Compartment-Specific Expression and Function of the Chemokine IP-10/CXCL10 in a Model of Renal Endothelial Microvascular Injury

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The recruitment of inflammatory cells into renal tissue, mainly T cells and monocytes, is a typical feature of various renal diseases such as glomerulonephritis, thrombotic angiopathies, allograft rejection, and vasculitis. T cells predominantly infiltrate the tubulointerstitium, whereas monocytes are present in the tubulointerstitial and glomerular compartment. Because chemokines play a pivotal role in leukocyte trafficking under inflammatory conditions, this study investigated whether a differential expression of chemokines contributes to the precise coordination of leukocyte subtype trafficking in a rat model of renal microvascular endothelial injury. Renal microvascular endothelial injury was induced in rats by selective renal artery perfusion with an anti-endothelial antibody. Induction of the disease led to severe glomerular and tubulointerstitial endothelial injury with subsequent upregulation of chemokines followed by inflammatory cell recruitment. Among the analyzed chemokine mRNA, IP-10/CXCL10 (119-fold), acting via CXCR3 on activated T cells, and MCP-1/CCL2 (65-fold), acting via CCR2 on monocytes, were by far the most strongly upregulated chemokines. In situ hybridization revealed that IP-10/CXCL10 mRNA was selectively expressed by endothelial cells in the tubulointerstitial area, co-localizing with infiltrating T cells. Despite extensive damage of glomerular vasculature, no IP-10/CXCL10 expression by glomerular endothelial cells was detected. MCP-1/CCL2 mRNA in contrast was detectable in the glomerulus and the tubulointerstitium. Treatment with a neutralizing anti-IP-10/CXCL10 antibody significantly reduced the number of infiltrating tubulointerstitial T cells without affecting monocyte migration and led to an improved renal function. Our study demonstrates a role of IP-10/CXCL10 on T cell recruitment in a rat model of renal endothelial microvascular injury. Furthermore, a differential chemokine expression profile by endothelial cells in different renal compartments was found. These findings are consistent with the hypothesis that functional heterogeneity of endothelial cells from different vascular sites exists and provide an insight into the molecular mechanisms that may mediate compartment-specific T cell and monocyte recruitment in inflammatory renal disease.


The recruitment of inflammatory cells from the circulation into renal tissue is a typical feature of various renal diseases. In particular, infiltrating activated T cells and monocytes are supposed to initiate renal tissue damage, eventually leading to progressive loss of renal function (1,2). It is interesting that in most cases, T cell recruitment is observed predominantly in the tubulointerstitium, whereas monocytes are detected in the tubulointerstitium and in the glomeruli (3).

Chemokines constitute a large family of mediators of inflammation and immunity (4,5). They are secretory proteins that are expressed by leukocytes and resident tissue cells. Their effects are mediated through seven membrane-spanning G protein–coupled chemokine receptors, predominantly expressed on leukocytes (6). Work over the past decade has established a central role of chemokines and their receptors as the main players in the precise coordination of inflammatory cell migration in renal inflammatory disease (7–10). The frequently observed appearance of different leukocyte subpopulations in different renal tissue compartments such as glomeruli and the tubulointerstitium, however, remains unexplained. We therefore analyzed the chemokine expression profile in a rat model of acute renal microvascular injury that is characterized by severe acute glomerular and tubular endothelial damage followed by massive glomerular and tubulointerstitial leukocyte infiltration and the development of acute renal failure (11,12).

The two most upregulated chemokines in kidneys of the nephritic animals analyzed were IP-10/CXCL10 and MCP-1/CCL2. IP-10/CXCL10 predominantly attracts T cells of Th1
specificity, which carry the corresponding receptor CXCR3 on their surface. The second chemokine, MCP-1/CCL2, is known to be responsible for attraction of cells that bear the corresponding receptor CCR2, which is found mainly on monocytes/macrophages (M/M).

We observed differential chemokine expression patterns and recruitment of distinct leukocyte populations in glomeruli and the tubulointerstitium. Our data show that IP-10/CXCL10 was merely expressed in the tubulointerstitium by peritubular capillaries (PTC), whereas no IP-10/CXCL10 expression by glomerular endothelial cells was detected. The IP-10/CXCL10 expression pattern was overlapping with the pattern of T cell influx. Massively tubulointerstitial T cell infiltration was observed, whereas no T cells were found inside the glomeruli.

In contrast, MCP-1/CCL2 was expressed in the tubulointerstitium and also intraglomerularly and correlated with the infiltration of monocytes into these compartments. We hypothesized that the damage of endothelial cells in different renal compartments leads to different chemokine expression patterns, which then specifically regulate infiltration of different leukocyte subsets.

Materials and Methods

Experimental Design

Four different groups of animals (total n = 68) were studied at 24 h and 4 d after uninephrectomy (controls) or uninephrectomy plus induction of renal endothelial microvascular injury (REMI). Control rats (n = 20) were uninephrectomized (UNX), and left kidneys were selectively perfused with 0.5 ml/100 g body wt nonantibody Ig. For induction of REMI, left kidneys of UNX rats (n = 48) were selectively perfused with the anti-endothelial cell antibody. Two subgroups of these nephritic rats were treated either with the anti-IP-10/CXCL10 antibody (0.5 ml/100 g body wt; n = 20), which was injected intravenously immediately and at day 2 after the induction of the nephritis, or with specific rabbit IgG (0.5 ml/100 g body wt) injected at the same time points (n = 8).

