Frontiers in Nephrology

How Does Proteinuria Cause Progressive Renal Damage?

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The possibility that proteinuria may accelerate kidney disease progression to end-stage renal failure has received support from the results of increasing numbers of experimental and clinical studies. Evidence indicating that this process occurs through multiple pathways, including induction of tubular chemokine expression and complement activation that lead to inflammatory cell infiltration in the interstitium and sustained fibrogenesis, is reviewed. Macrophages are prominent in the interstitial inflammatory infiltrate. This cell type mediates progression of renal injury to the extent that macrophage numbers in renal biopsy predict renal survival in patients with chronic renal disease. Chemoattractants and adhesive molecules for inflammatory cells are upregulated by excess ultrafiltered protein load of proximal tubular cells *via* activation of NF-κB–dependent and NF-κB–independent pathways. This mechanism is a potential target for therapeutic approaches, as shown by beneficial effects of manipulations with inhibitory molecules of NF-κB activation or of chemokine receptors in experimental studies. Targeting complement synthesis or activation in proximal tubule might offer novel therapeutic opportunities. Finally, proximal tubular cell receptors for uptake of plasma proteins that are under investigation may provide activation signals on excess tubular protein handling.

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The past 20 yr of research in nephrology have yielded substantial information on the mechanisms by which persisting dysfunction of an individual component cell in the glomerulus is generated and signaled to other glomerular cells and to the tubule. Spreading of disease is central to processes by which nephropathies of different types progress to ESRD. Independent of the underlying causes, chronic proteinuric glomerulopathies have in common a sustained or permanent loss of selectivity of the glomerular barrier to protein filtration. Glomerular sclerosis is the progressive lesion beginning at the glomerular capillary wall, the site of abnormal filtration of plasma proteins. Injury is transmitted to the interstitium favoring the self-destruction of nephrons and eventually of the kidney. This review's main focus is on underlying mechanisms of tubulointerstitial injury that are activated by ultrafiltered protein load of tubular epithelial cells.

It needs to be emphasized that this field is relevant to interpret clinical findings and to improve treatment of patients with nondiabetic or diabetic nephropathies. The opinion among nephrologists that proteinuria could be a marker only of injury largely has been challenged. The strong predictive value of proteinuria in chronic nephropathies now is firmly established. Baseline proteinuria was an independent predictor of renal outcome in patients with type 1 diabetes and nephropathy (1) and in patients who did not have diabetes and entered the Modification of Diet in Renal Disease (MDRD) study (2). In the Ramipril Efficacy In Nephropathy (REIN) trial (3), urinary protein excretion was the only baseline variable that correlated significantly with GFR decline and progression of nondiabetic chronic proteinuric nephropathies to ESRD. Similar evidence was provided recently in patients with type 2 diabetes and overt nephropathy (4). Other studies corroborated these data and extended the predictive value of proteinuria to risks for overall or cardiovascular mortality (5,6).

Clinical trials consistently showed renoprotective effects of proteinuria reduction and led to the recognition that the antiproteinuric treatment is instrumental to maximize renoprotection (2,3,7,8). The MDRD study revealed tight association between reduction of proteinuria and decrease in rate of GFR decline (2). Protection that was achieved by lowering BP depended on the extent of initial proteinuria. The renoprotection that was conferred by angiotensin-converting enzyme (ACE) inhibition in the REIN study was mediated by the drug's action of reducing urinary protein levels, to the extent that patients who were on ramipril had a better outcome paralleled by more reduction in proteinuria, whereas BP was comparable to that of control subjects (3). ACE inhibitor–induced reduction in proteinuria was the strongest time-dependent covariate predicting slower progression to ESRD. Finding that the rate of GFR decline correlated negatively with proteinuria reduction and positively with residual proteinuria provided further evidence for a pathogenetic role of proteinuria (9). Likewise, trials in type 1 (8,10) and type 2 diabetes (11,12) documented that whenever

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proteinuria is decreased by treatments, progression to ESRD is reduced. The Reduction of Endpoints in NIDDM with the Angiotensin II Antagonist Losartan (RENAAL) study (11) in 1513 patients with type 2 diabetic nephropathy confirmed that more reduction in proteinuria by losartan invariably was associated with more renoprotection at comparable levels of BP control. Beneficial cardiovascular effects of losartan also were driven by effects on urinary protein and largely depended on the amount of residual proteinuria. Similar results were found in the Irbesartan Diabetic Nephropathy Trial (12). Finally, the Angiotensin-Converting-Enzyme Inhibition and Progression of Renal Disease study (13,14) confirmed that proteinuria is a strong risk factor for progression of chronic renal disease and that patients with more severe renal disease benefit most from ACE inhibitor therapy. Importantly, in no case from a pooled analysis was there a worsening in proteinuria that subsequently was associated with an improved outcome (15).

