

# Proteinuria and Phenotypic Change of Proximal Tubular Cells

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Terminal renal failure is the final common fate of chronic nephropathies independent of the type of initial insult. The outstanding review by Brenner *et al.* (1) in 1982 introduced the idea that glomerular hemodynamic changes that develop as compensatory adaptation to irreversible nephron damage cause progressive deterioration of function and structure of the remaining nephrons. After reduction of renal mass in rats, remnant intact nephrons undergo sudden hypertrophy with concomitant lowering of arteriolar resistance and increased glomerular plasma flow that results from a differential decrease in vessel tone that is less in afferent than efferent arterioles. This fosters adaptive increase in glomerular capillary hydraulic pressure and more filtrate per nephron, changes, however, that are detrimental in the long term. The increase in intraglomerular capillary pressure alters the size-selective function of the glomerular barrier and causes protein ultrafiltration.

Abnormally filtered proteins have an intrinsic renal toxicity linked to their over-reabsorption by proximal tubular cells and activation of tubular-dependent pathways of interstitial inflammation and fibrosis (2,3). The functional importance of tubulointerstitial events in progressive renal disease is supported by evidence that the severity of tubular interstitial damage strongly correlates with the risk of renal failure, even better than do the glomerular lesions. The relationship between proteinuria and tubulointerstitial damage was suggested by early studies in renal biopsies of rats with age-related proteinuria (4) and adriamycin nephrosis (5), showing that protein reabsorption droplets accumulate in the cytoplasm of proximal tubular cells with focal breaks of the tubular basement membrane and extravasation of the tubular content into the renal interstitium, followed by an inflammatory reaction and tubulointerstitial lesions. Furthermore, in models of overload proteinuria, repeated injections of albumin in the rat increased glomerular barrier permeability and caused massive proteinuria (6,7) and tubular changes with heavy macrophage and T-lymphocyte infiltration into the renal interstitium (8). That excess protein reabsorption by proximal tubular cells may play a role in the development of interstitial inflammation has been documented by a recent study of our group in two different models of

proteinuric nephropathies (9). In rats with 5/6 nephrectomy, albumin and IgG accumulation in proximal tubular cells was visualized in the early stage, preceding the interstitial infiltration of major histocompatibility complex II-positive cells and macrophages. The infiltrating cells concentrated almost exclusively in regions containing IgG-positive proximal tubules and tubules with luminal casts. Similar patterns were found in the immune model of passive Heymann nephritis indicating that the interstitial inflammatory reaction develops at the sites of protein overload, regardless of the type of glomerular injury.

## Protein Traffic as a Fosterer of Tubular Cell Dysfunction

Filtered albumin and other proteins that accumulate within intracellular compartments of proximal tubular cells perturb cell function by several mechanisms. Studies have contributed to define the biochemical pathways specifically activated by excessive tubular reabsorption, and evidence is now available that protein overloading of proximal tubular cells in culture activates the transcription of a number of genes encoding for vasoactive, inflammatory, and fibrogenic molecules with potential toxic effects on the kidney.

## Protein Uptake by Proximal Tubular Cells

Proteins that have not been retained by the glomerulus are reabsorbed in the proximal tubules via processes that involve binding at the apical pole of the cells, vascular internalization, and subsequent lysosomal degradation into constituent amino acids and small peptides. The initial recognition step by receptor-mediated endocytosis involves at least two high molecular weight proteins, megalin and cubilin, which have multiligand properties and can therefore account for the wide variety of protein reabsorbed (10). Megalin—a transmembrane glycoprotein that belongs to the LDL receptor family—is the most abundant endocytic receptor in the proximal tubule epithelium, where it is concentrated in clathrin-coated pits and vesicles in the brush border region. It acts as a receptor for different ligands, including albumin, insulin, prolactin, and vitamin-binding proteins. Megalin's endocytotic function is regulated by heterotrimeric G protein-internalization signals (*Gαi3*, *GAIP*, and *GIPC*) (11) interacting with the cytoplasmic tail of megalin. This portion of the molecule also contains Src-homology domains, PDZ domains, and protein kinase phosphorylation sites, suggestive for a role of megalin in signal transduction. The potential for megalin to signal into the cell rests on the *in vitro* observation that the mitogenic effect of albumin in proximal tubular cells was mediated by the activation of phosphatidylinositol 3-kinase, which resulted in downstream

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phosphorylation and hence activation of p70 ribosomal protein S6 kinase (12,13).

Cubilin is another glycoprotein that is heavily expressed in the brush borders and intracellular endocytic compartments and that binds albumin, transferrin, IgG light chains, and receptor-associated protein (10,14,15). Cubilin is devoid of a transmembrane domain, and cubilin/ligand complexes are associated with megalin during internalization. That the cubilin internalization depends on megalin is supported by a recent report showing that cubilin-mediated endocytosis of transferrin did not operate in megalin knockout mice that excreted high amounts of transferrin (16).

