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The role of chemokines in progressive renal disease

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1. ABSTRACT

Tubulointerstitial damage followed by scarring and progressive loss of renal function is common to many forms of chronic proteinuric nephropathies. The severity of tubulointerstitial injury and in particular interstitial macrophage infiltration strongly correlate with the risk of renal failure. Proteins filtered through the glomerular capillary in excessive amount activate proximal tubular cells to upregulate chemokines mainly via activation of NF-kappaB-dependent pathway. Chemokines secreted toward the basolateral compartment of tubular epithelial cells incite local recruitment of mononuclear cells, that in turn interact with resident renal cells and extracellular matrix to create a proinflammatory microenvironment that amplifies tubulointerstitial inflammation and promotes renal scarring. The association between proteinuria and interstitial accumulation of inflammatory cells via activation of transcription factors and overexpression of chemokines has been established both experimentally and in human proteinuric nephropathies. Blocking leukocyte recruitment by interfering with transcription factor activity or chemokines and their receptors is envisioned as a strategy to retard kidney disease progression.

2. INTRODUCTION

Progressive renal diseases, independent of the type of initial insult, have in common persistent high levels of urinary protein excretion, tubulointerstitial lesions and scarring (1). The severity of tubulointerstitial injury is widely recognized to correlate with that of proteinuria and the rate of declining renal function in both experimental and human proteinuric nephropathies. Interstitial infiltrates of leukocytes, namely monocytes/macrophages and T cells, are a characteristic feature of progressive nephropathies (2, 3). Among the cellular mechanisms that may cause progression of kidney damage, excess of proteins filtered from the glomerulus, as the result of deleterious changes in glomerular permeability, may induce interstitial inflammatory reactions through activation of tubular epithelial cells (4). Experimental evidence shows that signals for leukocyte recruitment into the renal interstitium can be provided by the tubular synthesis of chemokines, a family of low molecular weight proteins, that interact with respective chemokine receptors expressed on the surface of leukocytes. Among chemokines that attract monocytes-macrophages and lymphocytes, monocyte chemoattractant protein-1 (MCP-1 or CCL2) (5) and regulated upon

activation normal T-cell expressed and secreted (RANTES or CCL5) (6) were upregulated by plasma proteins in cultured proximal tubular cells. Albumin overload induced in proximal tubular cells the synthesis of interleukin (IL-8), a chemokine of the C-X-C family with chemotactic activity for neutrophils and lymphocytes (7). The release of chemokines in response to protein load was polarized toward the basolateral compartment of the cells, which is indicative of a directional secretion potentially responsible for the tubulointerstitial inflammatory reaction as found in proteinuric nephropathies. Overexpression of chemokines by tubular cells has indeed been documented in experimental models of proteinuric nephropathies, in association with massive accumulation of inflammatory mononuclear cells in the renal interstitium (8-10). Those cells in turn become a source of local chemokine synthesis, leading to a positive amplification loop (11). In addition, infiltrating leukocytes may produce proapoptotic substances or release fibrogenic mediators that induce the accumulation of fibroblasts and the production of extracellular matrix (3, 12), thereby contributing to amplify and perpetuate renal injury.

A number of experimental studies has indicated that interfering with the chemokine pathway is protective in progressive proteinuric renal disease. The therapeutic blockade of the chemokine receptors appears to be an effective strategy to limit leukocyte recruitment (11, 13). In addition, investigation of the molecular mechanisms underlying chemokine gene induction in proximal tubular cells exposed to proteins has identified among transcription factors, NF-kappaB as potential target for therapeutic intervention to limit interstitial infiltration of inflammatory cells and reduce tubulointerstitial damage.

