Spatial and Temporally Restricted Expression of Chemokines and Chemokine Receptors in the Developing Human Kidney

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Abstract. The directed migration of cells, cell-cell adhesion, and the control of proliferation are key events during metanephric development. The chemokines are a family of proteins that selectively control aspects of cell migration, activation, proliferation, and adhesion. The expression of a series of chemokines and chemokine receptors during human renal development was investigated by using immunohistochemical analyses and real-time reverse transcription-PCR assays of defined laser-microdissected metanephric structures. The results demonstrate that mononuclear cell-like cells within the nephrogenic blastema focally express interferon-inducible protein-10/CXCL10, a ligand for CXCR3. Mononuclear-like cells dispersed through the developing organ express CX3CR1. Expression of CXCR4, the receptor for stromal cell-derived factor-1/CXCL12, is also limited to stromal CD34-positive cells. In contrast, the expression of stromal cell-derived factor-1/CXCL12, fractalkine, and CXCR3 is first observed in the comma- or S-shaped body stage. The intensity of this expression becomes stronger in the capillary loop stage, and expression is mainly observed in the mesangial stalk and endothelial cells of the glomeruli. These proteins may play modulatory roles in kidney development. Because genes that are expressed during ontogeny often play a role in tissue regeneration, these embryonal chemokine/chemokine receptor patterns may be important in renal injury and repair.

Early in metanephric development of the kidney, the ureteric bud emerges as a pouch-like structure from the mesonephric (Wolffian) duct and invades the nephrogenic blastema (1). The metanephric mesenchyme is then stimulated to form glomeruli and the proximal and distal tubules. Four arbitrary developmental stages have been used to describe the continuum of glomerulogenesis (1,2), i.e., the vesicle or comma-shaped body stage, the S-shaped body stage, the capillary loop stage, and the maturing stage. Although the processes involved in glomerulogenesis and tubule formation are not fully understood, cell-cell adhesion, cell proliferation, and directional movement of cells are clearly prominent features of nephrogenesis. Chemokines are a superfamily of structurally related cytokines that selectively promote the adhesion, chemotaxis, and activation of cells (3,4). The biologic actions of chemokines generally occur through their interactions with a family of seven-transmembrane domain, G protein-coupled receptors. Chemokines, which were initially regarded as mediators of inflammatory and immune reactions because of their ability to attract and activate leukocyte subpopulations, can also function in homeostasis (3,4).

Growth factors and adhesion molecules represent two major classes of genes that are expressed during kidney morphogenesis. Specific growth factors can influence cell division, differentiation, and cell survival or apoptotic processes thought to be essential for renal development. Adhesion molecules expressed during glomerulogenesis mediate cell-cell attachment and allow the direct attachment of cells to the cellular matrix (5–7). Chemokines have been demonstrated to control the activation of integrins and thus can help mediate cell-cell interactions and promote the direct binding of cells to the matrix (3,8). These phenomena have been demonstrated for mononuclear cells in their interactions with endothelial cells and stroma but have also been observed for epithelial cells (5–7). Some chemokines act as autocrine or paracrine growth factors (4). Functional chemokine receptor expression by non-hematopoietic cells and stem cells has suggested a more global role for these factors in wound healing, angiogenesis, and development (3,9–14). Considering these actions of chemokines and chemokine receptors, we postulated that they might play a role in renal ontogenesis.

We focused on the expression of representative chemokines that have been demonstrated to have either strong adhesive properties (fractalkine/CX3CR1) (15), proliferative activity on mesangial cells [interferon-inducible protein-10 (IP-10)/CXCR3] (13,14), or vascular effects [stromal cell-derived factor-1 (SDF-1)/CXCR4] (16–18). The immunolocalization of these chemokines and their corresponding receptors was char-
acterized during different stages of glomerulogenesis, using human kidneys of gestational age 14 to 22 wk. The expression of the “soluble” chemokine ligands by specific developing renal structures was verified using real-time reverse transcription (RT)-PCR analysis of laser-microdissected renal structures.