Glomerular Proteinuria as Signal for Interstitial Inflammation: Insights from Proximal Tubular Cells in Culture

Several lines of evidence suggested a role of glomerular ultrafiltrate of plasma proteins or protein-associated factors in chronic tubulointerstitial damage. *In vitro* studies using proximal tubular cells as a model to assess effects of apical exposure to plasma proteins proved highly valuable to approaching direct causal relationships. In monolayers of proximal tubular cells, the load with plasma proteins (albumin, IgG, and transferrin) induced the synthesis of the vasoconstrictor peptide endothelin-1 (ET-1), a mediator of progressive renal injury by virtue of ability to stimulate renal cell proliferation and extracellular matrix production and to attract monocytes (16). Since 1995, after this first report, independent investigators confirmed and extended the stimulatory effects of a diversity of plasma proteins on the expression of proinflammatory and profibrotic mediators in renal tubular cells (17–23). Among molecules that attract monocytes/macrophages and T lymphocytes, monocyte chemoattractant protein-1 (MCP-1) and RANTES were overexpressed in proximal tubular cells that were challenged with plasma proteins (17,24). Albumin upregulated tubular gene expression and production of IL-8, a potent chemotactic agent for lymphocytes and neutrophils (18). The release of ET-1 and chemokines in response to proteins was polarized mainly toward the basolateral compartment of the cell, as to mirror a directional secretion that favored the interstitial inflammatory reaction that was observed *in vivo*. Protein overloading of human proximal tubular cells induced the synthesis of fractalkine, which in its membrane-anchored form promotes mononuclear cell adhesion *via* CX3CR1 receptor (25). Fractalkine mRNA was overexpressed in kidneys of mice with protein overload proteinuria, and the gene product was detected in tubular epithelial cells mainly in the basal region. Treatment of mice with an antibody against CX3CR1 limited the interstitial accumulation of monocytes/macrophages (25).

Investigation of the molecular mechanisms underlying chemokine upregulation in proximal tubular cells on protein challenge had initial focus on the activation of transcriptional NF-κB (24). Other studies confirmed the pathway (26,27) and revealed reactive oxygen as a second messenger (18,28). Protein overload elicited rapid generation of hydrogen peroxide in human proximal tubular cells, an effect that, together with NF-κB activation, was prevented by antioxidants (28). Specific inhibitors of protein kinase C (PKC) prevented hydrogen peroxide generation, NF-κB activation (28), and MCP-1 and IL-8 gene upregulation that was induced by protein overload (18), suggesting a cascade of signals from PKC-dependent oxygen radical generation to nuclear translocation of NF-κB and consequent gene upregulation. A link also has been made between induction of NF-κB activity by protein load and mitogen-activated protein kinases, including p38 (25) and extracellular signal–regulated kinase 1 and 2 (ERK1/ERK2) (29) that are involved in chemokine synthesis. In support of the notion of protein overload as a key activator of signaling in proximal tubule is the finding that albumin activated the signal transducer and activator of transcription (STAT) proteins in cultured proximal tubular cells (30). Because the STAT pathway is the principal mechanism that converts the signal from a wide array of cytokines and growth factors into gene expression programs that regulate cell proliferation, differentiation, survival, and apoptosis (31), it was suggested that albumin may stimulate proximal tubular cells in the manner of a cytokine (32).

Which proteins play a predominant role as activator of tubular cells still is unanswered. Despite evidence that albumin overload elicits several responses by tubular cells *in vitro*, it has been argued that albumin *per se* may not be toxic to the proximal tubular epithelium. Compounds that are bound to albumin, such as free fatty acids (FFA), instead have been implicated to be causative in proinflammatory activation or injury of cultured proximal tubular cells (33). Kees-Folts *et al.* (34), by studying the specific response of cultured tubular cells to albumin-bound molecules, found that the tubular metabolism of albumin-bound fatty acids could generate macrophage chemotactic activity, whereas delipidated albumin produced little such activity. It was suggested that fatty acids can be released during degradation of albumin, HDL, or LDL. Lipid metabolites then would exert intracellular effects on second messenger systems with impairment or promotion of epithelial cell growth. Conversely, the exposure of proximal tubular cells to lipidated albumin resulted in chemokine overexpression at levels similar to delipidated albumin (17). Arici *et al.* (35) found that, among various fatty acids, oleic acid and linoleic acid exerted the most toxic and profibrogenic effects in human proximal tubular cells in culture. In studies of an *in vivo* model of overload proteinuria, animals that received an injection of FFA-replete BSA had higher levels of macrophage infiltration and tubulointerstitial damage as compared with the groups that received an injection of FFA-depleted BSA (36–38). Of note, potentially toxic substances, drugs, or haptens may act in a similar manner. Thus, compounds that are bound to freely ultrafiltered small proteins, such as cadmium to metallothionein, exert proximal tubule cell cytotoxicity upon receptor-mediated internalization and release into endosomal/lysosomal compartments (39). These studies collectively indicate that

the ability of albumin to act as a carrier enhances the proinflammatory activation of proximal tubular cells.

