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Why is proteinuria an ominous biomarker of progressive kidney disease?

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Why is proteinuria an ominous biomarker of progressive kidney disease? Progressive tubule injury and interstitial fibrosis frequently accompany glomerulopathies associated with proteinuria. Clinical experience indicates that higher levels of proteinuria prior to, as well as after initiation of treatment predict more rapid decline in renal function and more pronounced tubulointerstitial injury.

It has been proposed that filtration of potentially tubulotoxic plasma proteins is responsible for the observed correlations between proteinuria and progression (i.e., proteinuria is a cause and not only a consequence of progressive renal injury). Numerous attempts have been made to identify the species of putative tubulotoxic proteins in this progressive injury process, but much uncertainty persists. These uncertainties stem from nonphysiologic exposure of apical cell surfaces to proteins in vitro, the extremely high concentrations of various proteins tested in vitro, and the nonuniformity of end points measured. Furthermore, there is often a lack of correlation between in vitro and in vivo findings, and a lack of uniformity of results even for seemingly similar in vitro experiments.

Less controversy is evident in the potential pathways whereby injured tubules evoke a tubulointerstitial inflammatory and fibrotic response, with many in vivo models serving to incriminate excessive cytokine and chemokine production, infiltration of various inflammatory cells, and the balance between apoptosis and cell proliferation.

Despite many years of concerted efforts, we believe it is still unclear whether proteinuria is a cause (and if so, which species of protein), or only a consequence of progressive renal injury. Nevertheless, pending the resolution of these uncertainties by more decisive and unambiguous experimentation, the strongly predictive inverse relationship between level of proteinuria and long-term renal survival currently justifies aggressive antiproteinuric treatment strategies, with a goal of reducing protein excretion rate to the lowest level possible without the induction of symptoms or undue risk.

Chronic renal injury, irrespective of cause, often relentlessly progresses to end-stage renal disease (ESRD). Proteinuria is a common feature of chronic nephropathies, and in otherwise comparable groups of patients, those with higher levels of proteinuria are at greater risk of ESRD [1–3]. The recognition within the past two decades that proteinuria is an independent predictor and a possible contributor to progression rather than a mere marker of the severity of glomerular damage represents a major change in our concepts of progressive kidney disease. Furthermore, in studies of diabetic and nondiabetic kidney disorders, early reduction in proteinuria is associated with slower progression of chronic kidney disease (CKD) [4–9]. In the Ramipril Efficacy In Nephropathy (REIN) study, the severity of initial proteinuria was associated with more rapid decline in glomerular filtration rate (GFR) [10, 11]. A recent analysis of the REIN study data showed that the level of residual proteinuria was also a predictor of CKD progression, irrespective of blood pressure control and treatment randomization [4]. Furthermore, both the Modification of Diet in Renal Disease (MDRD) study and a recent meta-analysis of 11 studies involving 1860 patients with nondiabetic proteinuric renal disease demonstrated that a reduction in urine protein excretion was independently associated with a lower risk of progression of CKD [12, 13]. Data from the Reduction of End Points in NIDDM with Angiotensin II Antagonist Losartan (RENAAL) study also showed that the baseline urine albumin/creatinine ratio was the strongest independent predictor of reaching the combined end point of doubling of baseline serum creatinine concentration or ESRD among type 2 diabetic patients with nephropathy [14], and again, the level of residual proteinuria during therapy was as strong a marker of disease progression as baseline proteinuria [9]. Finally, there is an increasing body of evidence suggesting that proteinuria is also a cardiovascular risk marker [15, 16]. Based on these results, it is now widely accepted that reduction in proteinuria is both renoprotective and cardioprotective and, thus, must be considered as an independent therapeutic goal in the management of patients with CKD.

The most intuitive and obvious explanation for the relationship between proteinuria and progression of CKD

Key words: albuminuria, tubulointerstitial fibrosis, albumin filtration, protein uptake, proximal tubule cells, megalin, cubulin.

is that the magnitude of proteinuria is merely a reflection of the severity of the underlying glomerular disease. However, it has also been suggested that proteinuria directly contributes to renal injury and, in particular, to tubule and interstitial pathology. Three general mechanistic pathways have been suggested: one or more species of filtered proteins (*1*) directly cause tubule damage and apoptosis; (*2*) activate cellular response(s) that lead to interstitial injury; (*3*) stimulate production of fibrogenic molecules, such as transforming growth factor-β (TGF-β) or endothelin-1 (ET-1).

PROTEIN FILTRATION AND UPTAKE

Protein filtration

Each day more than 60,000 g of protein normally courses through the kidney, with less than 150 mg appearing in the final urine. The latter value refers to intact proteins only, as measured by readily available laboratory methods. If nonintact or partially degraded proteins are also considered, the protein content of the final urine has been estimated to be as high as 2 to 4 g/day [17, 18]. In this review we will consider intact proteins only, as evaluated by routine laboratory methods.

