BG, BW, SBP, and urinary volume in CCR2+/+ and CCR2−/− mice (control and diabetes groups; n = 8/group). The diabetic CCR2+/+ but not CCR2−/− mice had lower BW compared with nondiabetic mice. Importantly, there were no significant differences in SBP 6 wk after STZ-induction of diabetes between all groups.

### Table 2. Characteristics of CCR2+/+ and CCR2−/− mice (control and diabetes groups)

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<tr>
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<th>CCR2+/+ Controls</th>
<th>CCR2+/+ Diabetes</th>
<th>CCR2−/− Controls</th>
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<tr>
<td>Glucose, mg/dl</td>
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<tr>
<td>Week 0</td>
<td>162 ± 8</td>
<td>178 ± 5</td>
<td>183 ± 4</td>
<td>176 ± 6</td>
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<td>Week 3</td>
<td>184 ± 4</td>
<td>372 ± 41b</td>
<td>178 ± 6</td>
<td>317 ± 37d</td>
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<td>Week 6</td>
<td>148 ± 11</td>
<td>484 ± 3f</td>
<td>145 ± 8</td>
<td>482 ± 30p</td>
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<td>Week 6</td>
<td>25 ± 1</td>
<td>22 ± 1*</td>
<td>23 ± 1</td>
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<tr>
<td>Systolic BP, mmHg</td>
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<tr>
<td>Week 6</td>
<td>132 ± 3</td>
<td>135 ± 6</td>
<td>126 ± 4</td>
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<td>Urine volume, ml/24 h</td>
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<tr>
<td>Week 6</td>
<td>1.4 ± 0.2</td>
<td>1.9 ± 0.5</td>
<td>0.5 ± 0.1</td>
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Values are means ± SE; n = 8/group. *P < 0.05, †P < 0.001, #P < 0.0001 vs. control CCR2+/+ group; ‡P < 0.01, §P < 0.0001 to control CCR2−/− group.
Deficiency of CCR2 reduces albuminuria in diabetic mice. As expected, CCR2\(^{+/+}\) mice displayed a significant increase in UAE after 6 wk of diabetes compared with nondiabetic control mice (Fig. 4). In contrast, the increase in UAE was almost completely abrogated in mice lacking the CCR2 gene, similar to CCR2 antagonist treatment in Ins2Akita mice.

Deficiency of CCR2 reduces fibronectin mRNA expression in diabetic mice. DN has been shown to be associated with an increase in kidney matrix deposition and fibrosis. Therefore, we assessed the expression of fibronectin mRNA in diabetic kidneys as a marker for fibrosis. As shown in Fig. 5, diabetes led to a threefold increase in fibronectin mRNA in CCR2\(^{+/+}\) mice but not in CCR2\(^{-/-}\) mice. Thus the absence of CCR2 in diabetic mice correlates with reduced fibronectin mRNA expression.

Reduced urinary TNF-\(\alpha\) in diabetic CCR2\(^{-/-}\) mice. Increased inflammatory cytokines is a major feature of DN. Therefore, we assessed whether an absence of CCR2 is associated with alterations of the urinary levels of TNF-\(\alpha\) 6 wk after STZ-induced diabetes (Fig. 6). Urinary TNF-\(\alpha\) excretion increased significantly in the diabetic CCR2\(^{+/+}\) mice compared with control groups. Both CCR2\(^{-/-}\) control and diabetes groups exhibited lower levels of urinary TNF-\(\alpha\) after 6 wk of the study.