Further studies are required to assess the impact of reported changes in the expression of other genes in the proximal tubular cell, such as $\alpha V\beta 5$ integrin (40) and Na+/H+ exchanger (41) in response to albumin. *In vivo* gene expression profile analysis of proximal tubules from mice with protein overload proteinuria identified 2000 genes that were differentially regulated by excess proteins. More than half of them were upregulated (42). They included thymic shared antigen-1, the fibroblast-associated gene GS188, and glia maturation factor-B, a protein that originally was purified as a neurotrophic factor (43). The expression of glia maturation factor-B was induced in renal proximal tubular cells of mice with protein overload proteinuria (43). Proximal tubular cells that overexpressed glia maturation factor B acquired more susceptibility to death by sustained oxidative stress through p38-pathway activation.

A controversial issue is related to the concentrations of albumin that were used in various *in vitro* studies. Burton *et al.* (44) found that the apical exposure of human proximal tubular cells to 1 mg/ml albumin or transferrin did not increase MCP-1 or PDGF-AB release, an effect that instead was observed after exposure to a human serum fraction (40 to 100 kD) in the molecular weight range similar to albumin and transferrin. Studies that reported the effects of protein overload on NF-κB activation showed responses from 0.5 mg/ml in some experiments (17) and usually >2.5 (17) or >5 mg/ml (16,18). The latter concentration seems to far exceed the concentration reached in the proteinuric ultrafiltrate *in vivo* (45,46). Conversely, other studies found significant ET-1 and TGF-β upregulation (19) or enhanced collagen secretion (23) with as low as 0.1 mg/ml albumin (19,23) or 0.01 mg/ml globulins (23). A source of uncertainty also has been the suggestion that the normal proximal tubule might have great capacity to handle increasing amounts of proteins before tubular injury could develop (reviewed by Gekle [46]). More important, however, the interrelationship between protein uptake and gene expression in proximal tubule has been established (47). Low-affinity receptors for normal uptake of albumin have been identified on the brush border region of proximal tubular cells. Megalin, a 600-kD transmembrane glycoprotein that belongs to the LDL receptor family, binds albumin, insulin, prolactin, and vitamin-binding proteins and may act as a signal, at least partly, through phosphorylation of its cytoplasmic tail (48). Megalin is suggested to facilitate internalization and intracellular trafficking of cubilin, a 460-kD protein that binds albumin, transferrin, IgG light chains, and receptor-associated protein but lacks a transmembrane domain. Regulated intramembrane proteolysis, a process that links receptor-mediated endocytosis with intracellular signaling events, has been suggested to underlie the transcriptional regulation of specific genes in the proximal tubule (47). The first step in this process is the constitutive or ligand binding–dependent ectodomain shedding of the receptor by proteases. The transmembrane domain–containing fragment is the substrate for the γ-secretase activity of a multimolecular complex of proteins, including the so-called presenilins, that mediates intramembrane proteolysis. A soluble fragment of the receptor thereby is formed and trafficked to the nucleus to regulate gene expression. In the rat kidney, the brush border exhibits both γ-secretase activity and presenilin-1 expression (47,49). In a cell line that was derived from opossum proximal tubule (OK cells), metalloprotease activity mediated ectodomain shedding of megalin, producing a C-terminal fragment the same size as a major fragment of megalin found in kidney (47). Inhibition of endocytosis in proximal tubular cell lines did prevent increases in NF-κB DNA-binding activity (50) and in secretion of collagen caused by albumin overload (23).

The proximal tubule bears other receptors for ultrafiltered proteins, such as Ig (51) and complement molecules (52). The functional role of such receptors has not been established. It is likely that filtered proteins other than or in addition to albumin induce tubular dysfunction and injury in conditions of nonselective proteinuria, in which large molecular weight proteins are a significant component. In contrast, relatively selective albuminuria induces delayed mononuclear cell infiltration (53) and usually is associated with no or mild chronic tubulointerstitial injury. In this respect, the case of minimal-change disease has been considered sometimes an exception to the rule that interstitial infiltrates develop with time in proteinuric glomerulopathies. In addition, however, in minimal-change disease, a substantial percentage of patients respond to steroids, and the regression of proteinuria prevents inflammation and renal function deterioration (54). Patients who have this disease and the nephrotic syndrome and experience only a few relapses also are protected from renal damage, whereas patients who respond initially to glucocorticoids but have frequent relapses tend to develop interstitial injury and focal glomerulosclerosis. Finally, patients who have nephrotic syndrome with mild disease, are drug resistant, and have permanent proteinuria undergo renal function deterioration over time.