Under physiologic conditions low-molecular-weight (LMW) proteins (molecular weight of less than 40 kD and radius less than 30 Å) are freely filtered with reported glomerular sieving coefficients (GSC) of 0.75 to 0.99 [18–20]. In contrast, filtration of high-molecular-weight (HMW) proteins (molecular weight of more than 100 kD and radius greater than 55 Å) is almost completely restricted [20]. As for filtration of the intermediate-molecular-weight proteins, and albumin in particular, early micropuncture studies reported albumin concentration in proximal tubule fluid to range from 0.3 to 73 mg/dL [21–27]. More recently, Tojo and Endou found the concentration of albumin in the glomerular filtrate of rats to be 2.3 mg/dL with a GSC of 0.00062 (with average plasma albumin concentration of 3.7 g/dL) [18]. Low filtrate albumin concentration is consistent with the classic concept of glomerular permselectivity, based on molecular size, shape, and net charge [28]. However, other recent studies have estimated the amount of filtered albumin in rats to be much higher with a GSC of ~7.5%, which the authors extrapolated to a daily filtration of more than 400 g of albumin in humans [29, 30]. These studies employed cytotoxic chemicals to inhibit tubule protein reabsorption, and may also have unintentionally led to loss of normal glomerular barrier integrity. In support of this possibility, when tissue cooling was used to inhibit tubule protein uptake, Ohlson et al obtained results entirely consistent with the classic concept of glomerular permselectivity, and found a GSC for albumin of 0.0019 (corresponding to glomerular filtrate albumin concentration of about 7.0 mg/dL) [31]. In addition, a recent study measured the glomerular sieving coefficients of 12 different plasma proteins in human subjects with severe impairment of proximal tubule protein absorption due to early Dent's disease, Lowe's syndrome, or autosomal-dominant idiopathic Fanconi syndrome. The glomerular sieving coefficients for intact albumin and immunoglobulin G (IgG) were found to be 0.000077 and 0.000042, respectively. The average concentration of albumin in the glomerular filtrate was thus calculated to be ~0.35 mg/dL, which amounts to about 600 mg/day [32]. When added to estimates of nonintact albumin of ~1300 mg/day, we can infer that normal daily albumin filtration in humans does not exceed ~2000 mg/day (corresponding to ~1.1 mg/dL) [17]. An even lesser filtered quantity (more like 600 mg/day) emerges from the data in Fanconi syndrome where disordered apical uptake would be to preclude albumin uptake and degradation.

Albumin concentrations in glomerular filtrate in proteinuric conditions have also been estimated. In a micropuncture study of normal and puromycin aminonucleoside-induced nephrotic rats, the average intact albumin concentration of proximal tubule fluid in normal rats was ~2.5 mg/dL, whereas in nephrotic rats it ranged from 15.7 mg/dL to 233 mg/dL [33]. In another study in rats with immunologically mediated glomerular disease the proximal tubule intact albumin concentration averaged 7.4 mg/dL (up from 0.6 mg/dL in normal rats) [22].

Glomerular proteinuria has been divided into selective and nonselective categories based on the selectivity index (SI) [34]. Proteinuria with a ratio of IgG (a HMW protein) to transferrin (or albumin) of less than 0.1 is considered selective, whereas a ratio of more than 0.5 is considered nonselective [35]. For a long time the selectivity index of proteinuria was used to differentiate minimal change disease (with highly selective proteinuria) from focal segmental glomerulosclerosis (with nonselective proteinuria). Two recent studies showed a direct relationship between SI and the extent of tubulointerstitial damage, as well as the likelihood of future progression of renal disease [36, 37].

Protein uptake by proximal tubule cells

Oliver et al were among the first to appreciate that "when proteins pass the glomerular filter they are in part directly absorbed by the epithelial cells of the proximal convolution" [38]. Later, Straus, by using differential centrifugation, morphologic techniques, and horseradish peroxidase (as a probe protein), showed the segregation of absorbed proteins in endosomes (phagosomes) following their endocytic uptake by proximal tubule cells. He further demonstrated that subsequent fusion of these endosomes with lysosomes result in formation of phagolysosomes in which the absorbed proteins are ultimately

digested by acid hydrolyses [39–45]. These concepts received further support from Maunsbach, who showed that ^{125}I-labeled endogenous albumin injected into a single rat proximal tubule lumen is taken up by the proximal tubule cells, thereby providing further information regarding pathways of uptake and lysosomal digestion [46, 47].

The molecular mechanisms involved in the efficient tubule absorption and processing of filtered proteins have recently been elucidated. Megalin (gp 330) and cubulin, two multiligand endocytic receptors, bind together with high affinity and colocalize in many tissues, including renal proximal tubule cells, where they are significantly expressed in the brush border and the apical endocytic apparatus [48]. Megalin, a 600 kD transmembrane protein and a member of the low density lipoprotein (LDL) receptor family [49], is widely expressed in different cells, including podocytes, thyroid and parathyroid cells, type II pneumocytes, and endometrial cells, among others. Cubulin (intestinal intrinsic factor cobalamin receptor) is a 460 kD membrane protein which is believed to rely, at least in part, on megalin for internalization. Both megalin and cubulin are involved in protein absorption by proximal tubule cells, capable of binding albumin, hemoglobin, and immunoglobulin light chains [50]. In addition to their common ligands each has its own specific group of ligands (reviewed in [50, 51]).

A wealth of knowledge regarding specifications and functional characteristics of these receptors has emerged from studying megalin-knockout mice and dogs without functional cubulin [52, 53]. A study of five adult megalin-deficient mice showed increased proteinuria, particularly LMW proteinuria, and a threefold increase in urinary albumin/creatinine ratio (31 ± 12 vs. 11 ± 6 μg/mg in wild-type). In addition, dogs with functional cubulin deficiency showed a sevenfold increase in urinary albumin/creatinine ratio above wild-type [54].

POTENTIAL MECHANISMS OF PROTEINURIA-INDUCED TUBULE CELL INJURY

Misdirected filtration

This mechanism proposes that podocyte injury causes adhesion of the glomerular tuft to Bowman's capsule, followed by accumulation of the "misdirected" filtrate outside of Bowman's space and into the periglomerular space, which is then separated from the interstitium by a "layer of sheet-like fibroblast processes," ultimately resulting in glomerulosclerosis [55–60]. It further proposes that most damaged tubules are served by damaged glomeruli upstream. Similar adhesions were noted in up to 90% of glomeruli of rats recovering from puromycin aminonucleoside nephrosis (PAN) [61]. The extension of this process to involve the proximal tubule and its surrounding interstitium has been thought to result in the separation of proximal tubule cells from their adjacent basement membrane, with eventual tubule atrophy and interstitial fibrosis. This was first demonstrated in models of focal segmental glomerulosclerosis (FSGS), namely Fawn-hooded and Milan normotensive rats [59, 60]. Similar lesions were also noted in post-mortem kidney tissue samples from patients with FSGS and diabetic nephropathy [60], and in a rat model of adriamycin-induced nephropathy [62]. Further support for this hypothesis has come from tracer studies in rat models of FSGS demonstrating the presence of the tracers lissamine green, and horse spleen ferritin within tuft adhesions to Bowman's space, periglomerular space, at the glomerulotubular junction, and in peritubular subepithelial spaces, providing further evidence for a role for misdirected filtration of plasma proteins in renal injury [63].