Renal tissue-protective effect of CCR2\(^{-/-}\) is monocyte dependent following diabetes. Although there is clear evidence that CCR2 is critical for monocyte egress from the BM (36, 40), it remains unclear whether CCR2 is also required for monocyte migration from the circulation into tissues under pathological conditions. To answer this question directly, we transferred BM-derived monocytes, isolated by negative selection using magnetic beads, from CCR2\(^{+/+}\) or CCR2\(^{-/-}\) mice into diabetic CCR2\(^{-/-}\) mice 3 days after induction of diabetes (Fig. 7). Mice were then followed for an additional 6 wk. The characteristics of BG, BW, and glomerular macrophage counts of the monocyte transfer experiments are shown in Table 3. Our results show that the protective effects observed in diabetic CCR2\(^{-/-}\) mice as evaluated by UAE were reversed following adoptive transfer of either CCR2\(^{+/+}\) or CCR2\(^{-/-}\) monocytes. In contrast, reconstitution of monocytes to nondiabetic CCR2\(^{-/-}\) mice did not alter UAE (data not shown). These data demonstrate that monocytes directly contribute to the renal-protective effect of CCR2 deficiency in diabetes.

We next assessed kidney Mac-2-positive macrophages in the adoptive transfer experiments (Table 3). Induction of diabetes for 6 wk in CCR2\(^{+/+}\) mice resulted in significant increases in glomerular macrophages compared with nondiabetic mice. In contrast, the number of glomerular macrophages did not increase in both nondiabetic and diabetic CCR2\(^{-/-}\) mice. There were significant increases in glomerular macrophage numbers following adoptive transfer of either CCR2\(^{+/+}\) or CCR2\(^{-/-}\) monocytes into diabetic CCR2\(^{-/-}\) mice. To test whether the transferred monocytes were evident 6 wk after transfer, we injected monocytes that carry the CD45.1 antigen (from B6.SJL-Ptprca\(^{+}\)Pep3\(^{b}\)/BoyJ mice) into diabetic C57BL/6 mice (which express the CD45.2 antigen in leukocytes; \(n = 9\)). Using flow cytometry, we found that CD45.1-positive macrophages contributed \(\sim 11\%\) to total kidney macrophage recruitment after 6 wk (Fig. 8A). We also observed the expression of CCR2 in the kidneys of diabetic CCR2\(^{-/-}\) mice following adoptive transfer of monocytes from CCR2\(^{+/+}\) mice (Fig. 8, B and C), confirming the migration and persistence of monocytes into diabetic kidneys.

### DISCUSSION

Chemokines and cytokines are potent mediators of monocyte/macrophage movement that play a pivotal role in the pathogenesis of DN. CCR2 has a well-established role in...
recruiting monocytes to sites of inflammation, yet its direct role on monocyte/macrophage-induced diabetic kidney injury is not completely clear. This study shows that pharmacological blockade or genetic deficiency of CCR2 mediates renal tissue protection as evidenced by a reduction in albuminuria, BUN, plasma creatinine, histological changes, kidney fibronectin expression, macrophage recruitment, and inflammatory cytokine production during diabetes. We speculate that this renal-protective effect is mediated by a reduction in monocyte mobilization from the BM rather than from the circulation to diabetic kidneys since both albuminuria and kidney macrophages increased following monocyte transfer from CCR2\textsuperscript{+/+} or CCR2\textsuperscript{−/−} mice into diabetic CCR2\textsuperscript{+/+} mice. These findings reveal an important role for CCR2 in the pathogenesis of DN and provide an evidence for CCR2 inhibition as potential therapeutic modality for treating diabetic patients.

Kidney macrophage recruitment has been considered a cardinal feature in DN and correlates strongly with the progression of renal impairment (31). Several chemokines may facilitate macrophage infiltration, leading to kidney damage. In the kidney, CCL2/MCP-1 is produced by podocytes, mesangial, and tubular epithelial cells (27, 33, 37) and mediates renal interstitial inflammation, tubular atrophy, and interstitial fibrosis. CCL2/MCP-1 binds to and signals through CCR2 (9), but other MCPs (CCL8/MCP-2, CCL7/MCP-3, and CCL12/MCP-5) are also ligands for CCR2 with CCL7/MCP-3 having higher potency than CCL2/MCP-1 in maintaining circulatory monocyte homeostasis (40). In addition, both CCL2/MCP-1\textsuperscript{−/−} and CCL7/MCP-3\textsuperscript{−/−} mice have intermediate phenotypes compared with CCR2\textsuperscript{−/−} mice, suggesting that CCL2/MCP-1 and CCL7/MCP-3 contribute equally to monocyte recruitment (20). Interestingly, CCL7/MCP-3 can compensate for the lack of glomerular CCL2/MCP-1 signaling through CCR2 (39), indicating that deletion of CCL2/MCP-1 per se may not reflect its role in certain diseases. Whereas several similarities exist between CCR2\textsuperscript{−/−} and CCL2/MCP-1\textsuperscript{−/−} mice, important differences have also been described. For instance, CCR2\textsuperscript{−/−} mice have a marked impairment in IFN-γ production (6), Fig. 8.}