Induction of REMI

REMI was induced in male Wistar rats (200 to 220 g body wt; Charles River Wiga, Sulzfeld, Germany) according to a protocol published by Nangaku et al. (11). The technical procedure has been described by us previously (13). In brief, after a midline incision, rats were uninephrectomized (UNX), and left kidneys were selectively perfused with 0.5 ml/100 g body wt nonantibody Ig. For induction of REMI, left kidneys of UNX rats (n = 48) were selectively perfused with the anti-endothelial cell antibody. Two subgroups of these nephritic rats were treated either with the anti-IP-10/CXCL10 antibody (0.5 ml/100 g body wt; n = 20), which was injected intravenously immediately and at day 2 after the induction of the nephritis, or with specific rabbit IgG (0.5 ml/100 g body wt) injected at the same time points.

Production of Rabbit Antibodies

For production of the anti-endothelial cell antibody, rabbits were immunized four times at monthly intervals with rat glomerular endothelial cells (5 × 10^9 cells per injection) dissolved in Hunter Titer Max Gold (Serva, Wiesbaden, Germany). These glomerular endothelial cells have been described and characterized in detail previously (14). For the anti-IP-10/CXCL10 antibody, rabbits were immunized with 20 μg of recombinant human IP-10/CXCL10 (R&D, Wiesbaden, Germany) emulsified in Hunter Titer Max Gold four times at monthly intervals. One week after the last booster, rabbits were bled. Serum was complement inactivated by heating (30 min, 56°C in a water bath) and concentrated by high-pressure membrane filtration technique (Amicon stirred cell 8000 series; Millipore, Eschborn, Germany).

Functional Studies

Before being killed, rats were housed in metabolic cages without food but free access to tap water for 24 h for urine collection. At the time of killing, blood was drawn for creatinine and urea measurement. Urinary albuminuria was determined by standard ELISA analysis (Albumin [rat] EIA Kit, Cayman Chemical, Grünberg, Germany).

Preparation of Total RNA from Rat Kidneys

Total RNA was prepared by phenol-chloroform extraction after direct lysis of mechanically homogenized rat kidney tissue in 4 M guanidinium thiocyanate, 25 mM sodium citrate (pH 7), 0.5% sarcosyl, and 0.1 M 2-mercaptoethanol as described previously (15).

Tissue Laser Microdissection

Microdissection was carried out on 10-μm-thick cryosections of rat kidney tissue using the PALM Microbeam IP 230 V Z microscope for laser pressure catapulting (P.A.L.M., Bernried, Germany). Before the dissection procedure, tissue sections were stained with a short alcohol-based cresylviolet acetate protocol: Cryosections were air dried for 1 min and subsequently incubated for 2 min in precooled 75% EtOH. For staining, sections were dipped for 20 s in 1% cresyl violet acetate dissolved in EtOH. Finally, slides were washed in 75% and 100% EtOH for 30 s each and allowed 10 min to air dry. Four different tissue types were selectively cut out: (1) Glomeruli with Bowman’s capsule and the periglomerular region, (2) the glomerular tuft without Bowman’s capsule, (3) PTC, and (4) tubuli. RNA from microdissected tissue was prepared using the PALM RNA extraction kit.

Real-Time Reverse Transcription–PCR

RNA from freshly harvested kidneys and microdissected tissues was isolated as described above. Real-time reverse transcription–PCR was performed with ABI Prism N8650 using SYBR green as dye as described previously (16). Quantification was performed using glyceraldehyde-3-phosphate dehydrogenase as an internal control to correct for small variations in RNA quantity and cDNA synthesis essentially as described by ABI Prism.

The following rat-specific PCR primers were used in this study:

<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Forward Sequence</th>
<th>Reverse Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>CXCR3 forward</td>
<td>5'-TGC AAC CAC GTA CCT AAC ACA CT-3'</td>
<td>5'-TTG ATC TGG GTA AGT-3'</td>
</tr>
<tr>
<td>CXCR3 reverse</td>
<td>5'-CCA GCA CAA ATG TTA TGG GTA AGT-3'</td>
<td>5'-GGA TAC CAC ATT GGT-3'</td>
</tr>
<tr>
<td>CCR5 forward</td>
<td>5'-GCA TGC GAT TTC AGC ATC ACC-3'</td>
<td>5'-GGA TAC CAC ATT GGT-3'</td>
</tr>
<tr>
<td>CCR5 reverse</td>
<td>5'-ACA GAT TGG TGA AAT GCC AAG TAC TCA-3'</td>
<td>5'-AGC CCT ATG CCT TCT TCT TCT AT-3'</td>
</tr>
<tr>
<td>Mig/CXCL9 forward</td>
<td>5'-GGA CCA TAG AAA TTC AAA AAA-3'</td>
<td>5'-CAT TGT GCC AAT GAT CTC AAC AT-3'</td>
</tr>
<tr>
<td>Mig/CXCL9 reverse</td>
<td>5'-AGT TTC CCC CAA GCC CCT TTC AT-3'</td>
<td>5'-AGT TTC CCC CAA GCC CCT TTC AT-3'</td>
</tr>
<tr>
<td>CCR2 forward</td>
<td>5'-GGT CTA TAG GTC ATC TTC TGC TAC TCA-3'</td>
<td>5'-CAT TGT GCC AAT GAT CTC AAC AT-3'</td>
</tr>
<tr>
<td>CCR2 reverse</td>
<td>5'-GGG CCA TAC GTG ATG TTC TAC TCA-3'</td>
<td>5'-AGT TTC CCC CAA GCC CCT TTC AT-3'</td>
</tr>
<tr>
<td>RANTES/CCL5 forward</td>
<td>5'-GAT GTG CTC TCT CTG TTC CTT CTT CTT-3'</td>
<td>5'-GAT GTG CTC TCT CTG TTC CTT CTT CTT-3'</td>
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TGT CAC TCG AAG GA-3', RANTES/CCL5 reverse 5'-GAT GTA TTC TTG AAC CCA CTT CTT-3'; MIP-1α/CCL3 forward 5'-CGA AGT CTT CTC AGC GCC ATA-3', MIP-1α/CCL3 reverse 5'-GGA ATT TGC CTT CCA TAG GA-3'; MIP-1β/CCL4 forward 5'-CCG CCT TCT GCC ATT CAG T-3', MIP-1β/CCL4 reverse 5'-AGG TGT AAG AGA AGC AGC AGC AA-3'; and MCP-1/CCL2 forward 5'-CTG TCT CAG CCA GAT GCA GTC AA-3', MCP-1/CCL2 reverse 5'-TGG GAT CAT CTT GCC AGT GA-3'.