3. EXPRESSION OF CHEMOKINES IN PROXIMAL TUBULAR CELLS EXPOSED TO PROTEIN OVERLOAD

In vitro experiments with polarized proximal tubular cells, as a model to assess the effects of apical exposure to plasma proteins, thereby reproducing the conditions of luminal contact of proteins abnormally filtered through the glomerular capillary, have contributed to reveal mechanisms relevant to the tubulointerstitial inflammatory response and structural injury occurring in response to proteinuria. Thus, independent investigators showed that challenge of proximal tubular epithelial cells with high concentrations of proteins (albumin or IgG or transferrin) enhanced the release of CCL2/MCP-1, CCL5/RANTES or CXCL8/IL-8, mainly toward the basolateral side of the cell (5-7). As a follow up of those studies, using co-culture systems of proximal tubular epithelial cells and monocytes/T cells, it was documented that the release of CCL2/MCP-1 and CCL5/RANTES upon apical exposure of tubular cells to albumin was further increased when tubular cells were co-stimulated by monocytes or T cells, either through a cell-to-cell contact mechanism or mediated by soluble factors (14). In this setting, increased synthesis of soluble intercellular adhesion molecule-1 (ICAM-1) and IL-6 were also observed. IL-1 and TNF were identified as the humoral factors involved in the amplifying effects exerted by monocytes and T cells on chemokine synthesis from albumin-treated tubular cells, as indicated by the blocking effect of antibodies against TNF or IL-1, but not other cytokine-neutralizing antibodies. Distinct patterns of chemokine receptor expression were induced on T cells or monocytes upon exposure to conditioned medium from tubular cells activated by albumin (14). Thus, CD4+T cells showed increased expression of both CCR2 and CCR5, CD8+ cells of CCR2 only, whereas monocytes exhibited high CCR5 expression but reduced CCR2. This would imply that different arrays of chemokines may mediate the chemotactic activity of leukocytes in the tubulointerstitium during the proteinuric state.

Protein overload induces the tubular expression of CX3CL1/fractalkine, a chemokine with unique structural and functional properties (15). It has an extracellular chemokine domain anchored to the cell surface through an extended mucine-like stalk fused to a transmembrane helix and an intracytoplasmic tail. The transmembrane domain is cleaved proximal to the membrane by metalloproteinases of A disintegrin and metalloproteinase (ADAM) family to release a soluble species (16). The soluble domain of CX3CL1/fractalkine functions as a chemoattractant, whereas the transmembrane domain acts as a cell adhesion molecule for monocytes, natural killer cells and a subset of CD8+ T cells that express the specific receptor CX3CR1 (17). In this way, both domains promote leukocyte infiltration at sites of injury (18). There is evidence showing that renal proximal tubular epithelial cells stimulated by TNF are capable of expressing CX3CL1/fractalkine which may support the adhesion of leukocytes to tubular cells (19). Strong expression of CX3CL1/fractalkine mRNA by tubules close to inflammatory cell infiltrates has been detected in renal biopsies from patients with acute renal allograft rejection (20). In cultured proximal tubular epithelial cells, albumin induced upregulation of CX3CL1/fractalkine mRNA and the synthesis of both membrane-bound and soluble forms of the protein (21). Consistently, in a murine model of protein overload proteinuria induced by repeated injections of bovine serum albumin, CX3CL1/fractalkine mRNA was overexpressed in the kidney, and chemokine staining was detected in tubular epithelial cells in a focal distribution. Treatment of mice with an antibody against the CX3CR1 receptor limited the accumulation of monocytes/macrophages in the renal interstitium (21). These data suggest that in proteinuric conditions CX3CL1/fractalkine might drive mononuclear cells into the peritubular interstitium, and possibly enhance their adhesion property, thus favoring interstitial inflammation and disease progression.

Studies indicated that chemokines themselves may activate the tubular epithelium and contribute in an autocrine fashion to perpetuate the damaging effects of proteins. CCL2/MCP-1 induced basolateral secretion of IL-6 and the expression of ICAM-1 in human tubular epithelial cells via mechanisms dependent on Gi protein, protein kinase C (PKC) and intracellular calcium ions, and via sequence-specific DNA binding of NF-kappaB and activator protein-1 (AP-1), suggesting additional pathways of tubulointerstitial inflammation and injury (22).