Our results demonstrate that the chemokine receptor CXCR3 and the chemokines fractalkine/CX3CL1 and SDF-1/CXCL12 are expressed in different compartments of developing renal tissue. In contrast, single cells during renal development demonstrate focal expression of the chemokine IP-10/CXCL10 and are expressed in different compartments of developing kidneys. We conclude that chemokines and their receptors may be involved in the “fine tuning” of nephrogenesis. In addition, because embryonal expression patterns often reemerge in adult diseased tissue, elucidation of chemokine/chemokine receptor expression in developing kidneys may enhance our understanding of glomerular and interstitial disease.

Materials and Methods

Human Tissues Used

Inflamed adult kidneys removed because of reflux nephropathy were used to establish specific staining protocols for each antibody and to serve as positive control samples for RT-PCR experiments. Seven fetal renal tissue samples obtained after therapeutic abortions, representing 14 to 22 wk of gestation, were studied. Human tissue was used after approval by, and following the guidelines of, the Ethics Committee of the Medical Faculty of the University of Heidelberg (Heidelberg, Germany).

Antibody Reagents

CXCR3-specific mouse monoclonal antibody (MAB 160, 0.5 μg/ml), CXCR4-specific mouse monoclonal antibody (MAB171, 10 μg/ml), fractalkine-specific goat polyclonal antibody (AF 365, 0.2 μg/ml), and SDF-1/PBSF-specific goat polyclonal antibody (AF 310-NA, 0.2 μg/ml) were obtained from R & D Systems (Wiesbaden, Germany). Fractalkine/CX3CL1-specific rabbit polyclonal antibody (TP213, 20 μg/ml) was obtained from Torrey Pines Biolabs (San Diego, CA). Polyclonal rabbit antiserum directed against CX3CR1 extracellular loop 10 μg/ml (AB 1891) and N-terminus (AB1892) was obtained from Chemicon International (Temecula, CA). CX3CR1 rabbit antiserum obtained from Torrey Pines (TP502) appears to show significant cross-reactivity to unknown antigens. IP-10-specific mouse monoclonal antibody (6D4/D6/G2, 10 μg/ml), monokine induced by interferon-γ (Mig)-specific mouse monoclonal antibody (B8-11, 10 μg/ml), and CXCR4-specific mouse monoclonal antibody (12G5, 10 μg/ml) were purchased from Pharmingen (San Diego, CA). Anti-CD68 mouse monoclonal antibody was obtained from Dako (Hamburg, Germany).

For double-label immunohistochemical analyses, the anti-human myeloid/histiocyte antigen (1.5 μg/ml MAC387) was obtained from DAKO (Hamburg, Germany). Monospecific antiserum from rabbit against an 8 and 14 kD antigen of the S100 protein family, MRP8 (10 μg/ml) and MRP14 (10 μg/ml), respectively, was obtained from Biomedia (Baesweiler, Germany). Mouse monoclonal antibody 27E10 (5 μg/ml) directed against the heterocomplex of MRP8/MRP14 was obtained from Biomedia (Baesweiler, Germany). In addition, antibodies directed against endothelial antigen CD31 (mouse monoclonal antibody, 10 μg/ml; Dako), endothelial and stem cell antigen CD34 (mouse monoclonal antibody, 10 μg/ml; Dako), and smooth muscle actin (mouse monoclonal antibody, 5 μg/ml; Sigma, Diesenohenf, Germany) were used. Smooth muscle actin has been demonstrated to be a marker for mesangial cells in glomerular development (19).

Immunohistochemical Analyses

Immunohistochemical analyses were performed with 5-μm frozen tissue sections that had been fixed in acetone at −10°C for 10 min, essentially as described (20).

Alkaline Phosphatase/Anti-Alkaline Phosphatase Method. Tissue sections were incubated with the respective mouse monoclonal antibody for 18 h at 4°C. A rabbit anti-mouse Ig antibody (Z259, diluted 1:40; Dako) was applied at 22°C for 1 h, and alkaline phosphatase-specific mouse monoclonal antibody (diluted 1:40) was then applied at 22°C for 1 h. All dilutions were performed in phosphate-buffered saline (PBS) (pH 7.6). For staining, sections were exposed for 15 min to a solution of sodium nitrite (28 mM), new fuchsin (basic fuchsin, 21 mM), naphthol-AS-B1-phosphate (0.5 M), dimethylformamide (64 mM), and levansol (5 mM) in 50 mM Tris-HCl buffer (pH 8.4) containing 164 mM NaCl. When primary rabbit anti-human Ig antiseraum was used, the initial reaction was followed by a mouse anti-rabbit Ig antibody (M737, diluted 1:50; Dako), followed by antibody Z259 and the reactions described above.