In Vivo Evidence Linking Proteinuria and the Interstitial Inflammatory Reaction in Chronic Proteinuric Nephropathy

The link between proteinuria and mononuclear cell accumulation into the interstitium *via* activation of transcription factors and overexpression of chemokines has received consistent support from studies in experimental models. In rats with overload proteinuria, upregulation of MCP-1 and osteopontin in tubular epithelial cells was associated with an interstitial inflammatory reaction (55). NF-κB activity also increased in tubular epithelial cells (56). In this model, anti–MCP-1 gene therapy reduced interstitial inflammation and fibrosis and tubular damage (57). In nephropathies of nonimmune (five-sixths nephrectomy) or immune origin (passive Heymann nephritis), inflammatory cell infiltrates developed in the vicinity of tubules that were engaged in protein reabsorption and overexpressing osteopontin (58). In the same models, proteinuria over time was associated with increased NF-κB activity, paralleled by overexpression of MCP-1 mRNA that preceded the accumulation of monocytes/macrophages and T lymphocytes in the renal interstitium (59). Therapy with an ACE inhibitor, acting to reduce proteinuria, almost suppressed NF-κB activation and MCP-1 upregulation

and limited the interstitial accumulation of mononuclear cells in both models (59). The ACE inhibitor also reduced proteinuria, osteopontin upregulation, and interstitial macrophage infiltration in rats with Adriamycin nephrosis. The treatment, however, could not reverse established fibrosis in this model (60).

MCP-1 expression that is driven by excess plasma protein signaling consistently seems to represent an important pathway for progression of injury in humans (61). A strong relationship was found between proteinuria and MCP-1–mediated interstitial damage in a prospective study of patients who underwent renal biopsy for chronic renal disease (61). High levels of albuminuria, urinary MCP-1, and interstitial macrophages were predictive of doubling in serum creatinine and/or end-stage renal failure. In previous reports, the analysis of renal biopsy specimens from patients with severe proteinuria revealed NF-κB activation in tubular cells, which significantly correlated with the magnitude of proteinuria. Concomitant upregulation of MCP-1, RANTES, and osteopontin was found in tubular epithelial cells, with the strongest expression in patients with progressive nephropathy (62,63). These findings are in strong support of the prediction that the MCP-1 pathway, as suggested by *in vitro* studies, mediates interstitial macrophage accumulation that is responsible for further injury. Findings of NF-κB activation and MCP-1 upregulation in proximal tubular cells have been reported in patients with diabetes (64). The role of macrophage accumulation into the renal interstitium in human diabetes is not established. However, recent data in db/db type 2 diabetic mice (65) and in MCP-1−/− mice with streptozotocin-induced diabetes (66) provide evidence for chemokine-mediated macrophage accumulation and associated tubulointerstitial injury.

Targeting NF-κB activation seems to be an effective means of interrupting the process of tubulointerstitial injury, as documented in rats with Adriamycin-induced nephropathy that was treated long term with the NF-κB inhibitor pyrrolidine dithiocarbamate (67) and in rats that had overload proteinuria and underwent gene transfer of truncated IκBα (68). In addition, a series of reports indicated that interference with the chemokine pathway was protective in models of proteinuric nephropathies. Naked DNA vaccination against MCP-1 and RANTES ameliorated the progression of renal disease in rats with Adriamycin nephropathy (69). The protective mechanism possibly involved the production of autoantibodies against MCP-1 and RANTES, with consequent reduction in renal infiltration by and activation of effector cells. Blockade of chemokine receptors with specific small molecule antagonists was shown to reduce interstitial leukocyte accumulation and subsequent fibrosis in mouse models of chronic kidney disease (70).

Key Role for the Intrarenal Activation of Complement

Complement activation is a powerful mechanism underlying tubular and interstitial injury *via* cytotoxic, proinflammatory, and fibrogenic effects. Abnormal C3 and C5b-9 staining in proximal tubular cells and along the brush border is a long known feature both in human chronic proteinuric diseases and experimental models. *In vitro*, proximal tubular cells activate serum complement *via* an alternative pathway, leading to C5b-9 fixation on cell surface (71) as well as cytoskeletal changes and production of superoxide anion, hydrogen peroxide, and cytokines (IL-6 and TNF-α) (72). Studies in rats with puromycin aminonucleoside–induced nephrosis showed beneficial effects of complement depletion or C6 deficiency against tubulointerstitial damage associated with proteinuria (73,74). Evidence subsequently was provided that C3 and ultrafiltered plasma proteins co-localized to proximal tubular cells that were exposed to filtered protein overload in rats with remnant kidney since stages that preceded the accumulation of monocyte/macrophages into the interstitium (75). Treatment with ACE inhibitor limited the excess load of both C3 and plasma proteins in proximal tubular cells that may significantly contribute to promote the recruitment of inflammatory cells (75). Protective effects by C6 deficiency (76) revealed the role of activated complement as a key mediator of tubulointerstitial injury in this model.

