The proximal tubular cell, a key player in renal damage

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Chapter 1

General Introduction
**Chronic kidney disease**

Worldwide, increasing numbers of patients are affected by chronic kidney disease (CKD). Irrespective of the original cause, the degree of renal damage and loss of kidney function steadily progresses. A substantial part of the CKD patients ultimately progress to end-stage renal disease (ESRD) and therefore need renal replacement therapy, such as dialysis or renal transplantation [1]. In Europe the annual incidence of ESRD has raised to about 135 new patients per million of population, and this is expected to continue to rise at an annual rate of 5-8%. This is largely due to the ageing of the population, as the incidence of ESRD is higher in elderly people than in the general population, and the growing number of people with type 2 diabetes. CKD and ESRD are putting a substantial burden on global health-care resources. In Europe, dialysis alone uses about 2% of health-care budgets with only a small proportion (<0.1%) of the population needing such treatment. Also, ESRD is associated with high morbidity and mortality mainly due to cardiovascular complications. Cardiovascular mortality in patients with ESRD is 10- to 20-fold higher than in the general population and is the leading cause of death [2].

The progression of CKD is variable and depends on several risk factors. Non-modifiable factors include genetics, race, age and sex; the rate of progression of CKD is faster among patients who are elderly [3], male [4], or African-American [5]. Modifiable risk factors include systemic hypertension [6,7], proteinuria [8,9] and metabolic factors, for instance poor diabetes control in type 1 and type 2 diabetes [10]. Also hyperlipidaemia, obesity, cigarette smoking, caffeine consumption and analgesics consumption have been implicated in the initiation and progression of CKD [1]. Current treatment options for CKD are based on the control of known risk factors. The most effective interventions include the control of hypertension and proteinuria. Blockade of the renin-angiotensin-aldosterone system (RAAS) with angiotensin-converting enzyme (ACE) inhibitors and/or angiotensin type 1 (AT1)-receptor antagonists is currently the most powerful available antiproteinuric treatment [11,12]. Lipid modifications with statins have been proven to decrease the cardiovascular complications in CKD patients [13]. Despite these successful interventions, in many patients progressive loss of renal function towards ESRD still occurs. Therefore, new/additional therapies are needed. Hence, it is necessary to have a good understanding of the process of the initiation and the progression of renal damage.

**Proteinuria-induced renal damage**

In different clinical trials proteinuria is identified as an independent predictor of renal function decline and early reduction of proteinuria is associated with a slower progression of CKD [14-16]. The most intuitive explanation for the relationship between proteinuria and progression of CKD is that the magnitude of proteinuria is a reflection of the severity of glomerular damage. In other words, proteinuria is the result of pathophysiologic processes arising within the glomeruli themselves, i.e. intraglomerular hypertension, loss of capillary surface area, and altered permselectivity with consequently glomerular hyperfiltration as was proposed by Brenner [17]. However, morphological studies have demonstrated a stronger correlation between tubulointerstitial damage and poor renal outcome than between glomerular damage and poor renal outcome [18]. Also, numerous
experimental studies have shown that proteinuria itself is detrimental. Persistent proteinuria in rats induced in different ways, including overload proteinuria in which the animals are given large quantities of albumin, leads not only to glomerulosclerosis but also to interstitial inflammation and progressive tubulointerstitial fibrosis [19-22]. In vitro studies support these findings; proximal tubular cells exposed to high concentrations of protein show increased production of different chemokines, i.e. monocyte chemotactic protein 1 (MCP-1), endothelin-1, and RANTES (regulated on activation, normal T cell expressed and secreted) [23-25].