Avidin-Biotinylated Enzyme Complex Method. For mouse monoclonal antibodies, the following procedure was used. After blockade of endogenous peroxidase activity by incubation of the sections in 3% H2O2 at 22°C for 10 min, nonspecific binding sites were saturated with 4% skim milk in PBS (pH 7.6) at 22°C for 20 min. The primary antibody was added to the sections for 18 h at 4°C. Then, with the application of avidin followed by biotin (avidin/biotin blocking kit; Vector Laboratories, Burlingame, CA), endogenous biotin, biotin receptors, or avidin binding was labeled. This was followed by application of a biotinylated anti-mouse Ig secondary antibody (0202D; PharMingen), diluted 1:250 in 1% bovine serum albumin/PBS, at 22°C for 1 h. A horseradish peroxidase-streptavidin complex (SA 5004, diluted 1:200; Vector Laboratories) was then applied at 22°C for 1 h. 3-Amino-9-ethylcarbazole or 3,3’-diaminobenzidine substrate kits (SK-4200 or SK-4100, respectively; Vector Laboratories) were used for specific staining.

For goat or rabbit polyclonal antibodies, the reactions were performed essentially as described above, except that the secondary antibody used was a “pan-specific” biotinylated antibody recognizing goat and rabbit antibodies (BA-1300; Vector Laboratories). Counterstaining was performed with hematoxylin at 22°C for 4 min.

All antigens described, except those for which only goat antibodies were available, were documented by using both immunohistochemical methods, i.e., avidin-biotinylated enzyme complex (ABC) and alkaline phosphatase/anti-alkaline phosphatase (APAAP) methods, to confirm the specificity of the staining. Control experiments for the immunohistochemical demonstrations entailed immunohistologic analyses with nonimmune mouse, rabbit, or goat IgG or without primary antibody. Negative control samples for the APAAP and ABC stains, generated with nonimmune control rabbit and mouse antibodies, are presented in Figures 1 and 3, respectively.

Double-Label Immunohistochemical Analyses. In double-label immunohistochemical reactions, the first antigen was documented with the ABC method, as described above. Sections were then washed in water and 1× PBS (#1666 789, Roche, Mannheim, Germany), followed by incubation with fetal calf serum. The second antigen was then demonstrated by using the APAAP method, with development
with Vector Blue (Camon, Wiesbaden, Germany) (21). For the three chemokine receptors for which double-label experiments were performed, the receptor was labeled once with the ABC method and once with the APAAP method.

**Laser-Assisted Microdissection**

Frozen sections (nominally 5 μm) of fetal tissue were mounted on glass slides covered by polyethylene (1.35-μm thick). Sections were briefly stained with hemalum (30 min), dehydrated in three consecutive baths of 70, 90, and 100% ethanol, and then air-dried. Renal structures (comma and S shapes, glomeruli, and medullary collecting ducts) were dissected with a Palm microdissection apparatus (Palm GmbH, Wolftrathausen, Germany). In short, a pulsed nitrogen laser beam (wavelength, 337 nm), which was coupled to a microscope with a computer-controlled, two-axis stage, was activated to cut out the respective renal structures. The selected areas were then catapulted by a laser beam into a microscope cupe that was centered above the cut-out area and was covered with 1 μl of PCR oil. Eight comma- and S-shaped structures, eight glomeruli, and seven collecting ducts were pooled into one microfuge vial and immediately frozen at −80°C until processed for RT-PCR.

**Real-Time RT-PCR**

Because the general intensity of immunohistochemical staining can be attributable to multiple factors not linked to the level of protein expression (for example, the staining efficiency of antibody reagents), quantitative interpretation of these signals is not possible. In addition, secreted proteins (such as cytokines) can be produced by different tissues and yet accumulate on the surfaces of other cells. These issues can be addressed in part by mRNA studies. Laser-assisted tissue microdissection and quantitative RT-PCR can be used to corroborate, explain, and enhance protein expression data. In this regard, it is important to remember that mRNA expression data can be difficult to interpret because, during renal ontogeny, mRNA expression does not always parallel protein synthesis; that is, translation can also be controlled developmentally (22).