### Ultrafiltered Proteins Activate Proximal Tubular Cells to Express Vasoactive and Inflammatory Mediators

High concentrations of proteins (delipidated or lipid-enriched albumin, IgG and transferrin) induced in cultured proximal tubular cells a dose-dependent increase in the generation of endothelin-1 (17), a vasoconstrictor peptide deeply involved in progressive renal injury through its effect of stimulating renal cell proliferation and extracellular matrix production and its chemotactic property for monocytes (18).

Among other chemoattractants, monocyte chemoattractant protein-1 (MCP-1) and RANTES, chemokines with potent chemotactic activity for monocytes/macrophages and T lymphocytes, were upregulated in proximal tubular cells challenged with protein overload (19,20). Notably, protein secretion was polarized mainly toward the basolateral compartment of the cell, an event that could be relevant for the tubulointerstitial inflammatory response and structural injury observed *in vivo* in proteinuric nephropathies (2). Fractalkine, a unique chemokine that combines the property of both chemoattractant and adhesion molecules (21), was overexpressed upon albumin stimulation of proximal tubular cells, in a dose- and time-dependent manner (22). The induction of fractalkine mRNA resulted in increased synthesis of both the membrane-bound and soluble forms of fractalkine protein.

Molecular mechanisms that lead to chemokine gene induction as a consequence of enhanced protein uptake are being elucidated (20,23,24). From the most recent studies, it emerges that a candidate pathway is via NF- $\kappa$ B, a transcription factor of the Rel family that comprises protein homodimers or heterodimers (25,26). The prototype NF- $\kappa$ B is composed of p50–p65 subunits. NF- $\kappa$ B exists in an inactive form in the cytoplasm of cells bound to the inhibitory protein I $\kappa$ B. NF- $\kappa$ B activation by appropriate triggers, such as cytokines, viruses, and oxidants, promotes nuclear translocation of the DNA-binding subunits after they are released by I $\kappa$ B (25,26). We found that albumin and IgG caused a dose-dependent increase in NF- $\kappa$ B activation (p50–65 subunit) in proximal tubular cells followed by the upregulation of RANTES and MCP-1, which was fully suppressed by NF- $\kappa$ B inhibitors (20,24). Moreover, adenovirus-mediated gene transfer of I $\kappa$ B $\alpha$  reduced overexpression of fractalkine mRNA transcript levels in albumin-overloaded proximal tubular cells, thus supporting a role of

NF- $\kappa$ B activation in chemokine mRNA induction (22). A recent study has documented that reactive oxygen species act as second messengers in protein overload-induced NF- $\kappa$ B activation (24). Albumin and IgG dose-dependently elicited a rapid and sustained generation of hydrogen peroxide over time in human proximal tubular cells. The antioxidants DMTU and PDTTC prevented H<sub>2</sub>O<sub>2</sub> production and almost completely abolished the enhanced NF- $\kappa$ B activity induced by both proteins. An additional proof that H<sub>2</sub>O<sub>2</sub> could activate NF- $\kappa$ B rests on the data that stimulation of tubular cells with exogenous H<sub>2</sub>O<sub>2</sub> resulted in the activation of a NF- $\kappa$ B subunit pattern similar to that obtained after protein challenge (24). It is known that in other cellular systems, oxidant generation is upstream regulated by protein kinase C (PKC) (27). In protein-overloaded tubular cells, specific inhibitors of PKC prevented H<sub>2</sub>O<sub>2</sub> generation and inhibited the abnormal NF- $\kappa$ B–DNA binding activity (24). That PKC and oxygen radical generation induced by protein overload function as critical signals for the expression of NF- $\kappa$ B–dependent genes derives from data of real-time PCR experiments showing that oxidant scavengers and PKC inhibitors almost completely abolished the upregulation of the MCP-1 gene induced by albumin.