Luminal obstruction by protein casts

Tissue samples from kidneys associated with heavy proteinuria often demonstrate dilated tubules filled with proteineous casts. In adriamycin-induced proteinuric rats, cast formation was followed by tubule obstruction, breakage of tubule basement membranes, extravasation of tubule contents, and finally interstitial inflammation, and fibrosis [64, 65]. Furthermore, tubule atrophy (both proximal and distal to the obstruction) has been reported following proximal tubule obstruction by wax placed by micropuncture [66, 67]. However, the extent to which intraluminal casts contribute to tubule injury in proteinuric nephropathies is unknown, mainly due to lack of ability to prevent cast formation [62, 68]. Of note, the availability of mice with germ-line deletion of Tamm-Horsfall protein (uromodulin), a protein thought to be required for cast formation, could prove helpful in resolving the role of cast formation in tubulointerstitial injury in proteinuric conditions [69, 70].

Tubule uptake of filtered proteins

Oliver et al suggested that protein droplets in proximal tubule cells of kidneys with glomerular diseases might be due to abnormal proximal tubule absorption and degradation of filtered proteins (e.g., due to excessive filtration and an overwhelming of tubule mechanisms) [38]. A relationship between proteinuria and tubulointerstitial damage through *excessive* uptake by proximal tubule cells was proposed in the studies evaluating renal biopsies of rats with proteinuria due to advanced age [71] or adriamycin nephrosis [64]. These biopsies showed that the excessive amounts of filtered plasma proteins accumulated in the cytoplasm of proximal tubule cells, with eventual focal extravasation of cell contents into the neighboring interstitium, followed by an inflammatory reaction, and tubulointerstitial fibrosis [64, 71, 72]. In

addition, in studies of rats with overload proteinuria [73–75], interstitial infiltration of macrophages and T lymphocytes was noted [75] and in two models of proteinuric renal disease (5/6 nephrectomy and passive Heymann nephritis), interstitial inflammation was localized to the areas where proximal tubule cells contained IgG; this was preceded by albumin and IgG accumulation in these cells [72, 76]. Further support for this notion has come from studies using intraperitoneal protein injection in the axolotl (*Ambystoma mexicanum*), a primitive amphibian that has some open nephrons with ciliated peritoneal funnels (nephrostomes) connecting peritoneal cavity to proximal tubule lumen close to the glomerulus. Following intraperitoneal injection of fetal bovine serum (FBS), protein droplets were seen in tubule cells of these open nephrons along with significant peritubular accumulation of fibrous tissue. Other nephrons not open to the peritoneum failed to show these changes [77].

Nagase analbuminemic rats (NAR) offer further insight into the potential role of albumin versus other plasma proteins in mediating tubulointerstitial injury. Utilizing these rats, Osicka et al showed that the tubulointerstitial injury following the induction of PAN and antiglomerular basement membrane glomerulonephritis was similar to that seen in control albumin-sufficient Sprague-Dawley rats, thereby arguing for a pathogenetic role for nonalbumin proteins [78].

Proteinuria is a common but variable feature in patients with Imerslund-Grasbeck syndrome, an autosomal-recessive inherited cubulin deficiency which manifests as asymptomatic proteinuria from childhood onwards. These patients also exhibit low serum vitamin B_{12} levels, megaloblastic anemia, and neurologic symptoms [79, 80]. Treatment with vitamin B_{12} corrects the latter with no effect on proteinuria [80]. In a long-term follow-up study of 14 affected patients (including 10 from the original group reported in 1960), 10 had significant constant proteinuria (ranging from 300 to 1500 mg/day, average 750 mg/day), consisting mainly of low- and intermediate-molecular-weight proteins, with albumin making up about two thirds of the proteins. These levels of albuminuria add further support to the evidence cited above that under physiologic conditions albumin filtration is less than 2 to 4 g/day. Of note, during the follow-up of one to 39 years, the amount of proteinuria did not increase, hypertension did not develop, and all but two maintained normal kidney function (based on serum creatinine and creatinine clearance values) [79]. Another recent study has confirmed these data, and again showed normal kidney function despite consistency of proteinuria (mainly albuminuria) in these patients [81]. The relatively brief duration of heavy albuminuria in minimal change disease may be insufficient to evoke a "cytokine" response and, thus, explain the relative absence of tubulointerstitial changes in the classic form of steroid-sensitive, albeit, relapsing minimal change disease. It should also be mentioned that there are rare case reports of long lasting nephrotic range albuminuria without deterioration in kidney function or interstitial fibrosis [82]. Based on these reports, it may be reasonable to infer that nonabsorbed albumin, per se, is not tubulotoxic, and that only when uptake by tubule cells is excessive and chronic (due to chronic excessive filtration), does tubule cell injury occur. Furthermore, since nonalbumin proteins are present in the filtrate and urine of patients with cubulin deficiency, their effects on the tubule, at least in the normal quantities present, do not appear to be injurious.

WHAT IS THE POTENTIAL TUBULOTOXICITY OF SPECIFIC PLASMA PROTEINS IN VITRO?