For control experiments, total RNA was isolated from developing kidney homogenates by using a commercially available acid phenol procedure, as described previously (20). The mRNA species for all chemokine and chemokine receptor genes tested in this study (fractalkine, CX₃CR1, SDF-1, CXCR4, IP-10, and CXCR3) were first identified and analyzed by standard RT-PCR in adult and developing tissue (data not shown). This analysis was followed by characterization with real-time RT-PCR, to establish conditions and optimal housekeeping gene controls for subsequent experiments.

Real-time RT-PCR for laser-microdissected segments, tissue processing, and RT followed a protocol that had been previously established for single glomerular podocytes (23). In brief, microdissected samples or 2-μg samples of total RNA from whole tissue underwent random-primed RT, using a modified Moloney murine leukemia virus reverse transcriptase (Superscript II; Life Technologies, Karlsruhe, Germany), for 1 h at 40°C. Real-time RT-PCR was performed with a TaqMan ABI 7700 sequence detection system (PE Biosystems, Weiterstadt, Germany), using heat-activated Taq DNA polymerase (AmphiTaq Gold; PE Biosystems). After 2 min at 50°C and 10 min at 95°C, the samples were cycled 45 times at 95°C for 15 s and at 60°C for 60 s. mRNA expression for each signal was calculated by using the ΔCt procedure (24). Relative quantification was performed by using the following formula: \( \Delta\Delta CT = K (1 + E)^{Ct,R - Ct,T} \), where \( T_0 \) is the initial copy number of the target gene, \( R_0 \) is the initial copy number of reference (housekeeping gene) transcripts, \( E \) is the efficiency of amplification, \( C_{t,R} \) is the threshold cycle of the target gene, \( C_{t,T} \) is the threshold cycle of the reference (housekeeping gene), and \( K \) is a constant that is dependent on individual assay properties, as described (24). Similar amplification efficiencies for target genes and housekeeping genes were demonstrated by analysis of serial cDNA dilutions, which yielded an absolute value of <0.1 for the slope of log input cDNA amount versus μCT (i.e., \( C_{t,R} - C_{t,T} \)). By using the formula described above, the relative expression of the target gene, normalized to expression of the housekeeping gene, was calculated by subtracting the mean \( C_{t} \) of quadruplicates for the target gene from the mean \( C_{t} \) for housekeeping gene quadruplicates (ΔCt). Copies of the individual target transcripts were defined as \( K \times 2^{\Delta CT} \) copies of housekeeping gene transcripts. The amplification efficiency was defined as 1, because all compared analyses, including that of the control dilution series, were performed in the same run. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and β-actin were used as reference genes. Control samples, consisting of double-distilled water or non-reverse-transcribed samples, were negative for target and reference transcripts.

The following oligonucleotide primers (300 nM) and probes (100 nM) were used: human SDF-1/CXCL12 (gene bank number L36034, bp 119 to 194): sense, 5'-ACCGGCTCTGCTCCTC-3'; antisense, 5'-CATGGCTTTTCGAAGATCGG-3'; fluorescently labeled internal probe, 5'-TCAGCCTTGAAGCATAGGCCC-3'; human fractalkine/CX₃CL1 (gene bank number U84487, bp 209 to 276): sense, 5'-CTCGTACTGGCTCATACTCCATC-3'; antisense, 5'-TCCAA-GATGATTGCGCGTT-3'; fluorescently labeled internal probe, 5'-ACAGAACCAGGGCATCATGGGCA-3'. Commercially available, predeveloped, TaqMan reagents were used for IP-10/CXCL10, GAPDH, and β-actin. All primers and probes were obtained from PE Biosystems.