Glomerular permeability dysfunction of proteinuric nephropathies allows complement factors to be ultrafiltered abnormally across the altered glomerular barrier into the Bowman's space and tubular lumen. Plasma-derived C3 (molecular weight 180 kd) is likely to reflect more loss of glomerular permselectivity and to enhance cell dysfunction in the presence of abnormally filtered plasma proteins. Renal tubular cells also synthesize C3 and other complement factors (77) in ways that may have critical importance in disease, as found in experimental renal transplant rejection (78) and postischemic acute renal failure (79). Therefore, both excess ultrafiltration and proximal tubular cell synthesis of complement could underlie complement-mediated injury in chronic proteinuric renal disease (Figure 1). Recent findings of C3 mRNA upregulation and C3 accumulation in proximal tubular cells in kidneys of mice with protein overload proteinuria are in support of a role for the local synthesis of complement (80). Complement is an important effector of interstitial mononuclear cell infiltration and fibrogenesis in this model, as shown by significant attenuation of injury in C3-deficient mice (80). A direct role for protein overload as a stimulus was indicated by findings that the exposure of cultured proximal tubular cells to total serum proteins at the apical surface upregulated C3 mRNA expression and protein biosynthesis (20). Serum fractionation experiments identified substances that were responsible for such effects in the molecular size range of 30 to 100 kd. This fraction contains proteins that pass the glomerular barrier in proteinuric states. The addition of albumin alone did not reproduce the effects that were observed with whole serum, whereas the incubation with apical transferrin caused C3 mRNA overexpression and both apical and basolateral C3 secretion (20). C3 was upregulated to similar degrees in response to iron-poor transferrin or apotransferrin, indicating that transferrin protein rather than iron moiety could act as stimulus.

Renal parenchymal cells express a limited repertoire of receptors, including CR1, CR3, and CD88, that may bind complement proteins that are present in the ultrafiltrate. To which extent the stimulation of complement receptors on tubular cells

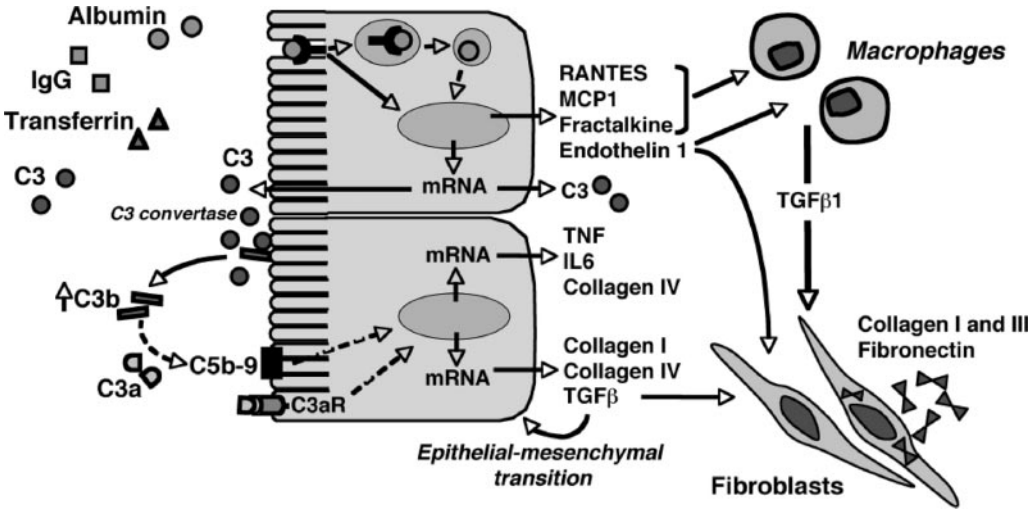

Figure 1. Mechanisms underlying the activation of inflammatory and fibrogenic pathways in proximal tubular epithelial cells by ultrafiltered protein load. As a consequence of proteinuria, the intrarenal activation of the complement cascade may promote injury through the formation of membrane attack complex and biologically active products, such as C3a, that interact with specific receptors. Monocytes/macrophages contribute to fibrosis by release of TGF-β, which stimulates myofibroblast formation and collagen deposition and epithelial mesenchymal transformation. The latter process could be induced in an autocrine manner by TGF-β of proximal tubular cell origin.