Amplicons of random samples for each primer pair were determined by automatic DNA sequencing to demonstrate the specificity of the PCR reaction (data not shown).

Western Immunoblot Analysis

The kidney samples were lysed in lysis buffer and subjected to brief sonication on ice. Equal amounts of protein (150 ng) were separated by 4 to 12% graded SDS-PAGE (NuPAGE; Invitrogen, Karlsruhe, Germany) and transferred onto polyvinylidene difluoride membranes (Immobilon-P; Millipore, Schwalbach, Germany) for immunoblotting. Nonspecific binding was blocked with 5% BSA in TBST (10 mM Tris-HCl [pH 7.4], 150 mM NaCl, and 0.05% Tween-20). Primary antibodies (polyclonal rabbit anti-mouse–IP-10) were diluted in Superblock (1:5000; Pierce, Bonn, Germany) and transferred onto polyvinylidene difluoride membranes (Immobilon-P; Millipore, Schwalbach, Germany) for immunoblotting. Nonspecific binding was blocked with 5% BSA in TBST (10 mM Tris-HCl [pH 7.4], 150 mM NaCl, and 0.05% Tween-20). Primary antibodies (polyclonal rabbit anti-mouse–IP-10) were diluted in Superblock (1:5000; Pierce, Bonn, Germany) and incubated for 3 h at room temperature on a rocking table. Washes and secondary antibody (1:5000; Jackson Immunoresearch, Hamburg, Germany) incubation were performed in blocking buffer for 1.5 h at room temperature on a rocking table. After removal of secondary antibodies, blots were developed with SuperSignal West Pico horseradish peroxidase substrate system (Pierce) and autoradiography (Biomax Light; Eastman Kodak, Rochester, NY). Blots were washed and reprobed with a mAb against β-Actin (Sigma, Taufkirchen, Germany) to control for small variation in protein loading and transfer.

Morphologic Examinations

Light microscopy and immunohistochemistry were performed by routine procedures as described in detail previously (17). Renal tissue was fixed in 4% buffered formaldehyde. Paraffin-embedded sections (2 μm) were stained either with an antibody directed against the monocye-specific marker ED-1 (Chemicon International, Temecula, CA) to evaluate glomerular M/M infiltration or with an antibody against the T cell marker CD3 (Clone 1F4; Biozol, Eching, Germany). Immunohistochemical complement staining was carried out using goat anti-rat C3c (Nordic Immunology, Tilburg, The Netherlands). Tissue sections were developed with the alkaline phosphatase anti-alkaline phosphatase technique. ED-1– and CD3-positive cells in 50 glomerular cross-sections (gcs) and 30 tubulointerstitial fields (hpf) per kidney were counted by light microscopy in a blinded manner by an investigator who was unaware of the groups (17). Four different kidneys of each group were evaluated at 24 h and 4 d. The cell numbers of ED-1– and CD3-positive cells are given per gcs and per tubulointerstitial hpf as means ± SEM.

In Situ Hybridization

In situ hybridization procedures were performed as described previously (16). The probes for rat IP-10/CXCL10 and MCP-1/CCL2 used for in situ hybridization were prepared by in vitro transcription of subcloned cDNA. The 307-bp IP-10/CXCL10 probe corresponds to nucleotides 124 to 430 of sequence U22520, and the 353-bp MCP-1/CCL2 probe corresponds to nucleotides 278 to 631 of sequence M57441 (GenBank accession numbers). Antisense and sense RNA transcripts were labeled with 35S-UTP (20 μCi/ml; Amersham, Freiburg, Germany) and served as hybridization probe and control, respectively. Free nucleotides were separated with a Sephadex G-50 column (quick-spin columns; Roche, Mannheim, Germany). In situ hybridization was performed on 12-μm cryosections of renal tissue using 5 ng of the 35S-labeled antisense and sense RNA probes, respectively. Sections were exposed overnight to Kodak Biomax MR x-ray films (Rochester, NY). Subsequently they were treated with Kodak NTB-3 nuclear track Emulsion and exposed for 3 wk, followed by development in Kodak D19 and fixation with Kodak Unifix. Finally, sections were stained with Mayer's Hemalum.