Fig. 8. Efficiency of monocyte transfer in diabetic kidneys. A: monocytes were isolated from bone marrow of B6.SJL-\textit{Ptprca Pep3b}\textsuperscript{+/−}BoyJ mice (which carry CD45.1 antigen) by negative selection and injected (5 \times 10\textsuperscript{6} cells at days 3 and 10 after STZ-induced diabetes) into C57BL/6 mice (which express CD45.2 antigen in leukocytes; \textit{n} = 9). After 6 wk, kidneys were harvested and processed for FACS. Macrophages were identified as CD11b\textsuperscript{high}F4/80\textsuperscript{low}. Graph shows representative contour plot of CD11b\textsuperscript{high}F4/80\textsuperscript{low}/CD45.1\textsuperscript{−} (donor macrophages) against CD11b\textsuperscript{high}F4/80\textsuperscript{low}/CD45.2\textsuperscript{+} (recipient macrophages). B and C: RT-PCR was performed on whole mouse kidney total RNA isolated after 6 wk of the study. B: gel analysis of PCR products. C: expression of CCR2 mRNA was normalized to GAPDH, and data were calculated as expression relative to control. Values are means \pm SE. ND, nondetectable. *\textit{P} \leq 0.05 vs. normal CCR2\textsuperscript{+/+}.

Fig. 9. Proposed scheme for the role of CCR2 in monocyte/macrophage-induced kidney injury in diabetes. Mobilization of monocytes from the bone marrow to the blood is CCR2 dependent under normal homeostatic conditions and in response to inflammation. Inflamed monocytes roll, extravasate, and migrate into kidney parenchyma to differentiate into macrophages (CCR2 independent). Kidney macrophages mediate diabetic renal injury indirectly by secreting proinflammatory cytokines and chemokines, leading to proteinuria, mesangial expansion, interstitial fibrosis, glomerulosclerosis, and finally end-stage renal disease (ESRD).
whereas CCL2/MCP-1−/− mice have a deficiency in the production of T helper cell type 2 cytokines and have normal IFN-γ production (17). Compensation by other MCPs such as CCL7/MCP-3 and/or CCL12/MCP-5 may explain this difference. In addition, there is increasing evidence for a functional CCL2/MCP-1 receptor other than CCR2 (18, 35). Therefore, blockade of CCR2, rather than CCL2/MCP-1, offers a more specific and perhaps potent means to evaluate the role of CCR2 in DN.

To directly examine the role of CCR2 in the setting of DN, we employed two approaches in two different models of type 1 diabetes by using a selective CCR2 antagonist in Ins2Akita and CCR2−/− mice (C57Bl/6; to avoid strain differences across all mice studied). To analyze the clinical significance of CCR2 in DN, we investigated the effects of inhibiting CCR2 on kidney dysfunction, glomerular histological changes, and CCR2 in DN, we investigated the effects of inhibiting CCR2 across all mice studied). To analyze the clinical significance of CCR2 antagonism in DN, we transferred BM-derived monocytes from CCR2−/− mice into CCR2+/+ mice. Taken together, our results provide support for blocking CCR2, using a CCR2 antagonist, as a therapeutic modality for the treatment of DN.