could influence progressive renal disease has not been established. The C3a receptor has been found to be highly expressed both by normal murine and by human kidney epithelial cells and to mediate altered gene expression in cultured proximal tubular cells (52). Among those genes, both TGF-β and collagen I were upregulated by C3a *in vitro*, suggesting the induction of pathways underlying inflammation and fibrosis. In kidneys of proteinuric mice with Adriamycin nephrosis, deposition of C3 and enhanced expression of collagen type IV and of its chaperone, heat-shock protein 47, showed topographic relationships at sites of tubulointerstitial damage (81). The gene expression of collagen and heat-shock protein 47 also was upregulated in primary cultures of mouse proximal tubular epithelial cells in response to C5b-9, further suggesting that complement activation on tubular cells can stimulate the fibrotic process directly. Other genes, such as pyrin (a gene that is responsible for familial Mediterranean fever) and Gulp were negatively regulated in tubular cells that were exposed to C3a, possibly reflecting modulatory effects on the inflammatory response and, respectively, on the clearance of apoptotic cells (52).

One important aspect of research in the complement field is the availability of complement inhibitory molecules that may block effector mechanisms even in the presence of persistent proteinuria. A strategy for specific delivery of complement inhibitors to the proximal tubule was designed using recombinant proteins that consisted of a carrier antibody against brush border antigen and a complement inhibitory molecule (sCrry and tCD59) in rats with puromycin aminonucleoside nephrosis (82). Inhibitors that were given intraperitoneally to proteinuric rats localized to proximal tubuli and protected against tubulointerstitial injury and renal dysfunction as assessed on day 11 after disease induction. This approach would obviate the need to inhibit complement systemically and its function in innate immunity.

Profibrogenic Signaling from Proximal Tubular Cells in Response to Protein Overload

Local recruitment of macrophages by tubular cells that are loaded with ultrafiltered plasma proteins may contribute to interstitial fibrosis by engaging matrix-producing interstitial myofibroblasts. Macrophages also regulate matrix accumulation *via* release of growth factors such as TGF-β and PDGF, ET-1, and PAI-1. TGF-β stimulates the transformation of interstitial cells into myofibroblasts. In addition, proximal tubular epithelial cells communicate with interstitial fibroblasts to promote fibrogenesis *via* paracrine release of TGF-β (Figure 1). In rats with remnant kidneys at day 14, after the onset of proteinuria, TGF-β mRNA was upregulated in proximal tubular cells in parallel with early accumulation of α-smooth muscle actin (α-SMA)-positive myofibroblasts, which were localized strictly to the peritubular interstitium, suggesting that interstitial fibroblasts are the initial target of profibrogenic signals elicited by protein overreabsorption (83). In remnant kidneys at day 30, staining for α-SMA also was visualized in proximal tubules, indicating that a phenotypic transformation of tubular cells occurs in a relatively late stage. Treatment of these rats with an ACE inhibitor at the same time limited excess protein overload and interstitial inflammatory cell infiltration and abrogated the abnormal TGF-β1 gene expression in tubular cells that in all likelihood was responsible for the recruitment of myofibroblasts in the surrounding areas. ACE inhibitor exerts beneficial effects in the glomerulus primarily by preserving the permselective barrier to proteins (84), thereby limiting proteinuria and filtered protein-dependent inflammatory and fibrogenic

signals. The ACE inhibitor also may act locally by preventing nonhemodynamic effects of angiotensin II (AngII) *via* apical angiotensin receptors on tubular cells, including renal cell proliferation and TGF-β1 expression (85). However, the toxic effect of protein trafficking on renal disease progression in mice with overload proteinuria is not dependent on AngII through the major angiotensin type 1 receptor isoform (86). Certainly, proteinuria and direct effects of AngII on tubular cells are not mutually exclusive targets of the drug's action.

TGF-β remains the most important cytokine for renal fibrogenesis, and it has been identified as a major stimulus in the process of epithelial-mesenchymal transition (EMT) of tubular epithelial cells (87). Studies have focused on the signaling pathways that are activated during TGF-β–induced EMT. It was shown that TGF-β induced Smad2 phosphorylation in a tubular epithelial cell line and that overexpression of an inhibitory Smad protein, Smad7, inhibited TGF-β–induced Smad2 activation, thereby preventing EMT and collagen synthesis (88). The same pathway was shown to mediate renal fibrosis in rats with ureteral obstruction (89). An endogenous antagonist of TGF-β1–induced EMT has been identified as bone morphogenic protein-7 (BMP-7), a member of the TGF-β superfamily whose genetic deletion in mice leads to severe impairment of kidney development (90). BMP-7 reversed TGF-β1–induced EMT through a Smad-dependent reinduction of E-cadherin, an adhesive junction protein that maintains the structural integrity and polarity of epithelial cells (91). Systemic administration of recombinant BMP-7 repaired severely damaged tubular epithelial cells and reversed renal injury in mice with nephrotoxic serum nephritis (91). Wu *et al.* (92) recently provided the interesting evidence that the immunosuppressive agent rapamycin was able to block EMT, as demonstrated by partial restoration of E-cadherin expression and inhibition of the *de novo* expression of α-SMA in cultured tubular epithelial cells after TGF-β1 stimulation. Importantly, in the rat model of unilateral ureteral obstruction, treatment with rapamycin attenuated tubulointerstitial damage and limited α-SMA expression and collagen deposition in the interstitium. Moreover, rapamycin decreased the infiltration of inflammatory cells and inhibited renal TGF-β1 expression (92). In rats with passive Heymann nephritis that was accelerated by severe renal mass reduction, rapamycin also was shown to ameliorate proteinuria-associated tubulointerstitial inflammation and fibrosis by preventing the overexpression of MCP-1, TGF-β, and PDGF in the kidney (93).