**Results**

**Expression of Fractalkine/CX₃CL1 and the Fractalkine Receptor CX₃CR1 during Human Renal Development**

Representative results from the immunohistochemical staining of a developing kidney with an anti-fractalkine antibody are presented in Figure 1 (A and B). Positive staining for fractalkine/CX₃CL1 expression was observed within the nephrogenic blastema and was accentuated around S-shaped structures. In developing glomeruli, the mesangium stained strongly positive (Figure 1B). Venous endothelia, medial smooth muscle cells, and arterial endothelial cells exhibited fractalkine/CX₃CL1 expression (data not shown). In the medulla, peritubular capillaries stained in a sheath-like pattern (Figure 1D). In contrast to the negative proximal tubules, distal tubules (e.g., macula densa segments of distal tubules, as demonstrated in Figure 1B) stained positively for fractalkine. The positive stromal cells did not stain for a monocyte/macrophage marker (CD68) (data not shown). The medulla and collecting ducts demonstrated positive basolateral staining (Figure 1D).

Fratalkine/CX₃CL1 can be cleaved from the surfaces of cells to generate a soluble protein. To address the possibility that protein was generated elsewhere in the developing tissue and accumulated on the filtration apparatus, RT-PCR was performed on samples of the tissue used for immunohistochemical analyses. Fratalkine/CX₃CL1 mRNA expression could be localized to comma- and S-shaped bodies, glomeruli,
and collecting ducts in laser-microdissected metanephric segments (Figures 5 and 6).

The pattern of expression for the fractalkine receptor CX₃CR1 is shown in Figure 2, A and B. Cells within the stroma of medulla and cortex stained positive for CX₃CR1. These cells were found in and around glomeruli and comma and S-shaped structures. The CX₃CR1-positive mononuclear cells were characterized by relatively large oval nuclei and varyingly broad granule-free cytoplasm. These cells could not be colabeled by a series of antibody reagents directed against monocytes/macrophage antigens (CD68, MAC387, MRP8, and MRP14), or the stem cell antigen (CD34). A very weak but

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*Figure 1.* Immunohistochemical localization of fractalkine/CX₃CL1 in human fetal kidney. (A) In an 18-wk-old female fetal kidney, staining for fractalkine is apparent in ureteric buds and S-shaped structures (long arrows). In an immature glomerulus with few capillaries, the endothelia demonstrate a fine positive cytoplasmic ring for fractalkine/CXC₃CR1 (short arrows). Stromal cells are also labeled. (B) In the same kidney, glomeruli exhibit pronounced positivity of the mesangial stalk for fractalkine. Slight staining was also observed in glomerular endothelial cells and in the media of arteries (not shown). Macula densa cells and respective distal tubules (DT), as well as stromal cells, are positively labeled with varying intensities. Proximal tubules (PT) are negative. (C) The negative control for the alkaline phosphatase/anti-alkaline phosphatase (APAAP) reactions, i.e., nonimmune rabbit IgG, does not yield a positive signal. (D) In the same kidney as in A, fractalkine/CX₃CL1 is demonstrated in the medulla in collecting ducts (arrows), with accentuation of the basolateral portion of the epithelia. Magnifications: ×400 in A to C; ×600 in D.
distinct staining for CX3CR1 was also seen in the media of preglomerular vessels and the mesangium (data not shown).

Broad Expression of the Chemokine Receptor CXCR3 in Developing Glomeruli and Limited Focal Expression of the CXCR3 Ligand IP-10/CXCL10 in Stromal Cells

Staining of developing kidneys with antibodies directed against IP-10/CXCL10 and CXCR3 demonstrated broad expression of CXCR3 during renal development but only focal expression of IP-10 (Figure 3). Figure 3C demonstrates positive staining for CXCR3 within the inner region of comma- and S-shaped bodies and in individual cells and vessels within the nephrogenic blastema. The general level of expression of CXCR3 seemed to increase with advancing renal development. A developing glomerulus with pronounced staining at the leading edge of the developing capillary convolute can be observed in Figure 3D. Double-label immunohistochemical analyses demonstrated coexpression of CXCR3 with the endothelial antigen CD31 (Figure 3, E and F) and with smooth muscle actin, a marker of developing mesangium (data not shown) (21). The epithelial collecting ducts demonstrated light staining, which was accentuated in the basolateral cytoplasm (Figure 3F). Figure 3F also demonstrates colocalization of peritubular capillary endothelia and CXCR3. Thus, CXCR3 was expressed in glomerular, capillary, and arterial endothelia, mesangium, and vascular smooth muscle cells.