In vitro studies of the apical uptake of protein by proximal tubule cells maintained in culture employed media protein concentrations vastly in excess of those found in proximal tubule fluid in vivo, and even far in excess of proximal tubule fluid values found in animals whose urine protein concentrations, are in the high nephrotic range. In addition, it is noteworthy that proximal tubule cells are routinely grown to confluence in media containing high protein concentrations (5% to 10% fetal calf serum or fetal bovine serum corresponding to ~250–500 mg/dL, respectively). Although there is usually a period of "growth arrest" with no serum added to culture media for 24 to 48 hours prior to stimulation with protein(s) under investigation, it is still possible that such high concentrations of serum proteins and/or growth arrest might have altered cell phenotype and function and, thus, the outcome of experiments. Indeed, it has recently been shown that when more physiologic cell culture conditions are employed (i.e., protein added to basolateral but not apical surfaces), cellular morphology and function differ drastically from patterns seen with protein added to both cell surfaces [83]. Unfortunately, all the studies reported below were performed under such nonphysiologic cell culture conditions (Fig. 1).

Albumin

Representative studies of albumin uptake by proximal tubule cells in culture are summarized in Table 1. While contamination with bacterial endotoxin [lipopolysaccharide (LPS)] was not rigorously excluded in all studies, it should be noted that in the majority of studies cited, the level of LPS was less than 0.1 ng/mg of albumin. In one study, high concentrations of albumin induced apoptosis in a proximal tubule cell culture model, whereas similar concentrations of transferrin and IgG failed to do so; the results were similar using either native or delipidated bovine serum albumin (BSA) [84]. In two other studies, pure yeast recombinant human serum albumin induced

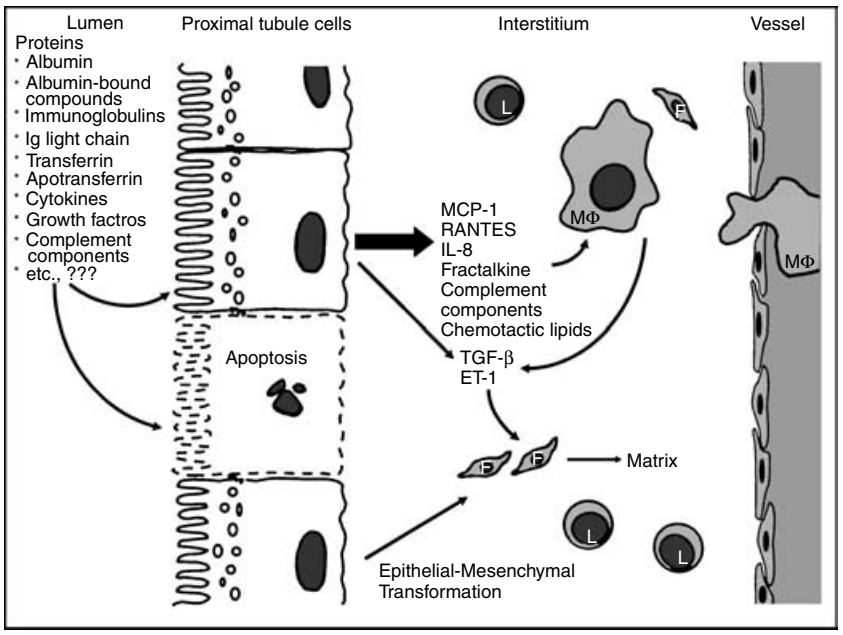

Fig. 1. Schematic summary of some of the pathways and mediators that are believed to be involved in proteinuria-induced tubulointerstitial injury. RANTES, regulated upon activation, normal T cell expressed and secreted; IL-8, interleukin-8; MCP-1, monocyte chemoattractant factor-1; ET-1, endothelin-1; TGF-β, transforming growth factor-β; MΦ, macrophage; L, lymphocyte; F, fibroblast; E, endothelial cell.

cell proliferation through phosphatidylinositide 3-kinase (PI 3-kinase)- and extracellular-signal-regulated kinase (ERK, a member of mitogen-activated protein kinase)-dependent pathways; of note, the response to albumin was several-fold less than the response to 10% fetal calf serum (FCS), whereas there was no response to ovalbumin. Although the maximum response was noted at albumin concentration of 100 mg/dL, significant positive results were noted with albumin concentrations as low as 10 mg/dL [85, 86]. Another study using primary culture of human proximal tubule cells showed a modest increase in thymidine uptake (a marker for cell proliferation) when exposed to 100 mg/dL normal unfractionated human serum. Further studies showed similar results following exposure to a similar high concentration of a plasma fraction of molecular weight of 40 to 100 kD. This is the fraction that contains albumin and transferrin; TGF-β was not detected in this fraction. However, use of pure human albumin (100 mg/dL) and pure partially iron saturated transferrin (5 mg/dL, similar concentration to unfractionated serum) failed to reveal evidence of proliferation. In addition, the fraction containing LMW proteins, including most of the cytokines and growth factors, also failed to have an effect on proliferation [87]. A similar study failed to show an increase in basolateral fibronectin (an extracellular matrix protein) production by proximal tubule cells exposed to albumin, whereas exposure to serum did provoke an increase. Addition of the serum was associated with increased lactate dehydrogenase (LDH) level, suggestive of cytotoxicity [88].

A group of studies using proximal tubule cells in culture showed that high concentrations of albumin induced the expression of chemokines RANTES (regulated upon activation, normal T cell expressed and secreted), MCP-1 (monocyte chemoattractant protein-1), and fractalkine via an NF-κB (nuclear factor kappa B)-dependent pathway subsequent to generation of intracellular reactive oxygen species (ROS) [89–92]. In addition, this up-regulation was associated with increased basolateral release of chemokines [90]. In one of these studies the renal level of fractalkine mRNA was increased 2.3-fold in mice with BSA-overload proteinuria [89]. In a recent study, albumin up-regulated interleukin-8 (IL-8) production by human proximal tubule cells in a time- and concentration-dependent (125 to 2000 mg/dL) manner. This up-regulation was again subsequent to intracellular ROS generation and NF-κB activation. Of note, boiled human serum albumin (HSA), trypsin-digested HSA, and IgG, at the concentration equal to intact HSA (1000 mg/dL), failed to elicit similar results, whereas transferrin induced a positive, albeit lesser, effect than albumin. Interestingly, exposure of cells to similar concentrations of albumin at their basolateral side also induced IL-8 production, although to a significantly lesser extent [93].