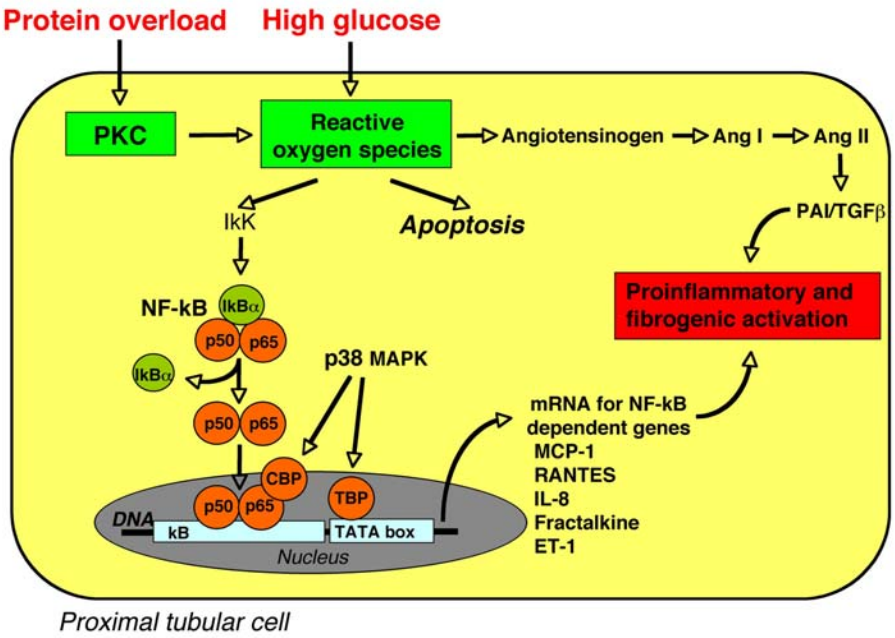

Figure 1. A simplified schema of mechanisms underlying proinflammatory and fibrogenic activation pathways in proximal tubular epithelial cells during non-diabetic or diabetic proteinuric settings. Major weight is given to the role played by reactive oxygen species (ROS) in response to protein overload and high glucose. Ultrafiltered protein load activates a cascade of signals from PKC-dependent ROS generation to nuclear translocation of NF-kappaB, via p38 MAPK activity, and gene upregulation of chemoattractants. Chemokines and endothelin-1 (ET-1) promote interstitial accumulation of mononuclear cells, that in turn may amplify local inflammation and promote fibrosis (ref.5-9, 21, 26, 27). In the diabetic state, the hyperglycemic condition increases ROS generation in proximal tubular cells followed by RAS activation through upregulation of the angiotensinogen gene. Increased angiotensin II production would contribute to injury by stimulation of PAI-1/TGFbeta pathways (ref. 30, 31). Induction of proapoptotic genes in diabetes may be mediated by ROS generation or be dependent of amplification loops via angiotensin II (ref. 31).

4. INTRACELLULAR SIGNALS UNDERLYING CHEMOKINE GENE UPREGULATION IN TUBULAR CELLS IN RESPONSE TO PLASMA PROTEINS

Molecular mechanisms underlying chemokine gene induction as a consequence of increased protein uptake by proximal tubular cells have been investigated. Nuclear transcription factors, such as NF-kappaB, have attracted attention as candidate pathway and are currently seen as potential targets of therapeutic intervention for proteinuria-induced tubulointerstitial injury. NF-kappaB is composed of homodimeric and heterodimeric complexes of the Rel/NF-kappaB family of proteins designed p50, p52, p65, c-rel, and RelB (23, 24). NF-kappaB dimers are present in the cytosol of unstimulated cells in an inactive form bound to the inhibitory protein IkB. Cell activation by triggers like cytokines, viruses, and oxidants leads to proteolytic degradation of IkB, allowing the translocation of NF-kappaB into the nucleus for binding to DNA motif in gene promoters (25). In cultured proximal tubular epithelial cells, protein overload activated NF-kappaB in a dose-dependent manner, followed by the upregulation of CCL5/RANTES, CCL2/MCP-1 or CXCL8/IL-8, that was abrogated by NF-kappaB inhibitors (6, 7, 26). Moreover, the role of NF-kappaB activation in chemokine mRNA induction by protein overload was supported by experiments showing that adenovirus-mediated gene transfer of IkBalpha or the dominant negative mutant of IkB kinase-2 (IKK-2), which fails to phosphorylate IkBalpha, reduced upregulation of CX3CL1/fractalkine mRNA in proximal tubular cells exposed to albumin (21). Reactive oxygen species served as second messengers in protein overload-induced NF-kappaB activation and subsequent chemokine gene upregulation (7, 27). Experiments in fact showed that in human proximal tubular cells, treatment with antioxidants while preventing the generation of hydrogen peroxide that followed the exposure of the cells to plasma proteins, almost completely abrogated the enhancement of NF-kappaB activity (27). There is evidence that oxidant generation is upstream regulated by protein kinase C (PKC), which once activated, translocates from the cytoplasm to cell membrane to mediate reactive oxygen species production and NF-kappaB activation (28). The finding that in protein overloaded tubular cells, specific inhibitors of PKC prevented hydrogen peroxide generation, NF-kappaB activation, and CCL2/MCP-1 and CXCL8/IL-8 upregulation, would imply in the proteinuric setting a cascade of signals from PKC-dependent oxygen radical generation to nuclear translocation of NF-kappaB and consequent chemokine gene upregulation (7, 27). A link has been proposed between induction of NF-kappaB by plasma proteins and mitogen-activated protein kinases, including p38 (21) and extracellular signal-regulated kinase 1 and 2 (ERK1/ERK2) (29) that are involved in chemokine synthesis (Figure 1).