In vitro studies have highlighted further the causal relation and mechanisms underlying the fibrogenic reaction. Albumin challenge of cultured proximal tubular cells resulted in increased gene expression and production of TGF-β (19). Of interest, albumin upregulated TGF-β receptor type II transcription, synthesis, and surface expression in proximal tubular cells, which could amplify the matrix-stimulatory actions of TGF-β on tubular cells, thereby contributing to the development of tubulointerstitial fibrosis (94). Another *in vitro* study demonstrated that fatty acid–free albumin stimulated the accumulation of extracellular collagen type IV, laminin, and fibronectin by proximal tubular cells through a posttranscriptional mechanism (95). Finding that albumin caused an increase in the levels of tissue inhibitors of metalloproteinases 1 (TIMP-1) and TIMP-2 suggested that a decrease in degradation rather than an increase in protein expression could be responsible for the increased accumulation of extracellular matrix protein components in response to albumin load. Recent findings suggested that dose-dependent TGF-β secretion by proximal tubular cells that were exposed to albumin requires activation of mitogen-activated protein kinase signaling pathway upon albumin binding to surface receptors (96). The response would be independent of albumin endocytosis, because inhibitors of endocytosis such as simvastatin and the megalin ligand, receptor-associated protein, failed to inhibit albumin-induced TGF-β secretion.

Role of Ultrafiltration of Cytokines and Growth Factors in Proteinuric Disease

In addition to albumin, transferrin, and Ig, glomerular proteinuria results in ultrafiltration of high molecular weight precursor forms or complexes of growth factor proteins such as insulin-like growth factor 1, hepatocyte growth factor, and TGF-β1 (97). Micropuncture studies in proteinuric rats and experiments in cultured proximal tubular cells allowed documentation that growth factors become activated in tubular fluid to interact with tubular cell receptors, thereby causing secretion of collagen types I and IV, MCP-1, and RANTES. Chemokines, upon secretion in the basolateral compartment of tubular cells, also may stimulate macrophages in the renal interstitium to secrete TGF-β, which in turn is a powerful stimulus for the expression of extracellular matrix proteins by interstitial myofibroblasts (98).

Intraglomerular cytokines may originate as a consequence of inflammation and be conveyed with proteins into the tubular lumen to amplify interstitial injury. The kidney of the axolotl, a primitive amphibian, has provided a tool whereby the effects of protein toxicity can be assessed in the absence of glomerular inflammation and possibly other, concurrent or alternative mechanisms of progressive injury, such as altered glomerular hemodynamics and misdirected glomerular filtration (99). Besides closed nephrons, the axolotl kidney contains nephrons that have ciliated peritoneal funnels (nephrostomes) that exhibit free access to the peritoneal cavity (100). Injection of bovine serum or human proteins (transferrin, IgG, or LDL but not albumin) into the peritoneal cavity caused massive accumulation of proteins and lipids within intracellular droplets in tubular epithelial cells of nephrons with nephrostomes. Progressive focal accumulation of fibrous tissue was noted around protein-storing tubules, with the presence of fibronectin and TGF-β both in tubular epithelial cells and in interstitial cells. The axolotl kidney differs significantly from the mammalian organ. However, these data are in support of the possibility that tubular injury and interstitial fibrosis in response to protein loading can ensue irrespective of the presence of glomerular damage.