In Vitro Chemotactic Assay

T cell chemotactic activity was determined in modified Boyden chambers (Neuro Probe, Cabin John, MD) using freshly prepared human peripheral blood mononuclear cells separated over Histopaque-1077 (Sigma). The T cell layer was washed with HBSS and resuspended in RPMI and 0.2% albumin to 3.5 × 106 cells/ml. The T cell chemotaxis was analyzed using 48-well Boyden chambers with 5-μm-pore-size polyvinylpyrrolidone-free polycarbonate membranes as described previously (18). Migration was allowed to proceed for 90 min at 37°C in 5% CO2. The membrane was then removed, washed on the upper side with PBS, fixed, and stained. All assays were performed in triplicate, and the migrated cells were counted in five randomly selected hpf at 1000-fold magnification. Spontaneous migration was determined in the absence of chemotacticant. Results are expressed as chemotactic index (control = 1). In a second in vitro chemotactic assay approach, the migration of murine pre-B cells (300-19) that were stably transfected with human CXCR3 (accession no. NM_001504) in response to recombinant IP-10/CXCL10 in the presence or absence of the neutralizing anti–IP-10/CXCL10 antibody was assessed using the same experimental setting as described above.

Statistical Analyses

Results are expressed as mean ± SEM. Differences between the individual groups were compared by Kruskal Wallis test with post hoc analysis by Mann-Whitney test. Statistical significance was defined as P < 0.05 or in case of k comparisons P < 0.05/k (Bonferroni adjustment for multiple testing).

Results

Characterization of the Model of REMI

We have established a rat model of REMI similar to a model described by Nangaku and Johnson (11). This model was induced by selective left renal artery perfusion with an antiglomerular endothelial cell antibody followed by right-side nephrectomy. Antibody binding to glomerular endothelium and endothelial cells of PTC was validated by immunohistochemical staining against rabbit IgG (Figure 1, G and H). The administration of the antibody resulted in severe acute renal failure concomitant with the development of glomerular endothelial cell injury. Glomerular thrombi and apoptotic glomerular endothelial cells were observed especially 24 h after antibody application (Figure 1B). Glomerular influx of inflammatory cells was already present at this early time point (24 h) but was more pronounced at 4 d after REMI induction (Figure 1C). Peritubular capillary endothelial cells were also severely damaged, which was associated with massive infiltration of mononuclear cells at both time points investigated (Figure 1, E and F), leading to pronounced tubular injury. Immunostaining against the proliferating cell nuclear antigen (PCNA) revealed a maximum of endothelial cell proliferation at day 4 after induction of the microvascular endothelial cell injury (data not shown). Anatomy of a healthy rat glomerulus and tubulointerstitium is shown in Figure 1, A and D.
Renal Chemokine and Chemokine Receptor mRNA and Protein Expression

For analyzing renal chemokine and chemokine receptor expression, RNA was isolated from whole kidneys at 24 h and 4 d after disease induction, and real-time PCR was performed. The intrarenal RNA expression of the CXCR3 ligands IP-10/CXCL10 and Mig/CXCL9 after REMI induction was significantly higher compared with the UNX control group at 24 h and 4 d (24 h: IP-10/CXCL10 118.6-fold, Mig/CXCL9 10.97-fold; 4 d: IP-10/CXCL10 11.5-fold, Mig/CXCL9 2.89-fold; \(P < 0.05\); Figure 2A). The intrarenal RNA expression of the CCR5 ligands MIP-1\(\alpha\)/CCL3, MIP-1\(\beta\)/CCL4, and RANTES/CCL5 was significantly upregulated 24 h after REMI induction (24 h: MIP-1\(\alpha\)/CCL3 16.6-fold, MIP-1\(\beta\)/CCL4 8.4-fold, RANTES/CCL5 8.9-fold; \(P < 0.05\); Figure 2A). After 4 d, MIP-1\(\beta\)/CCL4 had returned to normal. The expression of MIP-1\(\alpha\)/CCL3 and RANTES/CCL5 was still elevated, although not statistically significant (MIP-1\(\alpha\)/CCL3 4.0-fold, RANTES/CCL5 5.8-fold; NS). The RNA expression of CCR2 ligand MCP-1/CCL2 was significantly upregulated at 24 h and 4 d after REMI induction (24 h: MCP-1/CCL2 64.51-fold; 4 d: MCP-1/CCL2 34.65-fold; \(P < 0.05\); Figure 2A).

Analysis of the chemokine receptor RNA expression revealed a nonsignificant tendency for an upregulated CXCR3 and CCR5 RNA formation after disease induction (24 h: CXCR3 4.66-fold, \(P = 0.07\) [NS], CCR5 1.45-fold, \(P = 0.30\) [NS]; 4 d: CXCR3 3.78-fold, 

Figure 1. Histopathologic kinetics of glomerular and tubulointerstitial damage in the model of renal endothelial microvascular injury (REMI). The figure shows representative photographs of the light microscopy and immunohistochemistry performed. Glomerulus (A) and tubulointerstitial area (D) of a control, uninephrectomized (UNX) rat 24 h after renal perfusion with PBS. Glomerulus (B and C) and tubulointerstitial area (E and F) at 24 h and 4 d after the induction of REMI. Glomerular (G) and tubulointerstitial (H) rabbit IgG deposition at 24 h after induction of REMI. Magnification, \(\times 1000\) in A, through C and G; \(\times 200\) in D through F and H.