Evidence for a prominent role of ROS generated by proximal tubules as mediators of disease activity has derived from a recent study that applied transgenic technology to achieve selective overexpression of catalase –an enzyme that breaks down hydrogen peroxide to inactive components- in proximal tubular epithelial cells (30). In mice with streptozotocin-induced diabetes, catalase overexpression in proximal tubules attenuated diabetes-associated abnormalities in the proximal tubules such as increases in angiotensinogen expression and in the proinflammatory ROS-dependent, plasminogen activator inhibitor-1 (PAI-1), and tubular cell apoptosis. Proteinuria was not affected, thereby suggesting that excess protein continued to bombard the catalase-overexpressing proximal tubules in the diabetic transgenic mice (31). It was speculated that the results of limiting tubular generation of ROS could reflect prevention of the inflammatory and proapoptotic effects of excess albumin uptake by proximal tubular cells (31), which might support ROS-dependence of protein-induced tubulointerstitial injury (Figure 1).

5. RENAL UPREGULATION OF CHEMOKINES IN EXPERIMENTAL MODELS OF PROGRESSIVE PROTEINURIC NEPHROPATHIES

A number of studies in experimental nephropathies supported the association between proteinuria and interstitial accumulation of inflammatory cells via activation of transcription factors and overexpression of chemokines. In rats with protein-overload proteinuria, upregulation of CCL2/MCP-1 and osteopontin in tubular epithelial cells was strictly associated with an interstitial inflammatory reaction (8). In this model, NF-kappaB activity was increased, and localized mainly to tubular epithelial cells (32). In rats with 5/6 nephrectomy, proteinuria over time was associated with enhanced NF-kappaB DNA-binding activity in the remnant kidneys (9). Strong nuclear staining for the p50 NF-kappaB subunit was visualized in proximal tubular cells and in sparse cells in the renal interstitium. NF-kappaB activation was paralleled by a progressive increase in renal expression of CCL2/MCP-1 mRNA, with intense signals found in tubular epithelial cells and, to a lesser extent, in interstitial infiltrating cells. Chemokine upregulation preceded the accumulation of monocytes/macrophages and T lymphocytes in the remnant kidney interstitium, a cascade of events which was predicted by *in vitro* studies. As in remnant kidneys, renal NF-kappaB activation and CCL2/MCP-1 overexpression preceded the mononuclear cell infiltrates into the renal interstitium in the immune model of passive Heymann nephritis in the rat (9). In both models, early treatment with ACE inhibitor acting to reduce proteinuria, almost suppressed NF-kappaB activation, CCL2/MCP-1 mRNA overexpression and interstitial accumulation of inflammatory cells (9). The ACE inhibitor also reduced proteinuria, osteopontin upregulation and macrophage recruitment in the renal interstitium of rats with adriamycin-induced nephropathy (33).

That NF-kappaB activation has a role in tubulointerstitial injury in proteinuric rats has received support by data showing that injection of a recombinant adenovirus vector expressing the truncated form of IkBalpha into the renal arteries of rats with protein overload proteinuria prevented NF-kappaB activation in tubular cells, and attenuated tubular damage and interstitial infiltration of mononuclear cells (34). Targeting NF-kappaB appears to be a means of interrupting the process of tubulointerstitial damage. Thus, treatment of rats with adriamycin (ADR)-nephrosis with the putative NF-kappaB inhibitor pyrrolidine dithiocarbamate (PDTC) suppressed NF-kappaB activation, reduced interstitial monocyte infiltration, tubular injury and cortical production of lipid peroxides compared with vehicle-treated ADR rats (35). Moreover, in rats with 5/6 nephrectomy, chronic inhibition (60 days) of NF-kappaB with PDTC was associated with partial attenuation of proteinuria, interstitial inflammation and renal injury (36). PDTC has been widely employed as an inhibitor of NF-kappaB. However, the mechanisms underlying this effect are not clear; PDTC may in fact directly impede the degradation of IK-B, or it may also act through its antioxidant properties inhibiting the stimulatory effect of oxidative stress on the NF-kappaB system. Although no major collateral effects were recorded after PDTC treatment in the above models, studies on the clinical toxicity and safety of PDTC and other NF-kappaB inhibitors are obviously needed before considering their potential use as a therapy to incorporate into the armamentarium employed against the progression of chronic kidney disease. Systemic inhibition of NF-kappaB might in fact produce deleterious effects on inducible expression of important gene products involved in the inflammatory and immunologic response.

