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Expression of the fractalkine receptor (CX3CR1) in human kidney diseases

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Background. CX3CL1 (fractalkine) is a membrane bound chemokine that can function as an adhesion molecule for cells expressing the receptor CX3CR1. This receptor is involved in the recruitment of inflammatory cells in a rat model of crescentic glomerulonephritis, where blockade of CX3CR1 has been shown to be of benefit. Here we describe the distribution of CX3CR1 positive cells in a variety of kidney diseases and renal development.

Methods. A total of 84 formalin-fixed, paraffin-embedded specimens including fetal kidneys ($N = 12$), normal areas of kidneys uninvolved by neoplasia from tumor nephrectomies ($N = 4$), renal transplant nephrectomies ($N = 5$), renal transplant biopsies ($N = 19$), and kidney biopsies from patients with crescentic glomerulonephritis ($N = 7$), membranous nephropathy ($N = 7$), membranoproliferative glomerulonephritis ($N = 8$), focal and segmental glomerulosclerosis ($N = 10$), collapsing glomerulopathy ($N = 6$), and minimal change disease ($N = 6$) were studied. Immunohistochemistry was performed on consecutive tissue sections for CD3 positive T cells, CD68 positive monocyte/macrophages, CCR5 positive cells and CX3CR1 positive cells.

Results. The majority of inflammatory leukocytes infiltrating the kidney expressed CX3CR1. The distribution pattern was consistent with expression by both T cells and monocytes/macrophages. In contrast to the distribution of CCR5, which was expressed on a subset of infiltrating cells predominantly localized in the interstitium, CX3CR1 was present on both interstitial and glomerular infiltrating leukocytes. In developing kidneys CX3CR1 positive cells formed a small, scattered population of cells, consistent with the distribution of infiltrating leukocytes.

Conclusions. The high number of CX3CR1-positive inflammatory cells in various disease entities is consistent with its having a role in the accumulation of intrarenal inflammatory cells, but does not provide evidence of specificity of leukocytes bearing this receptor for specific types of injury. Other chemokine gradients, like those created by the ligands for the chemokine receptor CCR5, might subsequently guide leukocyte subsets to specific microenvironments.

Key words: chemokines, fractalkine receptor, inflammation, glomerulonephritis, transplant rejection, renal development.

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The recruitment of leukocytes toward the site of tissue injury involves two distinct phases [1]. First, a complex interaction between inflammatory cells and the endothelium of blood vessels leads to firm arrest and diapedesis of cells from the circulation [2, 3]. During a second stage, inflammatory cells migrate deeper into the tissue toward different microenvironments [4–6]. Chemokines play important roles in both stages of this process in inflammatory kidney diseases [6–8].

The large family of chemokines is divided into the four groups of CC, CXC, C and CX3C chemokines [9]. The first two of four conserved cysteine residues in the primary amino acid sequence, are either located next to each other (CC) or separated by one (CXC) or three amino acids (CX3C). An additional group is missing two of the four cysteine residues (C). Chemokines function via binding to G-protein coupled seven-transmembrane spanning receptors, which are named according to the subgroup of their chemokine ligands (CCRs, CXCRs, XCR, CX3CR1 [6, 9, 10]).

CX3CL1 (fractalkine) is currently the only chemokine described with three intervening amino acids between the first two cysteine residues. It is one of two chemokines that is tethered to the cell membrane via a mucin stalk [9, 11, 12]. Localized on the endothelial surface, CX3CL1 can function both as a chemoattractant and an adhesion molecule for cells expressing its receptor CX3CR1 [13, 14]. G protein signaling is necessary for CX3CR1 to induce migration, but not to support adhesion [14]. Expression of CX3CR1 and migration toward CX3CL1 has been demonstrated for a wide variety of cells including T cells, monocytes/macrophages, natural killer cells, neutrophils, neurons and microglia [14–18]. Among freshly isolated blood leukocytes about 14% of

CD3-positive T cells (predominantly CD8 positive cells), about 79% of CD14 positive monocytes and over 90% of CD16 positive natural killer cells expressed detectable amounts of CX3CR1 [14]. In a model of crescentic glomerulonephritis in Wistar Kyoto rats, CX3CL1 is upregulated and blockade of CX3CR1 by either a broad spectrum chemokine receptor antagonist (vMIP-II) or a CX3CR1 blocking antibody demonstrated a strong beneficial effect [19, 20]. The distribution of CX3CR1 in human kidney diseases including renal transplant rejection is currently unknown. Therefore, we conducted this study on various renal diseases and conditions, which differ in the localization of infiltrating leukocyte subsets, to describe the expression of CX3CR1 in relation to the inflammatory infiltrates. In addition we compared the expression pattern of CX3CR1 expression with the distribution pattern of the chemokine receptor CCR5.