Figure 2. Renal chemokine and chemokine receptor mRNA and protein expression. The figure demonstrates the relative intrarenal mRNA expression of the chemokines (A) and chemokine receptors (B) examined by real-time reverse transcription–PCR at 24 h and 4 d after the induction of REMI (x-fold of the UNX control group; \(n = 4\) for each group and time point). (C) Western Blot analysis showed that upregulation of IP-10/CXCL10 mRNA expression in kidneys of nephritic animals was paralleled by increased IP-10/CXCL10 protein expression.
P/H11005 0.09 [NS], CCR5 4.08-fold, P/H11005 0.053 [NS]). CCR2 mRNA, however, was significantly upregulated at both time points analyzed (24 h 2.25-fold; 4 d 4.95-fold; \( P < 0.05 \); Figure 2B).

To analyze the IP-10/CXCL10 protein expression in the model of REMI, we performed Western blot experiments. As demonstrated in Figure 2C, renal IP-10/CXCL10 protein expression was markedly upregulated 24 h and 4 d after nephritis induction in comparison with the UNX control animals.

Localization of Chemokine RNA Expression and Relation to Inflammatory Cell Infiltration

To analyze the localization of mRNA expression of the two most upregulated chemokines, IP-10/CXCL10 (acting via CXCR3 on activated T cells) and MCP-1/CCL2 (acting via CCR2 mainly on monocytes), we performed in situ hybridization. Induction of REMI led to a strong IP-10/CXCL10 and MCP-1/CCL2 positivity with a maximum at 24 h, mainly localized to the tubulointerstitial area. As shown in Figure 3, IP-10/CXCL10 and MCP-1/CCL2 transcripts were confined mainly to PTC (Figure 3, A and E). Furthermore IP-10/CXCL10 and MCP-1/CCL2 expression was detectable in the periglomerular region (Figure 3, C and G). It is interesting that in the glomerulus, no specific IP-10/CXCL10 signal was detectable despite antibody binding to and massive damage of glomerular endothelium. In contrast, MCP-1/CCL2 mRNA expression showed the expected distribution, as it was detectable in the periglomerular region (G+) was 0.23 compared with PTC. No signals were detected in glomerular tuft alone (G–) or tubuli (Tu).

Figure 3. Cellular localization of IP-10/CXCL10 and MCP-1/CCL2 expression by in situ hybridization. In situ hybridization revealed the strongest IP-10/CXCL10 expression by endothelial cells of peritubular capillaries (PTC; A). Furthermore, positive signals could be detected in the periglomerular region (C). Immunohistochemistry showed an infiltration of CD3-positive T cells exclusively to the same anatomic regions as those of IP-10/CXCL10 mRNA expression (B and D). Note that no significant glomerular IP-10/CXCL10 positivity or infiltration of CD3-positive T cells was detectable. MCP-1/CCL2 expression showed a similar pattern with strong positivity in endothelial cells of PTC (E) and the periglomerular region (G). However, an additional intraglomerular expression could be detected (G). Immunohistochemical staining for ED-1–positive monocytes/macrophages (M/M) revealed an infiltration exclusively to the same renal compartments in which MCP-1/CCL2 expression was found, including the glomerulus (F and H); \( n = 4 \) for each group and time point.

Figure 4. Relative mRNA expression of IP-10/CXCL10 in laser microdissected renal compartments. Different specific renal compartments were excised by laser microdissection: Glomerular tuft alone (G–; A), glomerular tuft, Bowman’s capsule, and periglomerular region (G+; C), and PTC (D). Glomerular tuft from A after excision is shown in B. (E) Relative mRNA expression of IP-10/CXCL10 was highest in PTC (normalized to 1). Relative expression in glomeruli with periglomerular region (G+) was 0.23 compared with PTC. No signals were detected in glomerular tuft alone (G–) or tubuli (Tu).
not only in the tubulointerstitial area but also in the glomerulus (Figure 3G). Chemokine RNA expression patterns perfectly matched infiltrating leukocytes. Large aggregates of CD3-positive T cells were found by immunohistochemistry exclusively in the same anatomic areas as IP-10/CXCL10 mRNA expression, namely around the PTC and in the periglomerular region (Figure 3, B and D). In contrast, no significant intraglomerular infiltration of T cells was found. ED-1–positive M/M were confined to the

**Figure 5.** Characterization of the rabbit anti IP-10/CXCL10 neutralizing antibody. To test the specificity of the IP-10/CXCL10 antibody, we performed the following experiments: Western blotting with a variety of recombinant mouse or human chemokines (A), Western blotting with increasing amounts of recombinant IP-10/CXCL10 (B), and IP-10/CXCL10–induced T cell (C) and CXCR3 transfected cell (D) chemotaxis assay in the presence or absence of the IP-10/CXCL10 antibody. (E) To demonstrate that the anti–IP-10/CXCL10 antibody does not interfere with REMI disease induction by the anti-endothelial antibody, we performed complement C3 staining.

**Figure 6.** Effects of IP-10/CXCL10 neutralization on T cell and monocyte recruitment. Immunohistologic staining of mononuclear cell infiltrates with an anti-CD3 and anti ED-1 antibody revealed large numbers of pericapillary (A) and periglomerular (C) tubulointerstitial CD3-positive T cells as well as pericapillary (E), peri- and intraglomerular (G) ED-1-positive M/M 4 d after induction of REMI. Application of an anti–IP-10/CXCL10 antibody after induction of the disease substantially reduced tubulo-interstitial and periglomerular CD3-positive cell recruitment (B and D). In contrast, recruitment of ED-1–positive M/M was not affected by IP-10/CXCL10 neutralization (F and H). Note that glomerular recruitment of T cells was only marginal in this model of microvascular injury. Magnification, ×400 in B and D.

not only in the tubulointerstitial area but also in the glomerulus (Figure 3G). Chemokine RNA expression patterns perfectly matched infiltrating leukocytes. Large aggregates of CD3-positive T cells were found by immunohistochemistry exclusively in the same anatomic areas as IP-10/CXCL10 mRNA expression, namely around the PTC and in the periglomerular region (Figure 3, B and D). In contrast, no significant intraglomerular infiltration of T cells was found. ED-1–positive M/M were confined to the
same renal compartments as MCP-1/CCL2 mRNA expression around PTC, peri- and also intraglomerally (Figure 3, F and H). The sense probes revealed only background signals. In animals that were treated with unspecific rabbit IgG after uninephrectomy, no chemokine expression could be detected (data not shown).