Due to the significant contribution of ROS to the over-expression of chemokines in the setting of progressive renal disease (7, 27), studies have been performed to evaluate whether the decreased synthesis of chemokines in response to antioxidant compounds had effects on the development of renal injury in chronic nephropathies. In this regard, it has been reported that in rats with streptozotocin-induced diabetes, oral administration of breviscapine, an antioxidant stress agent and PKC inhibitor, effectively inhibited renal upregulation of MCP-1 and ICAM-1 and suppressed macrophage infiltration along with reduction of albuminuria, glomerular hypertrophy and tubulointerstitial injury (37).

The use of mice deficient in chemokines provided a valuable means of determining the role of chemokines in promoting leukocyte recruitment and activation, and their importance in progressive kidney diseases. In a model of streptozotocin-induced type 1 diabetic nephropathy in mice, renal expression of CCL2/MCP-1 mRNA and urine levels of the chemokine increased progressively with the duration of diabetes and correlated with CD68+ macrophage accumulation in the glomeruli and interstitium (38). In diabetic mice deficient in CCL2/MCP-1, macrophage recruitment in the kidney was decreased by 50-90% between 2 and 18 weeks after streptozotocin injection compared to wild type mice. A smaller proportion of macrophages expressing markers of activation, as inducible nitric oxide synthase and

sialoadhesin (CD169), was present in the glomeruli and interstitium of these mice. Albuminuria, histological damage and renal fibrosis were markedly attenuated. These data strongly indicated that the overpression of CCL2/MCP-1 is a major factor in the sequence of events that promote the kidney macrophage accumulation and associated tubulointerstitial injury during the development of diabetic nephropathy. On the other hand, since CCL2/MCP-1 deficiency did not totally prevent macrophage accumulation, it was suggested that other chemoattractants, such as osteopontin, migration inhibitory factor and CCL5/RANTES, also overexpressed by renal cells during diabetes, could be involved in macrophage recruitment as well (38). In addition, macrophage colony stimulating factor produced at high levels in the diabetic kidney (39) (40) was proposed to contribute, at least in part, to the enhanced accumulation of macrophages by stimulating local proliferation.

Blockade of the chemokine signaling pathway was protective in models of proteinuric nephropathies (Table 1). In rats with protein overload proteinuria, a hydrodynamic-based gene transfer technique was used to introduce naked plasmid encoding 7ND, a deletion mutant of human CCL2/MCP-1 used as CCL2/MCP-1 antagonist, into the left kidney of rats given repeated injections of bovine serum albumin (41). This maneuver attenuated interstitial inflammation and fibrosis and tubular damage in the treated kidney but not in the controlateral one. Finding that anti-CCL2/MCP-1 gene therapy acted locally would envision a potential application for this strategy against tubulointerstitial injury in the clinical setting. In rats with adriamycin-induced nephropathy DNA vaccination with naked DNA encoding CCL2/MCP-1 and CCL5/RANTES significantly reduced cell infiltrates in renal interstitium, specifically macrophages, CD8+ and CD4+ T cells, and CD25+ cells, and protected against renal injury (42). The protective mechanism was proposed to possibly involve the production of autoantibodies against CCL2/MCP-1 and CCL5/RANTES, with consequent reduction in renal infiltration by and activation of effector cells. Whether this type of therapy may represent a pratical approach for the human situation deserves some considerations (43). Given the potential importance of CCL2/MCP-1 and CCL5/RANTES in host defence, knocking these chemokines out on a permanent basis could be unsafe, and if autoantibodies were produced they could induce immune complex renal disease. On the other hand, it cannot be completely ruled out the possibility that these vaccines induce over-production of MCP-1 and RANTES that may dissipate chemotactic gradients and ameliorate renal tissue injury on that basis.