So, during the past 2 decades it has become clear that proteinuria itself can have a pathogenic role, thereby contributing to tubulointerstitial damage. Different theories exist on the potential mechanisms of proteinuria-induced tubular cell injury. The excessive reabsorption of ultrafiltered proteins by proximal tubular cells can lead to tubular damage and apoptosis/necrosis by exhaustion of the lysosomal degradation pathway and spillage of lysosomal enzymes into the cytoplasm or the production of reactive oxygen species (ROS) [8,26,27]. Some compounds can directly be toxic to the proximal tubular cells, in particular complement factors [28]. In addition, several other compounds emerging from the catabolism of reabsorbed proteins have been considered to be harmful to the tubules, including ammonia [29] and heme [30]. In response to either excessive lysosomal protein degradation or toxic compounds the proximal tubular cells produce a variety of proinflammatory and profibrotic molecules, such as transforming growth factor-β (TGF-β) or endothelin-1, that initiate peritubular inflammation and fibrosis [31-34].

**Albumin in the progression of renal disease**

Proteinuria is not only a marker of renal damage, but ultrafiltered proteins are also toxic to the kidney, thereby contributing to tubulointerstitial damage. However, it is unclear whether all proteins of the ultrafiltrate affect the tubular epithelium cells to the same extent. Albumin is one of the most important proteins found in the ultrafiltrate and in nephrotic urine.

Albumin is synthesized in the liver and is the most abundant plasma protein. It is an anionic molecule with a molecular weight of ~65 kDa. It is not essential to life, but has various important functions, including the maintenance of the oncotic pressure and blood volume, acid/base buffer functions, and the transport of different substances, like fatty acids, bilirubin, ions, drugs and hormones [35]. The urinary albumin level is an important prognostic indicator in renal disease, it is directly related to the progression of renal disease [36]. Although albumin is a large anionic molecule, it is not completely retained by the glomerular filtration barrier and ends up in the ultrafiltrate. The amount of albumin normally filtered in the glomeruli has been estimated using various techniques, including micropuncture studies in rats and dogs, and is roughly estimated to lie between 0.1 and 10 g/day in humans. Albumin is reabsorbed along the proximal tubules by receptor-mediated endocytosis, which prevents occurrence of proteinuria. Receptor-mediated endocytosis mainly occurs via specific ligand binding to the multiligand receptors megalin and cubulin [37]. Once internalized, megalin is returned to the uptake pool via recycling
endosomes, whereas albumin is degraded in the lysosomes to its constituent amino acids that are then released into the bloodstream [37].

In vitro exposure of tubular cells to high albumin concentrations activates a wide array of diverse intracellular signalling pathways [38]. The transcription factor nuclear factor kappaB (NF-κB) is activated in proximal tubular cells incubated with albumin; this drives transcriptional activation of different chemokines such as MCP-1, RANTES and fractalkine [25,39,40]. Also, other inflammatory and fibrogenic mediators are induced upon albumin exposure, including interleukin-8 (IL-8) [41], tumor necrosis factor-α (TNF-α), endothelin [24], TGF-8 and collagen [42]. Albumin can also induce cell proliferation through phosphatidylinositide 3-kinase (PI 3-kinase) and extracellular-signal-regulated kinase (ERK, a member of mitogen-activated protein kinase)-dependent pathways [43,44]. High albumin exposure may also induce changes in tubular cell expression of surface integrins [45] or, eventually apoptosis [46,47].

In vivo studies using overload proteinuria in rodents have confirmed many of these effects. For example, tubular IL-8 [41], fractalkine [40], TGF-8, osteopontin and MCP-1 [22], and also interstitial collagens [22] were all upregulated after overloading rodents with albumin.