Lipids and fatty acids bound to albumin

It is well known that nephrotic-range proteinuria is associated with hyperlipidemia, and it has been shown that fatty acid content of albumin dramatically increases under nephrotic conditions [94], thereby exposing proximal tubule cells to large amounts of fatty acid-loaded albumin. The role of fatty acids in tubulointerstitial injury was first proposed in a rat model of overload proteinuria in which the urine of the rats contained a chemotactic

Table 1. Examples of studies investigating the effects of albumin on proximal tubule cells

Reference number	Protein(s) used	Concentration used *mg/dL*	Minimum concentration needed to elicit the response *mg/dL*	Concentration elicited the maximum response *mg/dL*	Duration of exposure for maximum response	Model used	Outcome studied	Result
84	BSA (lipidated)/BSA (delipidated)	0, 500, 1000, or 2000	500	2000	72 hours	LLC-PK1 cell culture	Apoptosis	+
85, 86	rHSA	0.1, 1, 10, or 100	10	100	26 hours	OK cell culture	Cell proliferation	+
89	HSA	100, 500, or 1000	100	1000	15 hours	Human Proximal tubule cells (HK-2)	Fractalkine production	+
90	BSA	100,1000, or 3000	1000	3000	24 or 48 hours	LLC-PK1 cell culture	RANTES production	+
90	FA-free BSA	1000	N/A	N/A	48 hours	LLC-PK1 cell culture	RANTES production	+
91	HSA	0, 100, 1000, or 3000	1000	3000	5 minutes	Human Proximal tubule cells (HK-2)	NF-κB activation	+
92	Delipidated BSA	0, 100, 250, 500, 1000, or 1500	250	1500	2 hours	Primary culture of rat PTC	MCP-1 production	+
93	HSA	0, 125, 250, 500, 1000, or 2000	125	500, 1000, and 2000	6 hours (3 hours for mRNA)	Primary culture of human PTC	IL-8 production	+
131	HSA	0, 10, 100, or 1000	10	1000	24 hours	Primary culture of human PTC	ET-1, TGF-β production	+
136	BSA (lipidated)/BSA (delipidated)	0, 10, 100, or 1000	1000	1000	6 hours	Rabbit proximal tubule cells	ET-1 production	+
87	Pure human albumin	100	N/A	N/A	48 hours	Primary culture of human PTC	Cell proliferation	–
88	Pure human albumin	100	N/A	N/A	48 hours	Primary culture of human PTC	Fibronectin production	–
111	HSA	Up to 3000	N/A	N/A	Up to 24 hours	SV40 immortalized human PTC culture	IL-6, IL-8, MCP-1 production	–

Abbreviations are: BSA, bovine serum albumin; OK cell, opossum kidney cell; rHSA, recombinant human serum albumin; HSA, human serum albumin; FA, fatty acid; PTC, proximal tubule cell; RANTES, regulated upon activation, normal T cell expressed and secreted; NF-κB, nuclear factor kappa B; IL-6, interleukin-6; IL-8, interleukin-8; MCP-1, monocyte chemoattractant factor-1; ET-1, endothelin-1; TGF-β, transforming growth factor-β.

factor for macrophages that proved to be a novel nonpolar lipid derived from the metabolism of these fatty acids. In addition, culture media from proximal tubule segments exposed to high concentrations of lipid-repleted BSA also showed chemotactic activity, whereas equivalent experiments with delipidated BSA produced little such activity [95]. Similarly, in studies of an in vivo model of overload proteinuria animals injected with free fatty acid (FFA)-repleted BSA had higher levels of macrophage infiltration and tubulointerstitial damage in comparison to the groups injected with FFA-depleted BSA [96, 97]. The proximal tubule uptake of albumin, however, was similar in FFA-repleted and FFA-depleted groups [96]. In an effort to differentiate the effect of different fatty acids, Arici et al studied the effects of four different fatty acids bound to albumin (palmetate, stearate, oleate, and linoleate) on cell toxicity and fibronectin production in human proximal tubule cells in culture. Oleic acid and linoleic acid were found to be the most profibrogenic and tubulotoxic fatty acids [98]. Interestingly, it has been shown that the urine albumin load of fatty acids in human minimal change disease is significantly lower in comparison to other nephrotic conditions; the difference was more pronounced for linoleic acid, followed by oleic acid [99].

Transferrin and iron

Initial studies held iron responsible for the tubulointerstitial scarring seen in proteinuric conditions because they showed a correlation between proximal tubule cell lysosomal iron concentration and tubulointerstitial damage [100, 101]. It has been suggested that upon entering the relatively acidic milieu of proximal tubule fluid, iron can be dissociated from transferrin, resulting in ROS production and subsequent damage particularly to brush border membrane [100, 102]. In addition, upon entering proximal tubule cells (along with transferrin), reactive iron can be released from transferrin inside the lysosomes, leave the lysosomes, and enter the cytoplasm as free reactive iron, where it can stimulate ROS production and subsequent cellular injury [101]. However, later experiments by the same investigators failed to show any benefit of iron deficiency in these conditions [103]. Further studies (Table 2) showed that both apotransferrin (iron-deficient transferrin) and holotransferrin (in extremely high concentrations from 100 to 800 mg/dL) could induce MCP-1 production by proximal tubule cells [104]. In human proximal tubule cells in culture both transferrin and apotransferrin (250 to 4000 mg/dL, maximum response reached at 1000 mg/dL) induced complement C3 production by the cultured cells, whereas similar concentrations of albumin failed to provoke a positive response. However, this conclusion regarding albumin's effect is questionable because C3 production in cells incubated with serum-free medium was significantly increased after 48 hours, reaching values close to those induced by 1000 mg/dL of transferrin. Thus, it is possible that a positive response could be noted if cells were exposed to albumin for longer periods [105]. In another in vitro study of proximal tubule cells, high concentrations of transferrin induced MCP-1, IL-8, and macrophage migration inhibitory factor (MIF) expression [106].