Specific chemokine receptor antagonists have been proven effective in retarding renal disease progression in different models of chronic kidney disease, even when given at a phase of established disease (13). In mice with adriamycin nephropathy that showed upregulation of the chemokine receptor CCR1 and its ligands CCL-3/MIP-alpha and CCL-5/RANTES, blockade of CCR1 with the small-molecule antagonist BX471 substantially reduced interstitial leukocyte recruitment and the subsequent renal fibrosis, without affecting the degree of proteinuria and glomerular sclerosis (44). Likewise, in $MRL^{lpr/lpr}$ mice with lupus nephritis, CCR1 blockade markedly reduced the numbers of interstitial macrophages, T cells and myofibroblasts, and decreased renal TGF-beta expression which was associated with less interstitial collagen deposits (45). Glomerular damage, macrophage infiltration into the glomerulus, and proteinuria were not instead attenuated by the CCR1 antagonist, thereby indicating that CCR1 does mediate leukocyte infiltration into the interstitium independently of glomerular effects (13).

6. CHEMOKINE EXPRESSION IN PATIENTS WITH CHRONIC PROTEINURIC NEPHROPATHIES

Induction of tubular chemokine expression in response to proteinuria consistently appears to represent an important pathway for progression of disease in humans.

Analysis of renal biopsies from 25 patients with idiopathic membranous nephropathy (13 progressive and 12 nonprogressive) showed a strong upregulation of CCL2/MCP-1, CCL5/RANTES and osteopontin, and the profibrogenic cytokines PDGF-BB and TGF-beta, mainly in tubular epithelial cells, with a significantly high intensity in patients with severe proteinuria and progressive disease. Chemokine overexpression was associated with an interstitial accumulation of mononuclear cells and an increase in myofibroblastic activity (46). In another study, activation of NF-kappaB was detected by Southwestern histochemistry in tubular epithelial cells of patients with minimal change disease and idiopathic membranous nephropathy, which significantly correlated with the magnitude of proteinuria (47). NF-kappaB was instead detected rarely in nonproteinuric IgA nephropathy patients. It is important to mention that the activation of NF-kappaB may require additional factors to effectively elicit an inflammatory response including the presence of nonselective proteinuria. Concomitant to NF-kappaB overactivation, tubular CCL2/MCP-1 and CCL5/RANTES mRNA and protein expression was observed in membranous nephropathy patients and was increased in those with progressive disease. NF-kappaB activation and CCL2/MCP-1 upregulation in proximal tubular cells were also described in patients with diabetic nephropathy (48).

In a large prospective study of 215 patients who underwent renal biopsy for investigation of chronic renal disease, a strong relationship was found between albuminuria, urinary CCL2/MCP-1, interstitial macrophage numbers, and renal damage (49). These factors were predictive of the renal outcome. In line with other studies, nephrotic range albuminuria was associated with the highest risk of disease progression and these patients had the highest levels of urinary CCL2/MCP-1 and interstitial macrophage numbers. A significant correlation was demonstrated between urinary CCL2/MCP-1 and CD68-positive macrophages that accumulated in the renal interstitium. Results from subset analysis of the different types of renal disease in this study (i.e. IgA nephropathy, focal segmental glomerulosclerosis, ischemic/hypertensive nephropathy) suggested that proteinuria-induced tubular

CCL2/MCP-1 expression is particularly important in the recruitment of macrophages during the initial phase of renal diseases, whereas other additional factors could be involved in more advanced stages (49).

Consistent with data obtained *in vitro* and *in vivo* models of protein overload (section 3), a recent study has provided evidence for a role of the CX3CL1/fractalkine-CX3CR1 system in the recruitment of inflammatory cells in the kidney of patients with progressive renal disease (50). By immunohistochemistry, the CX3CR1 receptor was expressed in the majority of cells, both T cells and monocytes/macrophages, infiltrating glomeruli and tubulointerstitium.

7. CONCLUSIONS AND PERSPECTIVES

During the last 20 years, research in experimental animals and humans has helped our understanding of the mechanisms by which chronic kidney disease progresses and has provided indication for therapies. In chronic renal disease, tubulointerstitial inflammation and injury is associated with infiltrating mononuclear cells. Chemoattractants and adhesive molecules for inflammatory cells are upregulated by ultrafiltered protein overload of proximal tubular cells mainly via activation of NF-kappaB-dependent pathway. This mechanism is a potential target for therapeutic interventions, as indicated by data of manipulations with inhibitory molecules of NF-kappaB activity or of chemokine receptors in experimental settings. Developments in these areas could help to design protocols in which drugs against secondary pathway of injury are given in combination with ACE inhibitors that limit the abnormal passage of proteins through the glomerular capillary barrier.

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