METHODS

Material

A total of 84 formalin-fixed and paraffin-embedded renal specimens were examined. These consisted of fetal kidneys ($N = 12$), normal areas of tumor nephrectomies ($N = 4$), renal transplant nephrectomies ($N = 5$), renal transplant biopsies ($N = 19$), and kidney biopsies from patients with crescentic glomerulonephritis ($N = 7$), membranous nephropathy ($N = 7$), membranoproliferative glomerulonephritis ($N = 8$), focal and segmental glomerulosclerosis ($N = 10$), collapsing glomerulopathy ($N = 6$), and minimal change disease ($N = 6$). The renal biopsies were from cases studied in the Department of Pathology, University of Washington (Seattle, WA, USA), and were included in this study after the diagnostic workup was completed. Normal areas from tumor nephrectomies and transplant rejection nephrectomies were collected over the years 1998 to 2001. No clinical data were available for morphological correlations as the approval of the University of Washington internal review board for human subjects prescribes that no patient identifiers may be linked to studies involving nephrectomy or biopsy tissue.

Immunohistochemistry

The protocols for immunohistochemistry have previously been described in detail [21, 22]. In brief, from each specimen serial sections were cut at 4 μm. Sections were deparaffinized and rehydrated. Endogenous peroxidases were blocked by hydrogen peroxide and antigen retrieval was performed by microwave treatment in Antigen Unmasking Solution (Vector, Burlingame, CA, USA). Endogenous biotin was blocked using the Avidin/Biotin Blocking Kit (Vector). Primary antibodies were applied for one hour, diluted either in phosphate-buffered saline (PBS) containing 1% bovine serum albumin (BSA; Sigma Chemicals, St Louis, MO, USA) or 10% non-fat dry milk. After subsequent washing in PBS the tissue was incubated with the biotinylated secondary antibody (Vector). For signal amplification the ABC-Elite reagent (Vector) was used. 3,3′-diaminobenzidine with nickel enhancement, resulting in a black color product, served as chromogen. Slides were counterstained with methyl green, dehydrated and coverslipped.

A polyclonal rabbit anti-human CX3CR1 antibody (AB1891; Chemicon International, Temecula, CA, USA) was used. In Western blots provided by the company, the antibody detects a band of approximately 50 kD, which can be blocked by preabsorption with the immunizing peptide. Controls used for this antibody in immunohistochemistry included omission of the primary antibody, substitution of the primary antibody by irrelevant rabbit IgG, and blockade of the signal by preabsorption of the primary antibody with the peptide used for immunization (Chemicon Int.). The antibodies MC5 (established and provided by M. Mack) against human CCR5 [23], against human CD3 positive T cells (rabbit anti-human, A0452; Dako, Carpenteria, CA, USA), and against human CD68 positive monocytes/macrophages (monoclonal mouse anti-human, Clone PG-M1; Dako) have previously been used for immunohistochemistry in formalin-fixed tissue [22, 24].

RESULTS

Expression of CX3CR1 in glomerular diseases

To describe the distribution of CX3CR1 positive cells in relation to infiltrating inflammatory cells we studied three groups of tissue samples, which differ by the predominant sites of tissue infiltration. Well preserved renal tissue from tumor nephrectomies and biopsies with minimal change disease was studied, as an example of a non-inflammatory disease without significant tubulointerstitial infiltrates. Glomerular diseases that usually show no prominent glomerular inflammatory cell influx, but that may have variable amounts of tubulointerstitial infiltrates and fibrosis depending on the disease stage, are represented by membranous nephropathy, focal and segmental glomerulosclerosis and collapsing glomerulopathy. Crescentic glomerulonephritis and mesangioproliferative glomerulonephritis (MPGN) are glomerular diseases that commonly show prominent glomerular macrophage influx and, with disease progression, typically demonstrate interstitial inflammatory infiltrates and fibrosis. Specimens were stained with hematoxylin and eosin (H&E), and by immunohistochemistry for CD3-positive T cells, CD68-positive monocyte/macrophages, and the chemokine receptors CX3CR1 and CCR5. Substitution of irrelevant rabbit IgG for the primary detecting antisera, and preabsorption of the primary antisera with