Immunoglobulins and light chains

Several studies have investigated the effects of IgG and light chains on proximal tubule cells (Table 3) (to the best of our knowledge, studies involving IgM, IgE, and IgA have not been reported). In porcine proximal tubule cells (LLC-PK1), IgG (100 to 3000 mg/dL) induced RANTES production and its mainly basolateral release by these cells [90]. In a similar study in human proximal tubule cells (HK-2), IgG incubation (100 to 3000 mg/dL, 5 to 60 min) induced ROS, which in turn activated NF-κB and its downstream inflammatory signals [91].

Light chains are relatively small proteins with a molecular weight of approximately 30 kD and, thus, freely filtered by normal glomeruli [107]. Certain conditions, such as multiple myeloma and other myeloproliferative disorders, can result in overproduction of these light chains with their subsequent appearance in urine (Bence-Jones proteins). In addition to cast nephropathy, which is the most common renal presentation, these light chains can cause light chain deposition disease (LCDD), Fanconi syndrome, and other tubulopathies. Upon entering the lumen of the proximal tubule, light chains are taken up by proximal tubule cells via a receptor-mediated pathway involving megalin and cubulin, where they may prove toxic to these cells [108–110]. A recent study using immortalized human proximal tubule cells in culture and eight different light chains showed suppressed proliferation and increased apoptosis, and even necrosis, in exposed cells. Cells were exposed to a range of 0 to 800 μmol/L (~0–2400 mg/dL) of light chains for up to 48 hours, and were grown in a medium containing 0.5% (~25 mg/dL) fetal bovine serum (FBS). There was a difference in the effect among light chains, with some being more toxic than others. In addition, a concentration-dependent response, irrespective of the light chain type, was noted, implying the importance of protein load [107]. Another recent study evaluated the effect of light chains versus HSA on production of different proinflammatory cytokines by human proximal tubule cells. The results showed that light chains (150 to 300 mg/dL) could induce the release of proinflammatory cytokines, including interleukin-6 (IL-6), IL-8, and MCP-1 in a NF-κB–dependent manner, whereas human serum albumin (up to 3000 mg/dL) failed to do so. NF-κB inhibitors and maneuvers interfering with light chain endocytosis abolished these responses [111].

Table 2. Examples of studies investigating the effects of transferrin on proximal tubule cells

Reference number	Protein(s) used	Concentration used *mg/dL*	Minimum concentration needed to elicit the response *mg/dL*	Concentration elicited the maximum response *mg/dL*	Duration of exposure for maximum response	Model used	Outcome studied	Result
104	Apotransferrin	100, 200, 400, or 800	100	800	8 hours	Primary culture of rat PTC	MCP-1 Induction	+
104	Holotransferrin	800	N/A	N/A	8 hours	Primary culture of rat PTC	MCP-1 Induction	+
105	Holotransferrin/ apotransferrin	250, 500, 1000, 2000, or 4000	250	1000	24 hours	Primary culture of human PTC	C3 production	+
106	Transferrin	125, 250, 500, 1000, or 2000	500 (IL-8), 250 (MCP-1), 2000 (MIF)	500 (IL-8), 1000–2000 (MCP-1), 2000 (MIF)	6–12 hours (IL-8), 3 hours (MCP-1), 24 hours (MIF)	Primary culture of human PTC	IL-8, MCP-1, and MIF production	+
93	Transferrin	1000	N/A	N/A	24 hours	Primary culture of human PTC	IL-8 production	+
88	Transferrin	5	N/A	N/A	48 hours	Primary culture of human PTC	Fibronectin production	–
87	Transferrin	5	N/A	N/A	48 hours	Primary culture of human PTC	Cell proliferation	–
84	Transferrin	2000	N/A	N/A	72 hours	LLC-PK1 cell culture	Apoptosis	–

For abbreviations, see Table 1.

Table 3. Examples of studies investigating the effects of immunoglobulins or their light chains on proximal tubule cells

Reference number	Protein(s) used	Concentration used *mg/dL*	Minimum concentration needed to elicit the response *mg/dL*	Concentration elicited the maximum response *mg/dL*	Duration of exposure for maximum response	Model used	Outcome studied	Result
90	IgG	1000	N/A	N/A	48 hours	LLC-PK1 cell culture	RANTES production	+
91	IgG	0, 100, 1000, or 3000	1000	3000	5 minutes	Human PTC (HK-2)	NF-κB activation	+
108	Ig light chains (8 different types)	~0–2400 (0–800 μmol/L)	~360–1100 (119–337 μmol/L) (IC_{50})	~360–1100 (119–337 μmol/L) (IC_{50})	48 hours	SV40 immortalized human PTC culture	Apoptosis	+
112	Ig light chains (6 different types)	~0, 75, 150, 300, 600, or 1200	~60	~1200	6 hours	SV40 immortalized human PTC culture	IL-6, IL-8, MCP-1 production	+
84	IgG	2000	N/A	N/A	72 hours	LLC-PK1 cell culture	Apoptosis	–
93	IgG	1000	N/A	N/A	24 hours	Primary culture of human PTC	IL-8 production	–