Quantification of IP-10/CXCL10 mRNA in Different Renal Compartments by Tissue Microdissection

To verify the observation from the in situ hybridization that IP-10/CXCL10 expression is found only in PTC and the periglomerular region but not inside glomeruli, we obtained mRNA from microdissected kidney tissue. Real-time PCR analyses revealed the strongest IP-10/CXCL10 expression by PTC of nephritic animals 24 h after REMI induction. Glomeruli that were microdissected together with Bowman’s capsule and the adjacent periglomerular region (G/H11001) also showed a strong IP-10/CXCL10 mRNA positivity (23% of relative PTC IP-10/CXCL10 mRNA expression). In contrast, analysis of mRNA from the glomerular tuft alone (G/H11002) was negative for IP-10/CXCL10 mRNA. Analysis of microdissected tubular tissue of nephritic animals did not show any IP-10/CXCL10 expression (Figure 4). Microdissected tissues from UNX control animals were all negative for IP-10/CXCL10 mRNA expression (data not shown). Taken together, these results from microdissection with subsequent semiquantitative real-time PCR analysis strongly support the in situ hybridization data.

Characterization of the Anti–IP-10/CXCL10 Neutralizing Antibody

To test the specificity of the anti–IP-10/CXCL10 antibody, the following experiments were performed: Western blotting with increasing amounts of recombinant IP-10/CXCL10 (Figure 5A), Western blotting with a variety of recombinant mouse or human chemokines (Figure 5B), and IP-10/CXCL10–induced T cell chemotaxis assay in the presence or absence of the IP-10/CXCL10 antibody (Figure 5C). To further characterize the functional specificity of the anti–IP-10/CXCL10 antibody, we performed chemotactic experiments using CXCR3-transfected murine pre-B cells (300-19) in response to recombinant IP-10/CXCL10 in the presence or absence of the neutralizing anti–IP-10/CXCL10 antibody (Figure 5D). Western blot analysis and functional chemotactic assays demonstrate specificity and neutralizing potency of the polyclonal rabbit anti–IP-10/CXCL10 antibody. Immunohistochemical staining for C3c as marker for complement activation showed equal distribution in REMI animals and REMI animals that were treated with the anti–IP-10/
**CXCL10 antibody in glomeruli as well as in PTC (Figure 5E).** Therefore, it seems very unlikely that application of the anti–IP-10/CXCL10 antibody interferes with the binding of the nephritis-inducing anti-endothelial antibody.

### Effects of IP-10/CXCL10 Neutralization on Renal Leukocyte Recruitment

Induction of renal endothelial microvascular injury resulted in a significant increase of tubulointerstitial T cell infiltration after 24 h (6.05 ± 1 cells/hpf) and 4 d (19.3 ± 2.19 cells/hpf). Glomerular T cell infiltration was only marginal at 24 h (1.08 ± 0.34 cells/gcs) and had returned to normal after 4 d. M/M recruitment, however, was significantly enhanced at 24 h in the tubulointerstitial area (9.15 ± 0.61 cells/hpf) and in the glomerular compartment (1.86 ± 0.48 cells/gcs) as well as on day 4 (8.98 ± 1.01 cells/hpf; 3.8 ± 0.84 cells/gcs). To test whether IP-10/CXCL10 plays a functional role in T cell and M/M recruitment, we treated animals with a neutralizing anti–IP-10/CXCL10 antibody directly after induction of REMI. Blocking of IP-10/CXCL10 selectively reduced tubulointerstitial T cell recruitment at day 4 (REMI+anti–IP-10 11.3 ± 1.38 cells/hpf versus REMI 19.3 ± 2.19 cells/hpf; P < 0.02), whereas recruitment of T cells at 24 h was not significantly reduced (24 h 4.65 ± 0.7 cells/hpf; P = 0.19 versus REMI). Glomerular T cell infiltration was not affected by IP-10/CXCL10 neutralization (24 h 1.38 ± 0.74 cells/gcs; 4 d 0.16 ± 0.03 cells/gcs; all NS versus REMI). The number of infiltrating M/M was not affected at any time point by IP-10/CXCL10 neutralization (24 h 9.35 ± 0.72 cells/hpf, 1.08 ± 0.15 cells/gcs; 4 d 5.78 ± 1.44 cells/hpf, 2.48 ± 0.77 cells/gcs; all NS versus REMI; Figures 6 and 7).