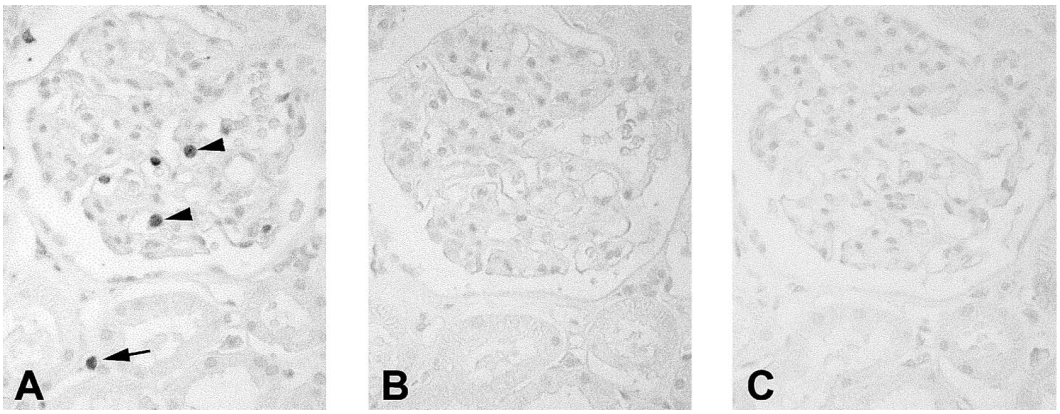

Fig. 1. Establishment of the antibody against human CX3CR1 for immunohistochemistry. Immunohistochemistry on consecutive sections of a tumor nephrectomy for CX3CR1 (*A*), preabsorption of CX3CR1 with the peptide used for immunization (*B*) and irrelevant rabbit IgG (*C*, original magnification ×400).

the peptide used for immunization, served as negative controls for CX3CR1 immunolocalization (Fig. 1).

In the group of cases from tumor nephrectomies and minimal change disease, only a small number of CD3-positive T cells and CD68-positive monocyte/macrophages could be detected in the interstitium. Small numbers of these cells also could be detected in lumina of peritubular and glomerular capillaries (Fig. 1 and Fig. 2 A-C). The distribution of CX3CR1 positive cells mirrored the combined distribution of these two infiltrating cell types. We found no clear evidence for CX3CR1 expression by intrinsic renal cells**,** with one exception. Expression of CX3CR1 by intrinsic cells was restricted to a single case of allograft rejection in which parietal epithelial cells in some glomeruli demonstrated positive immunostaining. CCR5 positive cells were only occasionally seen in a similar distribution like CX3CR1 positive cells. The number of CX3CR1 positive cells outnumbered the number of CCR5 positive cells in normal tissues as well as in all studied disease entities.

Biopsies of membranous nephropathy (Fig. 2 D-F), focal and segmental glomerulosclerosis and collapsing glomerulopathy contained variable degrees of interstitial inflammatory cell infiltration, consisting mainly of T cells and monocyte/macrophages. The number and distribution of CX3CR1 positive cells correlated well with both types of infiltrating cells. In contrast, CCR5 positive cells were present in lower numbers than CX3CR1 positive cells and the distribution pattern corresponded most to that of a subset of infiltrating T cells.

Biopsies of crescentic glomerulonephritis (Fig. 2 G-I) and MPGN (Fig. 2 J-L) demonstrated prominent glomerular macrophage influx. On consecutive sections glomeruli demonstrated a similar pattern of CX3CR1 positive cells as compared to the glomerular macrophage distribution (Fig. 2 H-K). The number of glomerular CCR5 positive cells was low in both entities and was clearly outnumbered by CD68 positive monocyte/macrophages and CX3CR1 positive cells. CCR5 positive cells principally were localized in the tubulointerstitium, consistent with our previous studies [23]. Although the percentage of CCR5-positive infiltrating cells was higher in the interstitium as compared to glomeruli, CX3CR1 positive cells also outnumbered CCR5 positive cells in the interstitium (Fig. 3). In areas of focal T cell infiltrates, there was an overlap in the distribution of CX3CR1 and CCR5, indicating T cells that expressed both chemokine receptors (Fig. 3 A, D). A population of large, round intratubular CD68 positive monocyte/macrophages was present in crescentic glomerulonephritis (Fig. 3 E-G). These cells were almost uniformly positive for CX3CR1, whereas they were usually CCR5 negative.