Evidence that proteinuria may determine the activation of transcription factors and the overexpression of chemokines *in vivo* is available both for experimental and human progressive nephropathies. In rats with protein-overload proteinuria, a model with interstitial inflammation and tubular upregulation of MCP-1 and osteopontin (28), NF- $\kappa$ B activity was increased, being mainly localized in tubular epithelial cells (29). In rats with 5/6 nephrectomy, we found that increased urinary protein excretion over time was associated with a remarkable increase in NF- $\kappa$ B activity in remnant kidneys (30). Intense nuclear staining for the p50 NF- $\kappa$ B subunit was localized to proximal tubular cells. NF- $\kappa$ B activation was paralleled by upregulation of renal MCP-1 gene expression, with strong signals being detected in tubular epithelial cells and to a lesser extent in interstitial infiltrating cells. MCP-1 mRNA upregulation preceded the accumulation of monocytes/macrophages and T lymphocytes in the remnant kidney interstitium, suggesting that in this model the initial mononuclear cell recruitment may occur at least in part by MCP-1–dependent mechanisms. In other models of proteinuric nephropathies, renal MCP-1 overexpression has been shown to precede or coincide with the infiltration of mononuclear cells into the renal interstitium (28,31). Moreover, administration of a neutralizing anti-MCP-1 antibody to rats with tubulointerstitial nephritis significantly decreased macrophage infiltration, supporting the possibility that MCP-1 is functionally important in eliciting interstitial inflammatory responses (31).

If the interstitial inflammatory reaction is indeed a feature of protein overloading, then limiting the enhanced protein traffic should also limit the biologic effect of excessive tubular protein reabsorption and slow renal disease progression. The best strategy to date in experimental animals and humans to reduce protein traffic rests on angiotensin-converting enzyme (ACE) inhibitors, which additionally limit renal injury (32–38). Administration of ACE inhibitor to rats with remnant kidneys

reduced urinary protein excretion, almost completely suppressed NF- $\kappa$ B DNA-binding activity, and reduced MCP-1 mRNA expression (30). The accumulation of mononuclear cells in the renal interstitium was also greatly limited by ACE inhibitor. Similar data were obtained in an immune model, passive Heyman nephritis (30). In fact, in rats with passive Heyman nephritis, proteinuria over time was associated with a remarkable increase in renal NF- $\kappa$ B activity, which was normalized by the early administration of ACE inhibitor. The decrease of NF- $\kappa$ B activation was associated with downregulation of MCP-1 expression and reduction of interstitial inflammation. Mezzano *et al.* (39) by analyzing renal biopsy specimens from patients with severe proteinuria detected NF- $\kappa$ B activation in tubular epithelial cells, which significantly correlated with the magnitude of proteinuria. There was a concomitant upregulation of proinflammatory chemokines MCP-1, RANTES, and osteopontin found mainly in tubular epithelial cells, with a stronger expression in patients with a progressive disease.

### Complement as a Pathogenic Component of Proteinuria

Among secondary processes that lead to interstitial damage in proteinuric conditions, the activation of complement proteins in the proximal tubule has proinflammatory potential and thus a major role to play (40,41). Complement components can be filtered across the glomerular barrier, and deposits (C3, C5b-9) were found both along the luminal side and within proximal tubular cells in kidneys of rats in which the disease was induced by protein overload, renal mass ablation, and aminonucleoside (8,41,42), a pattern also observed in kidneys of patients with nonselective proteinuria. Complement-depletion studies by Matsuo's (40,41) and Couser's (43) groups, using acute proteinuric models of glomerular injury, provided direct evidence for deleterious effects of urinary complement components on the tubulointerstitium in a relatively short period. In rats with proteinuria of glomerular origin as a result of 5/6 renal mass ablation, we found intracellular C3 staining in proximal tubules at 7 d after surgery in a stage closely preceding the appearance of inflammation (44). C3 colocalized with IgG to the same tubules in adjacent sections. Protein accumulation in proximal tubular cells was followed by interstitial infiltrates of major histocompatibility complex II-positive cells and ED-1 monocytes/macrophages that were first detectable in the peritubular interstitium at 14 d and then more evident at 30 d. More important, in double-stained sections, the infiltrating cells concentrated almost exclusively in regions containing C3-positive proximal tubules. Treatment with ACE inhibitor while preventing proteinuria limited both tubular accumulation of C3 and IgG and interstitial inflammation.

C3 is an essential component of both the classical and alternative pathways of complement activation (45). *In vitro* evidence is available that proximal tubular cells activate complement via the alternative pathway leading to fixation of the C5b-9 MAC neoantigen on the cell surface (46). This, in addition, was followed by cytoskeletal alterations, superoxide

anion and hydrogen peroxide production, and synthesis of proinflammatory cytokines such as IL-6 and TNF- $\alpha$  (47). Although the generation of C5b-9 on the apical surface can contribute to functional impairment of proximal tubular cells linked to proteinuria, the C3 component may have additional and independent proinflammatory actions in proteinuric settings. Actually, we found evidence of granular C3 staining in the basolateral region of proximal tubular cells of remnant kidneys, in addition to intracellular sites consistent with subapical and lysosomal compartments (44). Such reactivity together with the linear peripheral C3 staining may reflect polarized secretion of newly synthesized C3 into the interstitium, a pathway that would reinforce the role of proximal tubular cells as a trigger of tubulointerstitial injury. *In vitro* studies showed that proximal tubular epithelial cells synthesize complement components, including C3 (48). Moreover, exposure of human proximal tubular epithelial cells in culture to total serum proteins (49) or transferrin (50) at the apical surface upregulated C3 mRNA expression and enhanced the secretion of the protein predominantly at the basolateral site, providing *in vitro* evidence to suggest a role for locally synthesized complement in the process of tubulointerstitial damage.