### Effects of IP-10/CXCL10 Neutralization on Renal Function and Albuminuria

Induction of REMI significantly increased serum creatinine and urea serum level after 24 h (crea UNX 0.31 ± 0.02 mg/dl, REMI 0.78 ± 0.08 mg/dl; urea UNX 24.86 ± 2.5, REMI 63.1 ± 6.69; P < 0.001 UNX versus REMI) and 4 d (crea UNX 0.34 ± 0.02 mg/dl, REMI 1.57 ± 0.69 mg/dl; UNX urea 23.86 ± 1.87, REMI 114.67 ± 34.57; P < 0.001 UNX versus REMI). At day 4, serum creatinine and blood urea nitrogen levels were significantly lower in the group that had been treated with anti–IP-10/CXCL10 antibody (crea 0.58 ± 0.1 mg/dl, P = 0.012 versus REMI; urea 50.0 ± 12.34, P < 0.001 versus REMI), whereas after 24 h, only a nonsignificant tendency was observed (crea 0.65 ± 0.07 mg/dl, P = 0.123 versus REMI, NS; urea 63.1 ± 7.4, P = 0.029 versus REMI, NS as a result of Bonferroni adjustment;
Figure 8, A and B). All rats that received anti-endothelial antibody developed significant albuminuria after 24 h and 4 d (24 h \(527.63 \pm 113.03\) \(\mu\)g, 4 d \(1247.43 \pm 108.1\) \(\mu\)g; \(P < 0.001\) versus UNX). There was no difference in 24-h urinary albuminuria after anti-IP-10/CXCL10 treatment at both time points (24 h \(577.19 \pm 189.92\) \(\mu\)g/24 h, \(P = 0.393\) versus REMI; 4 d \(948.4 \pm 141.68\) \(\mu\)g/24 h, \(P = 0.118\) versus REMI; Figure 8C). The application of an equivalent amount of control rabbit IgG (instead of the polyclonal rabbit anti-IP-10/CXCL10 antibody) to nephritic rats did not significantly affect renal function of nephritic animals (serum creatinine and serum urea level; data not shown).

Discussion

Inflammatory cells at the site of tissue injury are a hallmark of almost every renal disease. Infiltrating leukocytes mediate the initiation and progression of damage by direct cytotoxicity, the secretion of soluble mediators, or the direct regulation of the immune response and probably also play a major role in tissue repair. Particularly, T cells and monocytes contribute to the development of renal tissue injury (1,2). It is interesting that in inflammatory kidney disease, T cell recruitment is observed predominantly in the tubulointerstitial area, whereas the infiltration of monocytes is usually detectable in both the glomerular and the tubulointerstitial compartment (3). The molecular mechanisms that lead to this distinct trafficking of T cells and monocytes are still not well characterized.

There is evidence from \textit{in vivo} and \textit{in vitro} studies that differential expression of chemokines and their receptors provides the molecular mechanisms that lead to the precise coordination of inflammatory cell migration in renal inflammatory diseases (7–10). We therefore hypothesized that different chemokine expression profiles between the tubulointerstitial and glomerular compartment might regulate the recruitment of different leukocyte subsets and thereby mediate specific site-directed tissue injury.

During inflammation, the endothelium is the side of initial contact with blood leukocytes. The endothelium thus may be functionally adapted to serve as an early and sustained source of chemokines that recruits specific leukocytes such as lymphocyte subsets and macrophages to the site of inflammation.

This difference in the temporal profile of chemokine expression may be one of several mechanisms by which endothelial cells regulate specific requirements of a local environment. We therefore chose to analyze possible differences in temporal and spatial expression patterns of chemokines by endothelial cells in a rat model of renal microvascular injury.

Induction of REMI increased renal mRNA expression of T cell–attracting and monocyte-attracting chemokines with a maximum at 24 h. Among the mRNA analyzed, IP-10/CXCL10 (119-fold), which acts primarily \textit{via} its receptor CXCR3 on activated T cells (19,20), was by far the most upregulated chemokine. The monocyte-attracting chemokine MCP-1/CCL2 (65-fold) was also significantly upregulated in REMI, thus resembling results from a number of earlier studies in different animal models (21–23). Western blot experiments confirmed that the increased IP-10/CXCL10 mRNA expression was paralleled by enhanced renal IP-10/CXCL10 protein formation.

To identify the cellular sources of the intrarenal IP-10/CXCL10 and MCP-1/CCL2 mRNA expression, we performed \textit{in situ} hybridization. IP-10/CXCL10 transcripts were confined to endothelial cells of PTC and infiltrating leukocytes. To a lesser degree, expression could be detected in the periglomerular area. Surprisingly, glomerular endothelium did not show any IP-10/CXCL10 expression despite strong endothelial antibody binding and extensive microvascular damage. MCP-1/CCL2 transcripts were detectable not only in endothelial cells of PTC and in the periglomerular region but also, to a lesser extent, inside the glomeruli. This intraglomerular positivity could be due to MCP-1/CCL2 expression by infiltrating M/M and/or by glomerular endothelial or mesangial cells. The tissue distribution of IP-10/CXCL10 was substantiated by quantification of IP-10/CXCL10 mRNA from different laser microdissected renal compartments. Although no expression was detected in the glomerular tuft alone, dissection of glomeruli with Bowman’s capsule and a small tissue stripe of the periglomerular region yielded strong IP-10/CXCL10 mRNA signals. In accordance with the \textit{in situ} hybridization, the most pronounced IP-10/CXCL10 expression was detected in PTC, whereas tubuli did not reveal any significant IP-10/CXCL10 expression.

These findings show that endothelial cells from different renal compartments respond in a site-specific manner to an antibody-mediated injury. This different chemokine expression pattern provides an explanation for the specific leukocyte subtype infiltration. A similar observation was made previously in hepatitis C virus–infected liver tissue. In this setting, IP-10/CXCL10 and Mig/CXCL9 were selectively expressed by sinusoidal endothelium, whereas CCR5 ligands MIP-1α/CCL3 and MIP-1β/CCL4 were largely confined to the endothelium of vessels within portal tracts (24). Differential expression of distinct chemokines by compartment-specific endothelial cells therefore might be an important general principle in inflammatory diseases.