Expression of CX3CR1 in renal transplants

As in the above description of glomerular diseases, the distribution of CX3CR1 positive cells correlated with that of the two major populations of infiltrating cells, T cells and monocyte/macrophages. The number of CX3CR1 expressing cells is higher in transplants with acute cellular and vascular rejection as compared to biopsies without signs of rejection. A prominent population of CX3CR1 positive cells was present in the intimal subendothelial regions of arteries involved in vascular rejection (Fig. 4 A-C). During cellular (interstitial) rejection, inflammatory cells infiltrating the tubular epithelium were generally CX3CR1 positive (Fig. 4 D, E). Additionally, CX3CR1 positive cells were present in and around peritubular capillaries (Fig. 4 E, F). As in glomerular diseases, the cellular infiltrates were uniformly positive for CX3CR1, whereas only a subset was CCR5 positive.

Expression of CX3CR1 during renal development

A total of 12 fetal kidneys ranging in age from 58 to 122 days were studied. CX3CR1 positive cells formed a small, scattered population of cells between developing

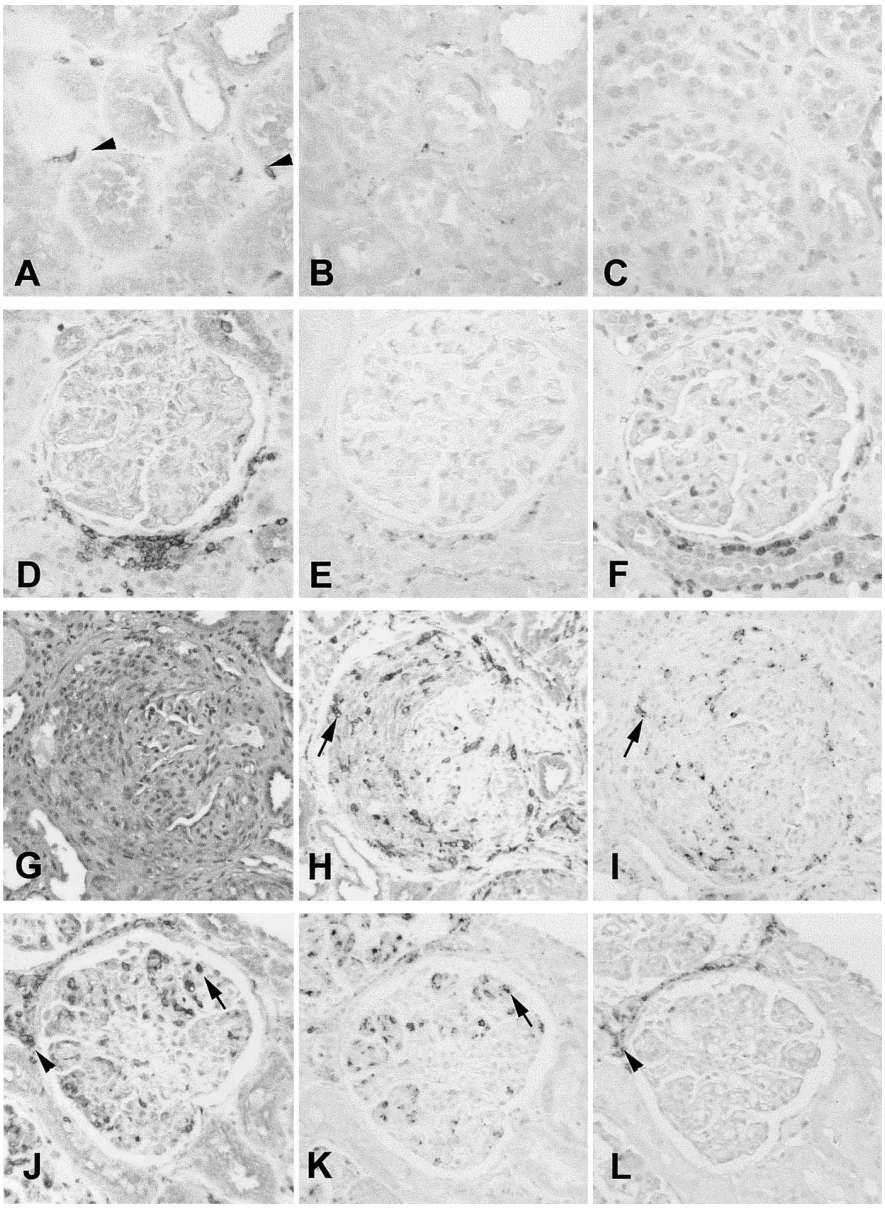

Fig. 2. CX3CR1 in well preserved renal tissue and glomerular diseases. Immunohistochemistry for CX3CR1 (*A*), CD68 positive monocytes/macrophages (*B*) and CD3 positive T cells (*C*, orig. ×400) on a normal area from a tumor nephrectomy. Scattered CX3CR1 positive cells are present in the interstitium (arrowheads). Immunohistochemistry for CX3CR1 (*D*), CD68 positive monocytes/macrophages (*E*) and CD3 positive T cells (*F*, orig. ×400) on a biopsy from a patient with membranous nephropathy. Biopsy from a patient with crescentic glomerulonephritis stained with H&E (*G*), as well as for CX3CR1 (*H*) and CD68 positive monocytes/macrophages (*I*, orig. ×200). The illustrated glomerulus demonstrated a cellular crescent containing a high number of monocytes/macrophages and CX3CR1 positive cells in a