In contrast to the broad pattern of expression observed for CXCR3, the distribution of the CXCR3 ligand IP-10 was focal and limited to isolated cells of the stroma within the cortex and medulla (Figure 3A). These mononuclear cell-like cells surrounded glomeruli and tubules. IP-10/CXCL10-positive cells were sometimes observed within the stromal regions of S-shaped structures, and IP-10/CXCL10-positive cells could also be detected at the vascular poles of glomeruli. In the medulla, IP-10/CXCL10-positive cells were observed in the peritubular interstitium. The expression of IP-10/CXCL10 within developing kidneys was confirmed by real-time RT-PCR analysis of total RNA isolated from fetal kidneys. TaqMan analysis demonstrated a mean IP-10/CXCL10/GAPDH signal ratio of 0.14 ± 0.05 for three kidneys analyzed (see the Materials and Methods sections for calculations). This was a significantly lower signal ratio than that observed for fractalkine/CX3CL1 (CX3CL1/GAPDH signal ratio, 2.69 ± 0.32) or SDF-1/CXCL12 (CXCL12/GAPDH signal ratio, 21.00 ± 1.45) (see below), with normalization to GAPDH levels, reflecting the rare focal IP-10/CXCL10 expression demonstrated by immunohistochemical staining (Figure 3A).

Expression of SDF-1/CXCL12 and Focal Expression of CXCR4

The chemokine SDF-1/CXCL12 was observed to be expressed by comma- and S-shaped bodies, as well as by the mesangium, blood vessels, and collecting ducts, in developing kidneys (Figure 4, A to D). Figure 4A demonstrates an S-shaped structure with faint positivity of the epithelial cells and distinct positivity of the inner stromal region. SDF-1/CXCL12 staining was observed within the mesangial area of a developed glomerulus (Figure 4C). Figure 4D demonstrates SDF-1/CXCL12-positive staining of an artery, with positively stained smooth muscle cells and unstained endothelium. Peritubular capillaries were also faintly labeled. Figure 4B demonstrates strong cytoplasmic staining of collecting duct epithelia. To
Figure 3. Immunohistochemical localization of interferon-inducible protein-10 (IP-10)/CXCL10 (A) and its receptor, CXCR3 (C to F), in human fetal kidney. (A) In a 22-wk-old fetal kidney, a few cells of mononuclear appearance (arrows) stain positively for IP-10 in the medulla. (B) A negative control for the avidin-biotinylated enzyme complex (ABC) reaction is shown. (A, C, and D) In the same kidney as in A, nonimmune mouse IgG does not yield a positive reaction. (C) In a 20-wk-old female fetal kidney, the IP-10/CXCL10 receptor CXCR3 stains strongly positively in ureteric buds and comma- and S-shaped structures (white arrow). Vessels in the nephrogenic stroma are also labeled for CXCR3 (black arrows). (D) In the same kidney as in C, a developing glomerulus demonstrates a positive CXCR3 signal in the glomerular stalk (white arrows) and endothelia (black arrow). (E and F) Double-label immunohistochemical analyses were performed for CXCR3 and CD31 in a glomerulus (E) and in a medulla with a vascular bundle, distal tubules, and collecting ducts (F). (E) CXCR3 (ABC method, red-brown label) can be observed in the mesangium (white arrows) and in endothelia, colocalizing with the endothelial marker CD31 (APAAP method, blue label). (F) In distal tubules as well as collecting ducts, epithelia stain for CXCR3 (ABC method, red-brown label). The vessels in the middle and lower parts of F (arrows) are colabeled for CXCR3 and the endothelial marker CD31 (APAAP method, blue label). Magnification: ×400 in A, C, and D; ×100 in B; ×600 in E and F.
Figure 4. Immunohistochemical demonstration of SDF-1/CXCL12 (A to D) and its receptor CXCR4 (E and F). (A) In a 22-wk-old female fetal kidney, SDF-1/CXCL12 staining is positive in an S-shaped body (arrowheads). (B) In the same kidney, collecting ducts are strongly stained by the anti-SDF-1 antibody, which also reacts strongly with renal pelvic urothelium (data not shown). (C) A developing glomerulus demonstrates positive labeling for SDF-1/CXCL12 in the mesangium (arrows). A cortical collecting duct is also labeled. (D) In the same kidney, an artery demonstrates strong staining for SDF-1/CXCL12 in the media (arrows). Developing glomeruli and cortical collecting ducts (arrowheads) again are positive for SDF-1/CXCL12. (E) CXCR4 is present on cells of the nephrogenic zone (arrowheads) and, to a lesser extent, in the medulla (data not shown) of a 17-wk-old male kidney. The CXCR4-positive cells surround comma- and S-shaped structures, as well as more developed glomerular structures. (F) Double-label immunohistochemical analyses for CXCR4 (ABC method, with red-brown 3-amino-9-ethylcarbazole substrate label) and the stem cell antigen CD34 (APAAP method, with Vector Blue APAAP label) were performed. A CD34- and CXCR4-positive cell (arrow) appears beside an unstained tubule. See Results and Discussion sections for interpretation. (A to E) ABC method. (F) Double-label immunohistochemical analyses, ABC with red-brown 3-amino-9-ethylcarbazole substrate and APAAP method with Vector Blue. Magnification: ×400 in A to E; ×1200 in F.
verify that SDF-1/CXCL12 was produced by these structures, the tissues were analyzed by using laser-assisted microdissection and real-time RT-PCR. The results demonstrated that SDF-1/CXCL12 mRNA was expressed in comma- and S-shaped bodies, glomeruli, and, at low levels, collecting ducts (Figures 5 and 6).