More direct *in vivo* evidence for a role of complement in late stages of the disease has been provided by a recent study (51) using C6-deficient PVG rats with 5/6 nephrectomy. A marked improvement of tubulointerstitial injury and renal function with respect to normocomplementemic rats was in fact demonstrated in the remnant kidney when the ability to form C5b-9 was abrogated. It was suggested that treatment to reduce C5b-9 attack in tubular cells may slow disease progression and facilitate recovery of renal function (51).

### Protein Overload Activates Fibrogenic Pathways in Proximal Tubular Cells

Interstitial fibrogenesis can be viewed as a multifactorial process mediated by a diversity of biologically active molecules, such as cytokines, growth factors, and vasoactive substances, resulting in abnormal accumulation of extracellular matrix collagen types, fibronectin, laminin, and other components. However, how progression factors can lead to fibrogenic responses is less well understood. Studies in experimental models indicate that the interstitial accumulation of myofibroblasts (52,53) and phenotypic changes of tubular epithelial cells (54–56) play major roles. In this context, there is evidence to suggest that the excess exposure of proximal tubular cells to ultrafiltered proteins can contribute to initiate and/or enhance fibrogenesis by at least two pathways. First, as implicated by the results of several studies that have been mentioned above, the proinflammatory activation of the tubular cells promotes local recruitment of macrophages and lymphocytes, which in turn via release of TGF- $\beta$ , PDGF, and other cytokines (57) can stimulate the transformation of interstitial cells into myofibroblasts. Furthermore, the proximal tubular epithelial cells can interact with surrounding interstitial fibroblasts directly to promote fibrogenesis via paracrine release of profibrogenic molecules. This has been suggested by the *in vitro*

observation that proximal tubular cells, presumably by virtue of ability to synthesize PDGF-AB and TGF- $\beta$ 1, stimulate renal cortical fibroblasts in co-culture to grow and synthesize collagen (58). That both the inflammatory cell-dependent pathway and the tubular paracrine pathway can be activated *in vivo* has been recently documented by evidence that in remnant kidneys of rats, after the onset of the proteinuric stage, cells expressing the myofibroblast-associated marker  $\alpha$ -smooth muscle actin ( $\alpha$ -SMA) in the interstitial areas colocalize with macrophages surrounding proximal tubular cells that were engaged in excess protein reabsorption. Importantly, the finding that TGF- $\beta$  mRNA was upregulated in proximal tubular cells since 14 d after surgery, in concomitance with the accumulation of the inflammatory cells and the  $\alpha$ -SMA-positive myofibroblasts, was consistent with the effective induction of a fibrogenic response by protein overreabsorption (59). Evidence of focal  $\alpha$ -SMA expression in proximal tubuli in a subsequent stage at 30 d supports the possibility that the same pathway may play a role in the epithelial-mesenchymal transformation, or trans-differentiation, of proximal tubular cells. TGF- $\beta$  has been shown to act as a single factor in inducing  $\alpha$ -SMA expression in proximal tubular cells in culture (60). Of major interest, high concentration of albumin did upregulate TGF- $\beta$ 1 mRNA expression in proximal tubular cells (61). Other mediators in this response include PDGF (62) as well as endothelin-1, which was shown to activate  $\alpha$ -SMA gene expression (63). Moreover, complement components and protein-bound molecules in the ultrafiltrate, such as hepatocyte growth factor or TGF- $\beta$ 1 itself (64), may further contribute to the induction of fibrosis *in vivo*. Finally, together with the concomitant inhibitory effects on excess protein accumulation and interstitial inflammatory cell infiltration, the antiproteinuric action of ACE inhibitor was associated with abrogation of abnormal TGF- $\beta$ 1 gene expression in tubular cells and of myofibroblast formation (59). It seems likely that if the primary site of ACE inhibitor's action were at the level of glomerular permselective barrier as suggested by several studies (65–68), then excess proteins in the ultrafiltrate act at least as a contributory factor to elicit the interstitial fibrogenic events that can be prevented by the antiproteinuric action of the drug. Importantly, the detection of abnormal  $\alpha$ -SMA-positive cells into the interstitium can be used as a morphologic predictor of progression in both experimental and human nephropathies (69–71), further indicating that the prevention of myofibroblast formation can be an instrumental step in the ACE inhibitor's renoprotective action.

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