similar distribution (arrow). Immunohistochemistry for CX3CR1 (*J*), CD68 positive monocytes/macrophages (*K*) and CCR5 positive cells (*L*, orig. ×200) on a biopsy from a patient with MPGN. A high number of CX3CR1 positive cells were present in the glomerulus (arrow) and additionally in the interstitium (arrowhead). CD68 positive monocytes/macrophages are the infiltrating cell type in the glomerulus (arrow), whereas CCR5 positive cells are mainly found in the interstitium (arrowhead).

nephrons and in developing glomeruli (Fig. 5). These cells did not follow typical patterns of cells forming specific parts of the developing nephron. Furthermore, these cells demonstrated no association with the stage of differentiation of the developing nephron, nor with the age of the kidney. The distribution was consistent with the scattered distribution of CD68 positive cells, although we cannot exclude a CX3CR1 expression by a small subset of stromal cells. CCR5 positive cells were very rare in developing kidneys and the number of CD68 positive cells greatly outnumbered CCR5 positive cells.

DISCUSSION

Inflammatory infiltrates are composed of different subsets of infiltrating leukocytes, influenced in part by the type of tissue injury, the involved organ, genetic factors of the host and the time course of the insult (acute vs. chronic). The discovery of the chemokines, functioning as chemoattractants specific for subsets of inflammatory cells, helped explain an apparent discrepancy between the previously known chemoattractants that lacked specificities for discrete cell types, and the complexity of inflammatory infiltrates [4, 25]. During recent years it became apparent that subsets of lymphocytes, monocytes and dendritic cells express patterns of different chemokine receptors according to their stage of maturation and activation [2]. This enables them to follow simultaneous or successive chemotactic gradients in order to migrate toward specific microenvironments [5, 26].