In contrast to the widespread expression of SDF-1/CXCL12 during glomerulogenesis, few cells were observed to express the only known receptor for SDF-1/CXCL12, i.e., CXCR4. Only scattered mononuclear cell-like cells stained positively for CXCR4 (Figure 4E). In double-label immunohistochemical analyses, these cells were generally positive for CD34, a marker for stem cells. Some intravascular CXCR4-positive cells were also colabeled for the monocytic markers MRP14 and MAC387 (data not shown). The results regarding chemokine/chemokine receptor expression during human renal ontogeny are summarized in Figure 7.

Discussion

Chemokines, a family of chemotactic cytokines, were first identified on the basis of their ability to induce the migration of different cell types, particularly those of lymphoid origin (3,4). To date, little is known regarding the role these factors may play in kidney development. We used immunohistochemical analyses, laser-assisted microdissection of developing renal structures, and real-time RT-PCR to monitor the expression of a series of chemokines and their respective chemokine receptors during glomerulogenesis and tubule development of the human kidney (Figure 6).

The results help demonstrate the effectiveness of combining real-time PCR technology with laser-assisted microdissection for the localization and quantitation of specific gene expression in defined nephron segments. The information received exceeds that obtained with in situ hybridization with respect to sensitivity and quantification. This approach allows simultaneous histologic characterization and sample collection and holds promise for future studies in renal development.

Unlike other chemokines, the CX₃C chemokine fractalkine/CX₃CL1 is tethered directly to the cell membrane, via a long mucin stalk. Observation of a soluble fractalkine/CX₃CL1 isoform suggests that fractalkine/CX₃CL1 may mediate adhesive and chemoattractive events. Outside the kidney, fractalkine/

Figure 5. TaqMan reverse transcription (RT)-PCR expression profile of stromal cell-derived factor-1 (SDF-1)/CXCL12 and fractalkine in laser-microdissected developing nephron segments. Developing glomerular structures show higher levels of expression of SDF-1/CXCL12, compared with fractalkine (mean ± SD). Original amplification data (for triplicates) are shown for glomeruli (A) and comma- and S-shaped bodies (B). The threshold cycles (Ct), measured as fluorescence intensity above background (ΔRn) for triplicates, are consistently lower for SDF-1/CXCL12 than for fractalkine.
CX3CL1 expression has been observed on endothelial cells and neurons and demonstrated strong upregulation by endothelium during inflammation (25). Subpopulations of T cells and natural killer cells have been demonstrated to express the only known receptor for fractalkine, i.e., CX3CR1. This ligand/receptor pair has been demonstrated to play a role in the modulation of apoptosis and the selective attraction of cell types (26,27). Fractalkine/CX3CL1 is strongly expressed during glomerulogenesis (Figure 1). Mesangial cells and collecting duct cells express this ligand. Some interstitial cells and mononuclear-like cells were also found to express fractalkine/CX3CL1. The CX3CR1-positive mononuclear-like cells found in the stroma of medulla and cortex did not show colabeling with a series of monocytes/macrophage antigens or with CD34 a marker for stem cells. The CX3CR1-positive cells were seen in and around glomeruli (Figure 2). The presence of fractalkine/CX3CL1 on endothelial cells in developing kidneys could be advantageous for the attraction and adhesion of CX3CR1-bearing cells from the blood flow in developing glomeruli.