Role of Proteinuria in Tubular Apoptosis

Emerging evidence suggests that proteinuria causes tubular cell apoptosis. In cultured proximal tubular cells, delipidated

Table 1. Activating factors and molecular pathways underlying tubular epithelial cell dysfunction and interstitial inflammation and fibrosis in progressive proteinuric nephropathies[a]

Factor/Pathway	References
Transcription factor–mediated upregulation of inflammatory, vasoactive, and fibrogenic genes in proximal tubular cells by protein overload	
NF-κB–dependent pathways for synthesis and polarize secretion of MCP-1, RANTES, IL-8, fractalkine, ET-1	(16–18,24,25)
STAT activation	(30)
TGF-β upregulation	(19,96)
Activation of the complement cascade in proximal tubule	
upregulation and basolateral/apical secretion of C3 by proximal tubular cells in response to plasma protein overload	(20)
deposition of C3 (newly synthesized, ultrafiltered) on apical surface; activation *via* alternative pathway	(71,72,75,83)
C5b-9–mediated upregulation of extracellular matrix proteins (collagen IV, fibronectin)	(74,76)
C3a receptor–mediated TGF-β and collagen synthesis	(52)
interaction of C3 with ammonium generated as a result of excess protein degradation leading to formation of monocyte-activating products	(109)
Tubular cell activation by protein-bound circulating molecules	
release of albumin-bound chemoattractant lipid factor	(34)
IGF-1, HGF, and TGF-β receptor–mediated synthesis of collagen type I and IV, MCP-1, RANTES	(97)
Apoptotic response to protein overload	
activation of Fas pathways in proximal and distal tubular cells	(105,106)
PPAR-γ–mediated proximal tubular cell apoptosis	(102)

[a]ET-1, endothelin-1; HGF, hepatocyte growth factor; IGF-1, insulin-like growth factor 1; MCP-1, monocyte chemoattractant protein-1; PPAR-γ, peroxisome proliferator–activated receptor-γ; STAT, signal transducer and activator of transcription.

albumin induced apoptosis in a dose- and duration-dependent manner (101). This phenomenon was associated with activation of Fas-FADD-caspase 8 pathway. Peroxisome proliferator activated receptor-γ seemed to play a role in the apoptotic response to high concentrations of albumin-bound fatty acids in human proximal tubular cells (102). However, the role of albumin as an inducer of apoptosis has not been established clearly, in that the effect may not occur unless a high-protein dose is used (103,104). A recent study showed that the exposure of proximal tubular cells to a 100- to 440-kD human plasma fraction but not to the 30- to 100-kD albumin-rich fraction showed strong upregulation in the expression of Fas and Fas ligand and apoptotic response, suggesting that protein load–induced apoptosis can be mediated by higher molecular weight protein species that are filtered upon more severe permselective defect (105).

Evidence of apoptotic responses to protein load is not confined to cultured tubular epithelial cells. Kidneys of rats with albumin overload proteinuria indeed showed increased numbers of terminal dUTP nick-end labeling–positive apoptotic cells both in the tubulointerstitial compartment and in the glomeruli. Most of the positive tubular cells belonged to profiles that expressed AngII type 2 receptor. Findings of reduced phosphorylation of ERK and Bcl-2 were suggested to reflect an AngII type 2 receptor–mediated mechanism underlying tubular cell apoptosis (106). Proximal tubular cell apoptosis also was found and may contribute to glomerular–tubule disconnection and atrophy in response to proteinuria in the accelerated model of passive Heymann nephritis (107). Apoptotic cells were detected recently in both proximal and distal tubular profiles in biopsy specimens of patients with primary FSGS. In support of the pathophysiologic significance of such observation, a strong positive correlation was found between proteinuria and incidence of tubule cell apoptosis, which was identified as a strong predictor of outcome in these patients (108). The prolonged incubation of Madin-Darby canine kidney epithelial (distal/collecting) cells with albumin also resulted in the activation of the Fas pathway and apoptosis, extending to the distal nephron the pathogenetic potential of excess exposure of tubular cells to ultrafiltered proteins and of the epithelial apoptotic response (108).

Conclusion

In progressive nephropathies, severe dysfunction of the glomerular capillary barrier to circulating proteins causes protein overload of tubular epithelial cells and intrarenal activation of complement that is responsible for spreading of injury to the tubulointerstitium. These pathways are summarized in Table 1 along with other postulated mechanisms of tubulointerstitial injury. Drugs that block AngII limit the abnormal passage of plasma proteins and are renoprotective. The podocyte is the

primary site of antiproteinuric action through stabilization of podocyte–podocyte contacts and prevention of permselective dysfunction at the slit diaphragm. Although the abnormal passage of plasma proteins across the glomerular capillary wall is likely to be a factor that is responsible for further podocyte injury and progression to glomerulosclerosis (84), most of the available data highlight the mechanisms underlying proximal tubular cell activation and interstitial inflammation and fibrosis. The toxicity of albumin seems to be mediated by its initial endocytic uptake, although the importance of albumin itself *versus* protein-bound molecules in the induction of irreversible tubular damage is not clear. Other proteins, including ultrafiltered transferrin and Ig, and the intrarenal complement pathway could play a predominant role. Developments in these areas yield further support to design protocols in which drugs against secondary pathways of injury should be tested in association with drugs that limit the abnormal passage of proteins across the glomerular capillary barrier.

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