Experimental data in animal models suggest an impor-

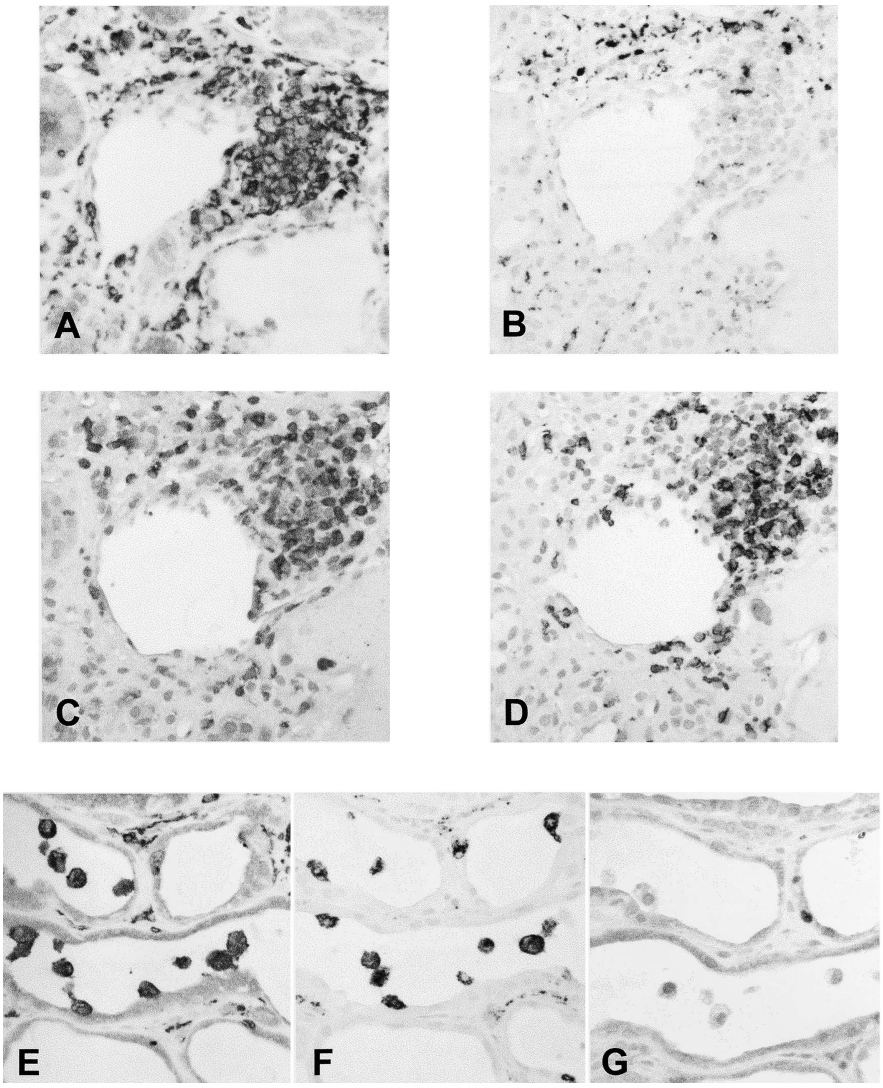

Fig. 3. CX3CR1 expression by interstitial infiltrates in cases of crescentic glomerulonephritis. Immunohistochemistry for CX3CR1 positive cells (*A*), CD68 positive monocytes/macrophages (*B*), CD3 positive T cells (*C*) and CCR5 positive cells (*D*; orig. ×400) on consecutive sections of a biopsy from a patient with crescentic glomerulonephritis. The right upper part of the picture shows a focal T cell infiltrate (C). This area shows an overlapping positivity for CX3CR1 and CCR5 (A, D). CX3CR1 shows an additional cell population in the left upper part of the panel (A). Immunohistochemistry for CX3CR1 positive cells (*E*), CD68 positive monocytes/macrophages (*F*), and CD3 positive T cells (*G*, orig. ×400) on consecutive sections of a biopsy from a patient with crescentic glomerulonephritis illustrating intratubular monocytes/macrophages positive for CD68 and CX3CR1.

tant role of the chemokine-receptor pair of CX3CL1-CX3CR1 in various inflammatory diseases and make it an attractive target for therapeutic interventions [19, 20]. CX3CL1 is up-regulated during crescentic glomerulonephritis induced in Wistar-Kyoto rats as well as during mouse cardiac allograft rejection [19, 27]. The blockade of CX3CR1 demonstrated beneficial effects in both experimental systems. However, in contrast to the data obtained in rats with crescentic glomerulonephritis, CX3CR1 deficient mice did not demonstrate amelioration of the disease course in nephrotoxic serum nephritis [28]. Differences in the model system and the types of intervention might account for these results, as speculated by the authors [28]. CX3CR1 deficient mice demonstrated a significant prolongation of heart transplant survival when treated with low amounts of cyclosporine, whereas untreated mice demonstrated no differences in allograft survival as compared to CX3CR1 expressing controls [28].

A recent report localized the expression of CX3CL1 to endothelium of peritubular capillaries but not glomeruli in humans, although this was demonstrated in a very small number of vessels [29]. However, a study by Cockwell et al demonstrated expression of fractalkine in glomeruli, tubular epithelial cells, and peritubular capillaries in settings of acute crescentic glomerulonephritis or acute renal allograft rejection accompanied by prominent parenchymal infiltration by mononuclear leukocytes, but not in normal kidneys or in biopsies of patients with non-inflammatory diseases such as minimal change disease [30]. This is quite complementary to our finding of leukocytes bearing the appropriate fractalkine receptor in similar disease settings.

CX3CR1 is expressed on the two most common groups of infiltrating cells in inflammatory kidney diseases and renal allograft rejection, namely T cells and monocyte/macrophages [14]. In the current study we dissected the patterns of CX3CR1 positive cells by using disease enti-