Filtered cytokines and growth factors

In addition to albumin, transferrin, and immunoglobulin light chains, high-molecular-weight plasma proteins can enter the urine under nonselective proteinuric conditions. In serum, insulin growth factor-I (IGF-I) exists in 50 or 150 kD forms, preventing it from normally appearing in glomerular ultrafiltrate [112]. However, it has been detected in the urine of nephrotic diabetic rats [112, 113]. In these rats, IGF-I is biologically active and capable of increasing secretion of types I and IV collagen by proximal tubule cells in culture; this effect is partially ameliorated by IGF-I receptor antibody [113]. TGF-β and hepatocyte growth factor (HGF) are other proteins incapable of entering urine under physiologic conditions due to their high molecular weight. Receptors for both of these ligands are present on the apical surface of renal epithelial cells [114]. The majority of TGF-β in the plasma is biologically inactive due to its association with different proteins, namely latency associated peptide (LAP, 100 kD), tertiary protein complex of LAP, and latent TGF-β binding protein (LAP-LTBP, 220 kD), or α_2 microglobulin (~900 kD). If filtered in disease, transformation of the inactive to active form may occur in the tubule by increased urea concentration, urokinase, or thrombospondin-1 [114–116]. In a rat model of diabetic nephropathy, mature HGF and active and latent TGF-β were detected in early proximal tubule fluid. In the same study, incubation of mouse proximal tubule cells in culture with fluid collected from the proximal tubule of diabetic rats induced up to threefold greater expression of MCP-1 and RANTES (both at mRNA and protein levels). Incubation with recombinant human HGF and TGF-β produced similar results, whereas high glucose (450 mg/dL), BSA (10 mg/dL), rat albumin (10 mg/dL), and IGF-1 (10 nmol/L) failed to evoke positive responses [117]. Furthermore, in contrast to these findings, a large group of studies have pointed toward an antifibrotic role for HGF [118–120].

Complement

Several lines of evidence point to a role for complement in initiating tubulointerstitial damage in proteinuric states. In two studies in proteinuric rats, complement inhibitors were able to reduce tubulointerstitial damage and preserve renal function [121, 122]. In addition, piebald viral glaxo (PVG) rats unable to produce C5-9 membrane attack complex (MAC) due to a genetic C6 deficiency were protected against tubulointerstitial damage associated with proteinuria [123, 124]. Furthermore, blocking the expression of Crry, a complement regulatory protein in rats, was associated with increased tubulointerstitial damage [125, 126]. In proximal tubule cells in culture, MAC induced expression of proinflammatory cytokines IL-6 and tumor necrosis factor-α (TNF-α) [127]. Complement components, including the C5-9 complex, were detected in proteinuric urine, although the large size of this complex argues against entry via filtration. Although in certain conditions, namely human membranous glomerulopathy and rat Heymann nephritis, the source of MAC in the urine can be glomerular shedding, it has also been detected in the urine of proteinuric patients without a source in their glomeruli [126, 128]. This implies the possibility that MAC can be assembled in the lumen following glomerular filtration of individual complement components. In fact, it has been shown that apical membranes of proximal tubule cells are capable of activating the complement system directly and through the alternative pathway, further supporting this possibility [129].

HOW MIGHT FILTERED PROTEINS INITIATE TUBULOINTERSTITIAL FIBROSIS?

Effects on cytokine and chemokine production by proximal tubule cells

The infiltration of inflammatory cells following renal injury is dependent on chemotactic factors and adhesion molecules. The most potent chemoattractants are chemokines, small proteins with molecular weights of approximately 10 kD that can be produced by a variety of cells, including renal epithelial cells and activated immune cells. Chemokines are further categorized such that CC chemokines are mainly involved in chemoattraction of monocytes and lymphocytes, whereas chemokines of C-X-C family usually attract neutrophils. A host of in vitro and in vivo studies has shown induction of MCP-1 and RANTES (members of CC subfamily) by proximal tubule cells upon exposure to extremely high concentrations of proteins (Fig. 1) [90, 92, 130]. Of note, the resultant chemokine secretion was polarized mainly toward the basolateral aspect of the cell. In vitro studies have shown similar results for fractalkine, a chemokine from the C-X_3-C subfamily with both chemokine and adhesion molecule properties [89], and IL-8, a chemokine from the C-X-C family that is chemotactic for both neutrophils and lymphocytes [93]. In addition, these studies have shown that generation of ROS and activation of NF-κB are involved in the chemokine secretory process [89–93].

TGF-β is believed to be one of the mediators through which proximal tubule cells are capable of directly stimulating adjacent interstitial fibroblasts, in addition to any indirect stimulatory effect via infiltrating inflammatory cells (e.g., macrophages). An in vitro study of proximal tubule cells in culture has shown an increased expression of TGF-β in response to high concentrations of albumin (10–100 mg/dL) [131]. In a study of the rat remnant kidney model, up-regulation of TGF-β mRNA in proximal tubule cells followed the uptake of filtered proteins (IgG) by these cells [132]. In addition, a study of kidney biopsy samples from patients with heavy

proteinuria (8.4 ± 3.0 g/day) found proximal tubule cells as the main site of TGF-β synthesis and expression [133].

Endothelin-1 (ET-1) is a peptide with chemotactic (for macrophages) and pro-proliferative characteristics in addition to its ability to induce vasoconstriction [134–136]. In an in vitro study, high concentrations (100 to 1000 mg/dL) of various proteins (albumin, transferrin, and IgG) induced ET-1 expression by rabbit proximal tubule cells [136]. Further evidence supporting the role of ET-1 in the progression of renal damage has come from transgenic mice over-expressing ET-1 in their kidneys. These mice develop renal lesions despite normal systemic blood pressure. In addition, results from studies using an ET receptor antagonist support a role for ET-1 in the progression of renal scarring [137].

Effects on apoptosis

One of the pathways to tubule atrophy is apoptosis, or more accurately, an increased rate of apoptosis of proximal tubule cells relative to their rate of proliferation. A cell culture study using very high concentrations of albumin (up to 2000 mg/dL) has shown an increased level of apoptosis (in comparison with appropriate controls) due, at least in part, to a Fas-FADD-caspase 8 pathway. The authors hypothesized that apoptosis may be a means by which sublethally injured cells can be removed by either neighboring cells (in a Fas-dependent pathway) or by inflammatory cells infiltrating the adjacent interstitium, clearing the path for tubule regeneration [84]. Indeed, in proximal tubule cells in culture, activated macrophages induced apoptosis through release of an as yet unidentified soluble factor, and in the absence of direct cell-to-cell contact [138]. In addition, studies in an in vivo model of overload proteinuria (using fatty acid-carrying BSA) showed both increased tubule cell proliferation and apoptosis, with the latter being the dominant feature [97, 139]. In contrast, other studies have shown that cell proliferation predominates. Studies on human kidney biopsies from patients with proteinuric glomerulopathy (e.g., membranous nephropathy and minimal change nephropathy) have shown increased proliferation and hyperplasia of proximal tubule cells [87, 140]. Studies of proximal tubule cells in vitro have shown similar results [85–87, 141]. In an effort to reconcile the conflicting results of different studies, Erkan et al have proposed that if exposure to high protein concentrations is of short duration, proliferation will dominate and tubules will regenerate, whereas in chronic severe proteinuria apoptosis will prevail, resulting in tubular atrophy [84]. Further studies will be required to clarify this issue.