The renal-protective effect of CCR2 antagonists correlates with a significant reduction of kidney macrophage infiltration. This suggests that macrophages expressing CCR2 are pivotal during the pathogenesis of DN. These results are consistent with recent reports that CCR2 blockade preserved renal function with an associated reduction in kidney macrophage infiltration in type 2 diabetic mice (23, 34). To address directly whether monocytes play a direct role in the CCR2-mediated effects in DN, we transferred BM-derived monocytes from CCR2+/+ or CCR2−/− mice into CCR2−/− mice 3 days after establishing diabetes. Our data show that transfer of monocytes from either CCR2+/+ or CCR2−/− into CCR2−/− mice reversed the kidney protection after diabetes as shown by increased albuminuria and kidney macrophage recruitment. Furthermore, increased expression of CCR2 mRNA in diabetic CCR2−/− mice following adoptive transfer of monocytes from CCR2+/+ confirms the migration and persistence of monocytes into diabetic kidneys. Taken together, these results suggest that CCR2 is not required for monocyte migration from the circulation into diabetic kidneys and that other factors, such as chemokines/chemokine receptors and/or complement, may play a role in this process. Our data are therefore in agreement with Serbina and Palmer (36), who reported that CCR2 is only required for monocyte mobilization from the BM but not from the circulation into tissues.

Once recruited to the kidney, macrophages have been shown to release lysosomal enzymes, growth factors, and inflammatory cytokines (38) that may play an important role in the development and/or progression of DN. We have shown previously that urinary TNF-α is increased in a rat model of type 1 diabetes (1). The current study confirms these results in murine STZ-induced diabetes and shows that diabetic CCR2+/- mice exhibit a profound increase in urinary TNF-α levels, an effect significantly diminished in CCR2−/− mice. TNF-α is produced mainly by monocytes, macrophages, T and B lymphocytes, and glomerular mesangial cells (21). TNF-α has been associated with increasing vascular endothelial permeability and has been detected in isolated glomerular basement membrane in diabetes (29). Recently, infliximab, a chimeric type TNF-α monoclonal antibody has been shown to reduce UAE in a STZ-induced rat model (28).

Blockade of CCR2 resulted in less interstitial fibrosis and glomerular hypercellularity after diabetes induction. In addition, deletion of CCR2 was associated with significantly reduced kidney fibronectin mRNA expression. Increased expression of fibronectin is an early event in the pathogenesis of diabetic renal disease and its accumulation in the kidney is thought to lead to the development of glomerulosclerosis (26). In diabetes, fibronectin stimulates matrix production and blocks matrix degradation. These findings are similar to a previous report indicating that the CCL2/CCR2 system plays an important role in high glucose-induced fibronectin and type IV collagen synthesis in cultured mesangial cells (33).

In conclusion, our study demonstrates three novel observations. First, we demonstrated a beneficial effect of a CCR2 antagonist as a new therapy in the treatment of DN. This finding may ultimately result in novel therapeutic interventions designed to attenuate the CCR2 signaling pathway in the treatment of diabetic kidney disease. A number of orally active CCR2 pharmacological antagonists are now in phase II clinical trials in other diseases and could be considered as a potential therapeutic approach in the treatment of DN. Second, we showed that a deficiency of CCR2 ameliorates monocyte/macrophage-induced diabetic renal injury. Third, using a monocyte reconstitution strategy, we show the first evidence of the direct role of macrophage CCR2 in diabetic nephropathy. A possible scheme to account for the role of CCR2 in DN is presented in Fig. 9.

ACKNOWLEDGMENTS

The authors gratefully acknowledge Dr. Peter Lobo (University of Virginia) and Dr. W. Brian Reeves (Penn State Hershey Medical Center) for helpful discussions and Dr. Timothy Cooper (Penn State Hershey Medical Center) for taking the histological pictures.

GRANTS

This study was supported by National Institutes of Health Grants DK077444 to A. S. Awad, DK56223 and JDRF1–2005-10751 to M. D. Okusa, and DK076095 to W. K. Bolton.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

REFERENCES

4. Awad AS, Ye H, Huang L, Li L, Foss FW, Jr, Macdonald TL, Lynch KR, Okusa MD. Selective sphingosine 1-phosphate 1 receptor activation...
Role of CCR2 in Diabetic Nephropathy