CXCR3 is expressed at low levels on mesangial cells, vascular smooth muscle cells, and endothelial cells in normal adult kidneys and is upregulated by mesangial cells during mesangial proliferative disease (13,14). The endothelial expression of CXCR3 is limited to the S/G2-M phase of the cell cycle (14). In addition, CXCR3 is angiostatic in endothelial cells (14). CXCR3-positive mesangial cells exhibit Ca2⁺ flux, migrate, and proliferate in response to IP-10/CXCL10 or Mig/CXCL9 stimulation (13). Of the three ligands identified for CXCR3 (IP-10/CXCL10, Mig/CXCL9, and interferon-inducible T cell chemokine/CXCL11) (4), antibodies directed against two (IP-10/CXCL10 and Mig/CXCL9) were available for this study. An antibody specific for Mig/CXCL9 was observed to stain inflamed adult tissue, but no Mig/CXCL9-specific signal could be detected in developing human kidneys (data not shown). In contrast, focal expression of IP-10/CXCL10 was observed in developing kidneys (Figure 3A). IP-10/CXCL10-positive cells were observed at the vascular pole of developed glomeruli and were distributed focally in the nephrogenic stroma. Extrapolation from the functional study results and expression patterns observed for adult kidney tissues suggests that the focal expression of IP-10/CXCL10 during renal development may provide selective control of proliferation by CXCR3-positive cells, specifically glomerular endothelial and mesangial cells (3,13,14).

The chemokine SDF-1/CXCL12 is an important regulator of leukocyte and hematopoietic precursor migration (28,29). SDF-1/CXCL12 stimulates the proliferation of B cell progenitors and is strongly expressed in developing glomeruli (26). In collecting ducts, the expression of fractalkine is greater than that of SDF-1/CXCL12. Original amplification data are shown in A. The expression data for the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) are shown in inset B, with the lowest threshold cycle in collecting ducts, followed by comma- and S-shaped bodies and glomeruli. The overlay of three different analyses for individual target/housekeeping transcripts reflects the low intra-assay variability.

**Figure 6.** In collecting ducts, the expression of fractalkine is greater than that of SDF-1/CXCL12. Original amplification data are shown in A. The expression data for the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) are shown in inset B, with the lowest threshold cycle in collecting ducts, followed by comma- and S-shaped bodies and glomeruli. The overlay of three different analyses for individual target/housekeeping transcripts reflects the low intra-assay variability.
Development of human kidneys remains unknown, it is tempting to speculate that these factors may play a role in organogenesis (10). CXC chemokines have been demonstrated to act as both chemotactic and chemokinetic factors in the development of the kidney. The expression of specific chemokines may help control microvascularization within the tissue. SDF-1/CXCL12 and CXCR4 play essential roles in the interactions of hematopoietic cells, bone marrow stroma, and the stromal vascular supply (30,31), and the CXC chemokines macrophage inflammatory protein-2/CXCL1, epithelial neutrophil-activating protein-78/CXCL5, and interleukin-8/CXCL8 have been demonstrated to enhance liver regeneration (32,33).

The complexity and apparent redundancy of this system are thought to provide a high degree of effectiveness and flexibility in vivo (3,4). An analogous redundancy may also exist in renal development and may add to the degree of flexibility during renal ontogeny. This also suggests that targeted disruption of these genes might be less informative in this respect. Indeed, it has been demonstrated that genes important in renal development do not always exhibit renal phenotypes in knockout animals (2).

These results may also facilitate the characterization of disease processes. Genes involved in renal ontogeny are often re-expressed during glomerular and tubulointerstitial regeneration in adults, as demonstrated for CXCR3. Select chemokines and chemokine receptors may play a role in regenerative and reparative processes in adult kidneys. These results suggest that some caution should be used when chemokine antagonists are applied in the treatment of renal or systemic inflammatory disorders.

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