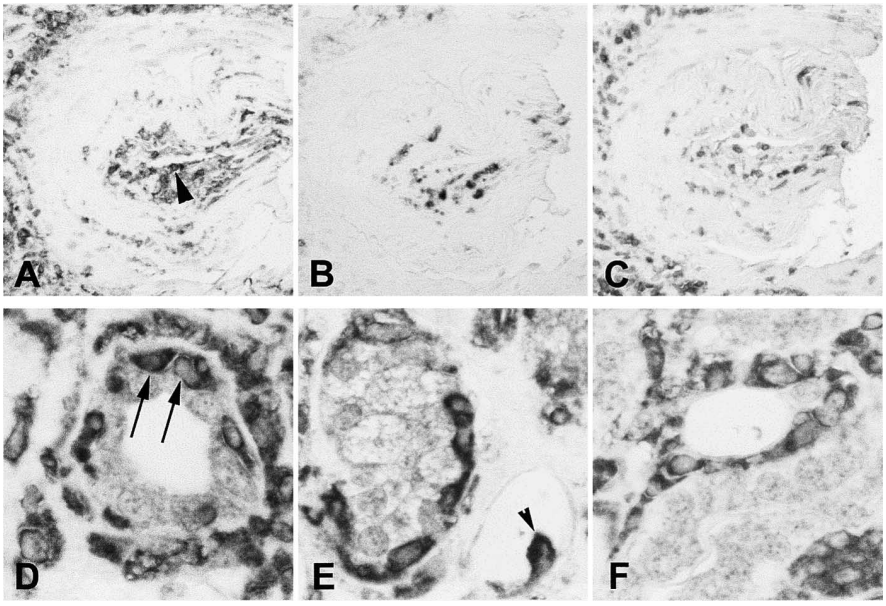

Fig. 4. CX3CR1 expression in renal transplant rejection. Immunohistochemistry for CX3CR1 positive cells (*A*), CD68 positive monocytes/macrophages (*B*), and CD3 positive T cells (*C*, orig. ×400) on consecutive sections of a biopsy from a patient with vascular rejection. A strong infiltrate of CX3CR1 positive cells is present in the subendothelial area of the illustrated artery (arrowhead). (*D–F*) Immunohistochemistry for CX3CR1 positive cells in a biopsy from a patient with cellular (interstitial) allograft rejection with tubulitis (all orig. ×1000). (D) CX3CR1 positive cells were infiltrating the tubular epithelium (arrows). (E) A CX3CR1 positive infiltrating cell was adherent to the endothelium of a peritubular capillary (arrowhead). (F) Infiltrating cells within the interstitium were seen surrounding a peritubular capillary, and were uniformly positive for CX3CR1.