Role of inflammatory cells

Interstitial infiltration of mononuclear inflammatory cells (mainly macrophages and lymphocytes) can be seen in a variety of immune- and nonimmune-mediated kidney disorders, including remnant kidney and protein-overload proteinuric models (reviewed in [142]), where cell infiltration is thought to play a significant role in tubulointerstitial damage and fibrosis. The mechanisms involved in the recruitment of mononuclear inflammatory cells and their role in induction of fibrosis is complex and beyond the scope of this paper (reviewed in [142, 143]). Briefly, it is thought that renal tubule cells, upon exposure to high protein concentrations, produce a host of chemokines, vasoactive mediators, and adhesion molecules (vide supra), resulting in interstitial infiltration and activation of mononuclear inflammatory cells, which, if they persist, may then contribute to interstitial fibrosis. It should be remembered that infiltrating cells are not always harmful, and may in fact be beneficial by helping to repair the tissues they infiltrate [68]. Support for a pathogenetic role of mononuclear inflammatory cell infiltration has come from studies in which a reduction in infiltration has been associated with reduced renal injury and preserved renal structure and function [144, 145]. In a model of unilateral ureteral obstruction, blocking of chemokine receptor CCR-1 by an antagonist was associated with reduced infiltration of lymphocytes and macrophages and less interstitial fibrosis [146]. Recently, studies have examined the potential benefit of mycophenolate mofetil (MMF), a reversible inhibitor of de novo purine synthesis essential for lymphocyte proliferation. In the remnant kidney model, MMF reduced mononuclear cell infiltration and interstitial fibrosis without affecting the magnitude of proteinuria, glomerular hypertension, or hypertrophy [147]. In a similar study in the remnant kidney model, MMF reduced the infiltration of mononuclear inflammatory cells and abolished the progressive renal damage, again with no effect on systemic hypertension or glomerular hypertrophy [148]. However, it has been shown that MMF has favorable effects on single nephron GFR (SNGFR), glomerular injury, and magnitude of proteinuria, and therefore, the extent to which its beneficial effects can be attributed solely to its role in attenuating interstitial infiltration remains unclear [149].

CONCLUSION

Progressive tubule injury and interstitial fibrosis frequently accompany glomerulopathies associated with proteinuria. Clinical experience indicates that higher levels of proteinuria prior to, as well as after initiation of treatment predict more rapid decline in renal function and more pronounced tubulointerstitial injury. In addition, the composition of the abnormal protein excretion (e.g., IgG content) has a powerful predictive influence on progression, perhaps reflecting the greater nonselective glomerular wall damage. It has been proposed that filtration of potentially tubulotoxic plasma proteins is

responsible for the observed correlations between proteinuria and progression (i.e., proteinuria is a cause and not only a consequence of progressive renal injury). Numerous attempts have been made to identify the species of putative tubulotoxic proteins in this progressive injury process, but much uncertainty persists. These uncertainties stem from nonphysiologic exposure of apical cell surfaces to proteins in vitro, the extremely high concentrations of various proteins tested in vitro, and the nonuniformity of end points measured. Furthermore, there is often a lack of correlation between in vitro and in vivo findings and a lack of uniformity of results, even for seemingly similar in vitro experiments. Although many long-term human follow-up studies support little or no tubulotoxic potential for albumin, several in vivo and in vitro studies have been taken to suggest otherwise. For other plasma protein species, similar variability in injury potential exists. Less controversy is evident in the potential pathways whereby injured tubules evoke a tubulointerstitial inflammatory and fibrotic response, with many in vivo models serving to incriminate excessive cytokine and chemokine production, infiltration of various inflammatory cells, and the balance between apoptosis and cell proliferation.

Despite many years of concerted efforts, we believe it is still unclear whether proteinuria is a cause (and if so, which species of protein) or only a consequence of progressive renal injury. It remains possible that progression (i.e., the loss of GFR) in chronic proteinuric glomerulopathies is the direct result of pathophysiologic processes arising within the glomeruli themselves (e.g., capillary hypertension, loss of capillary surface area, mesangial expansion, etc.). In this scenario, the magnitude and compositional characteristics of the proteinuria are merely manifestations of the extent of the underlying glomerular wall injury. Alternatively, events occurring downstream from the glomerular capillary circulation, arising from altered permselectivity, may indeed be responsible for progression. These alternative views of the pathogenesis of progression are not mutually exclusive; however, experiments reported thus far do not reliably distinguish between them. It should also be noted that in vivo and in vitro experiments yielding positive findings are far more likely to be accepted for publication than those yielding negative results. Despite the publication bias, our review of available literature justifies our cautious interpretations and conclusions. Nevertheless, pending the resolution of these uncertainties by more decisive and unambiguous experimentation, the strongly predictive inverse relationship between level of proteinuria and long-term renal survival currently justifies aggressive antiproteinuric treatment strategies, with a goal of reducing protein excretion rate to the lowest level possible without the induction of symptoms or undue risk.

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Due to length restrictions, we were unable to quote and discuss every pertinent reference dealing with the subject under review. Instead, our intent has been to provide a fair and balanced summary of the main factors currently believed to be involved in the complex interaction between proteinuria and renal injury.

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