Indeed, recent studies by the Schlöndorff group support the importance of compartment-specific chemokine expression. Anders \textit{et al.} (25) showed that application of the CCR1 antagonist BX471 in the MRL/lpr mouse model of lupus nephritis reduced the amount of macrophage and lymphocyte migration into the interstitium but not into the glomeruli, indicating a compartment-specific function of CCR1 and its ligands. Using the same nonpeptide CCR1 antagonist in a murine model of Adriamycin-induced focal segmental glomerulosclerosis with nephrotic syndrome and interstitial inflammation, Vielhauer \textit{et al.} (26) demonstrated a reduced amount of tubulointerstitial macrophage and T cell recruitment, whereas the extent of proteinuria and glomerular sclerosis was not affected. Finally, Segerer \textit{et al.} (27) showed earlier a prominence of CCR5-positive cell infiltration into the tubulointerstitium in an immunohistochemical study of renal biopsies from patients with different forms of glomerulonephritis, whereas the number of CCR5-positive cells within the glomeruli was very low.

The upregulation of chemokines in the early phase after induction of microvascular renal injury was followed by an increased recruitment of T cells and monocytes into the kidney with a maximum at day 4. Significant T cell infiltration was found only in the same renal compartments as IP-10/CXCL10.
mRNA expression around the PTC and in the periglomerular area, whereas no significant glomerular infiltration of T cells was detectable. Infiltration of monocytes was restricted to the same anatomic regions of upregulated MCP-1/CCL2 mRNA expression around PTC, peri- and also intraglomerular.

To investigate further whether IP-10/CXCL10 might play a functional role in T cell and monocyte recruitment in this model of REMI, we treated animals with a neutralizing anti–IP-10 antibody. Treatment of REMI rats with the anti–IP-10/CXCL10 antibody reduced tubulointerstitial infiltration of T cells by approximately 40% without affecting tubulointerstitial M/M infiltration. The effect on IP-10/CXCL10 neutralization on tubulointerstitial T cell recruitment was incomplete, suggesting that other chemokines are of functional relevance. In fact, we could detect a significant but less pronounced intrarenal upregulation of Mig/CXCL9, the second CXCR3 ligand identified in the rat so far, as well as an enhanced expression of the CCR5 ligands MIP-1α/CCL3, MIP-1β/CCL4, and RANTES/CCL5 in whole-kidney lysates from animals after REMI induction. Because CCR5 is expressed on T cells, these chemokines could also participate in the T cell recruitment.

Reduction of tubulointerstitial T cell infiltration was paralleled by improved renal function in animals that had microvascular injury and were treated with the neutralizing IP-10/CXCL10 antibody. In contrast, albuminuria was not significantly altered by blocking IP-10/CXCL10, which strongly argues against an important functional role for IP-10/CXCL10 in mediating glomerular damage.

In recent years, a pathophysiologic link between proteinuria and tubulointerstitial injury was established. The pathologic glomerular filtration of proteins leads to an increased protein uptake in proximal tubular cells (28). This in turn may be of functional relevance for the induction of inflammatory cytokines (e.g., chemokines) that might regulate the tubulointerstitial cell recruitment. However, in our model of microvascular renal injury, three points strongly argue against a major pathophysiologic role of proteinuria in mediating tubulointerstitial lesions: (1) the amount of albuminuria was very low in all nephritic animals tested, (2) chemokine RNA expression was undetectable in tubular cells by in situ hybridization and by microdissection, and (3) strong binding of anti-endothelial antibody to the PTC was detectable in co-localization with IP-10/CXCL10 and MCP-1/CCL2 expression. This indicates that indeed the antibody binding with subsequent complement activation (12) is the primary event leading to endothelial injury and chemokine production followed by leukocyte recruitment into the tubulointerstitial compartment.

Increased glomerular IP-10/CXCL10 expression was demonstrated by immunohistochemistry in human membranoproliferative glomerulonephritis, IgA nephropathy, and crescentic glomerulonephritis (29). Double staining indicated that mesangial cells were the main source of IP-10/CXCL10 production. In addition, in a rat model of anti–Thy-1 glomerulonephritis (30), it was suggested by immunohistochemistry that IP-10/CXCL10 is expressed by podocytes and might have a function in maintaining the glomerular filtration barrier. In contrast, we and others could not detect significant glomerular IP-10/CXCL10 and CXCR3 expression by in situ hybridization or immunohistochemistry (16,31).

In our hand, immunohistochemical staining for IP-10/CXCL10 and CXCR3 on paraffin and cryosections of rat renal tissue failed to produce convincing results. In our opinion, IP-10/CXCL10 and CXCR3 protein staining in the rat still awaits definitive confirmation using different antibodies and techniques.

Conclusion

Our findings demonstrate a differential chemokine expression pattern by endothelial cells from different intrarenal compartments in a rat model of acute renal endothelial microvascular injury. These findings are consistent with the hypothesis that functional heterogeneity of endothelial cells from different vascular sites exists (32). This may provide an insight into the molecular mechanisms that mediate compartment-specific T cell and monocyte recruitment in renal inflammatory diseases. Furthermore, we could show for the first time an important functional role of the CXCR3-specific chemokine IP-10/CXCL10 in renal microvascular damage, thus providing a rationale for considering IP-10/CXCL10 blockade as a future therapeutic option.

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