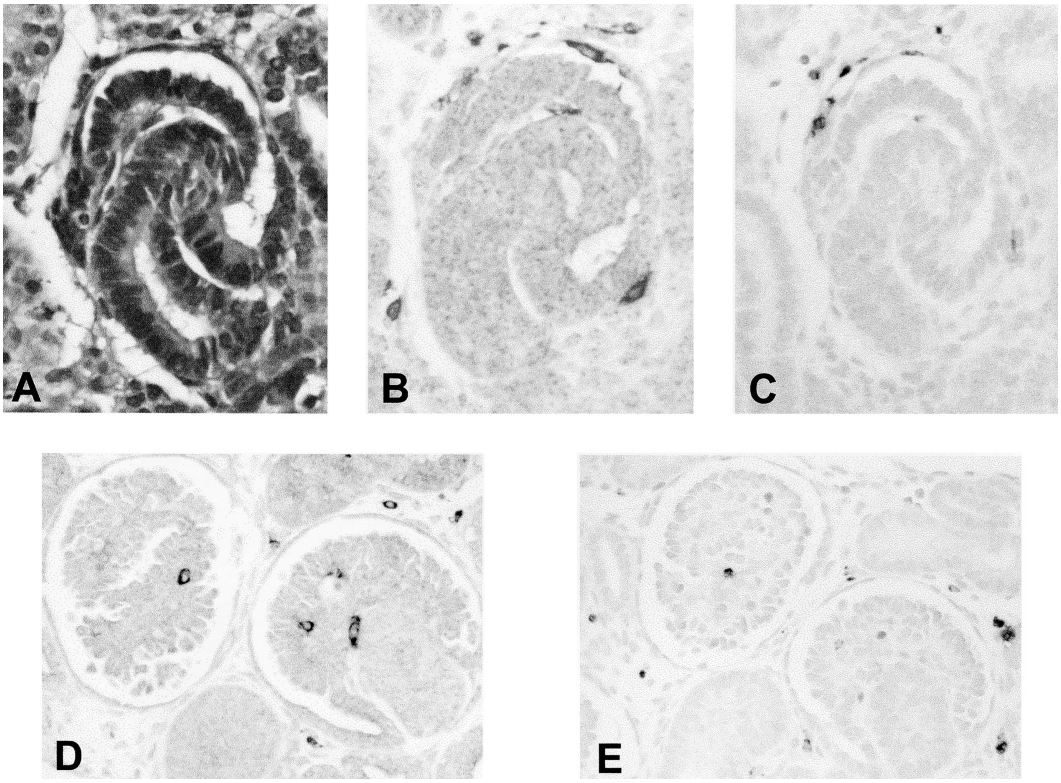

Fig. 5. CX3CR1 expression in renal development. Kidney from a 72-day-old fetus, stained with H&E (*A*), and for CX3CR1 (*B*) and CD68 (*C*, orig. ×400). A scattered population of cells surrounding the illustrated S-shaped body was CX3CR1 positive. Immunohistochemistry for CX3CR1 (*D*) and CD68 (*E*, orig. ×400) on a kidney from a 103-day-old fetus, showing a small scattered population of cells expressing these markers within the vascular clefts of developing glomeruli and in the interstitium.

ties with different distribution of inflammatory cells, while performing concurrent localization of T cells, monocyte/macrophages, and CCR5 positive cells. The main finding in this study is that the vast majority of cells infiltrating glomeruli as well as all parts of the tubulointerstitium in various kidney diseases seem to be capable of CX3CR1 expression. CX3CR1 expression is not limited to certain subsets of infiltrating cells or to localization to specialized environments (for example, interstitial vs. glomerular) in inflammatory kidney diseases. This is in contrast to our previous findings on two chemokine receptors, CCR2 and CCR5, both of which seem to be expressed on specialized subsets of inflammatory cells and are preferentially localized in different renal compartments [22, 23]. In peripheral blood 13% of CD4-positive T cells, 32% of CD8-positive T cells, 7.8% of monocytes, and 4% of natural killer cells were CCR5 positive [31]. CCR5 is expressed by a large subset of tubulointerstitial inflammatory cells, predominantly T cells, but is rarely expressed by infiltrating cells in glomeruli [23]. CCR2, on the other hand, is commonly expressed by leukocytes infiltrating glomeruli and its distribution fits best with the distribution of monocytes/macrophages [23]. We now present evidence that CX3CR1 is expressed on both T cells and monocytes/macrophages in renal inflammation. In a study comparing the effects of CX3CR1 and CCR5 blockade, it was found that both approaches showed significant benefits but the antagonism of CX3CR1 was superior to CCR5 blockade in ameliorating rat crescentic glomerulonephritis [19].

Integrating these data into a working model of renal injury, the widespread expression of CX3CR1 at various sites of inflammation suggests that this chemokine receptor is not an important mediator of the process by which localization of specific leukocyte subsets to specific microenvironments during renal inflammation occurs. It appears more likely that CX3CR1 functions predominantly as an adhesion molecule, functioning in the processes of firm adhesion and extravasation of leukocytes from the circulation. Subsequently, other chemokine gradients, such as those created by the ligands for CCR5 and CCR2 might then function to recruit subsets of leukocytes bearing these specific receptors to the target microenvironments.

A note of caution has to be raised about the interpretation of chemokine receptor localization by immunohistochemistry. Chemokine receptors can become desensitized and internalized upon ligand binding, and therefore detection of receptor expression may not necessarily correspond with the ability of these cells to migrate toward their appropriate ligands in vivo [32]. This caution notwithstanding, we have previously predicted a role for CCR5 in allograft rejection that was extrapolated from the CCR5 pattern detected by immunohistochemistry, and this conclusion has recently found support from a study that demonstrated improved allograft survival in patients deficient in CCR5 [33].

A question that remains unanswered by the present study concerns the rare appearance of CCR5 positive cells in glomeruli in some inflammatory states. Hypothetically this might be due to differences in ligand concentration and/or presentation between glomerular and peritubular endothelium, differences in expression of adhesion molecules and their ligands on endothelium of these two compartments, and differences in shear stress. Recently, the active movement of T cells away from a chemokine has been described [34]. Chemokines might not only recruit subpopulations of inflammatory cells to special microenvironments, but also repulse subsets of cells from entering other compartments.

In summary, our current study as well as correlative animal data imply an important role for CX3CL1-CX3CR1 during renal inflammation. We predict that the effect of engagement of this receptor by its endothelial bound ligand occurs early during the process of leukocyte extravasation. Mice deficient in either CX3CL1 or CX3CR1 appear to be phenotypically normal, but temporary blockade of this ligand/receptor system has shown significant beneficial effects in renal injury [35, 36]. Although the compensatory mechanisms in mice deficient in CX3CL1-CX3CR1 are currently incompletely understood, it raises hopes that therapeutic interventions aimed at this ligand/receptor system in human renal disease might be safe.

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