**Lipids bound to albumin in the progression of renal disease**

Despite experimental evidence that albumin can elicit an inflammatory and fibrotic response, it has been argued that not albumin per se, but rather compounds that are bound to albumin, such as fatty acids, may be toxic to tubular epithelial cells. Long chain fatty acids, such as oleic acid and palmitic acid, are crucial intermediates in lipid metabolism. They circulate in plasma and have a very rapid turnover time. Most circulating fatty acids (over 99.9%) are bound to albumin as non-esterified fatty acids (NEFA) [48]. Nephrotic-range proteinuria is associated with hyperlipidaemia and already in 1958 Shafir et al [49] have shown that the fatty acid content of albumin is dramatically increased in nephrotic conditions. Circulating albumin normally contains < 1 fatty acid per albumin molecule, while during nephrotic syndrome albumin is loaded with 5-6 fatty acids [49]. Interestingly, in human minimal change disease – which has a relatively mild disease progression – the urine albumin load of fatty acids is significantly lower in comparison to other nephrotic conditions [50]. 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 factor for macrophages that appeared to be a lipid derived from the metabolism of these fatty acids. In addition, supernatant from proximal tubular cells that were cultured in high concentrations of lipid containing albumin showed chemotactic activity, whereas proximal tubules cultured with delipidated albumin (containing no lipids) produced little activity [34]. Similarly, in studies of an in vivo model of overload proteinuria animals injected with lipid containing albumin had more macrophage infiltration and tubulointerstitial damage in comparison to the groups injected with delipidated albumin [51,52]. Arici et al [53] have studied the effects of four different fatty acids bound to albumin in cultured proximal tubular cells. Oleic acid and linoleic acid were found to be the most profibrogenic and tubulotoxic fatty acids. Also, oleic acid complexed albumin has
been shown to induce more oxidant stress via increased mitochondrial production of ROS than delipidated albumin [54].

**Experimental models of overload proteinuria**

*In vivo*, the model of overload proteinuria is very suitable to study the effects of albumin and albumin-bound ligands on induction of renal damage. Already in the second part of the 20th century, the first reports on protein-overload appeared [55,56]. Excellent studies on the pathophysiology were performed by Weening et al [57] and Eddy et al [20]. Repeated intraperitoneal injections of albumin lead to increased transcapillary movement of albumin to the urinary space. This is accompanied with degenerative changes of glomerular epithelial cells characterized by swelling, vacuolization, increased reabsorption droplets, loss of foot processes, and lifting from the underlying glomerular basement membrane, ultimately resulting in defects in the glomerular sieving barrier. These defects allow macromolecules other than albumin to enter the urinary space as well. Within 24 hours after the first injection, proteinuria comprised of the injected albumin as well as endogenous proteins is already prominent. The extent of proteinuria and glomerular lesions are dependent on the amount of albumin administered, and it has been shown to remit approximately 48 hours after the last albumin injection [57,58]. The first published studies mainly focused on the acute events that occur within the glomerulus. However, degenerative changes were also described in tubules, including casts, cytoplasmic swelling, hyaline droplets and disruption of the brush border. Next, protein-overload has been shown to induce mRNA and protein expression of different pro-inflammatory proteins, such as IL-8 [41], RANTES [25], fractalkine [40], MCP-1, TGF-β, and osteopontin [22]. Different mitogen-activated protein (MAP) kinase pathways, at least ERK [43,59] and p38 MAP kinase [40], appear to be involved in the production of these pro-inflammatory molecules. The p38 MAP kinase pathway was activated by human serum albumin in proximal tubular cells and was involved in NF-κB-dependent transcription of fractalkine [40]. NF-κB activation in response to protein overload (in vivo and in vitro) was also shown by Gomez-Garre et al [60], and the production of different chemokines, i.e. RANTES, MCP-1, was dependent on activation of NF-κB [23,25,40]. Horita et al [61] showed an increase of glomerular vascular endothelial growth factor (VEGF) expression and its receptors, fit-1 and KDR/flk-1. Interstitial fibrosis occurring as a consequence of protein-overload has been described in detail by Eddy et al [20,22]. Interstitial fibrosis is characterized by an initial interstitial influx of inflammatory cells, i.e. mainly macrophages and T-cells, which trigger interstitial fibroblast proliferation and activation. A steadily increasing expression of renal matrix proteins (protein and mRNA level), such as collagen I, III and IV, laminin and fibronectin, was shown after protein-overload [22]. Increased production of extracellular matrix proteins leads to an accumulation and this will lead to fibrosis and ultimately to loss of renal function [20,62].

Strain and gender related differences in magnitude of the proteinuria in response to albumin overload have been reported in rats. Lawrence et al [63] have compared male and female rats from four inbred strains, i.e Wistar (albino), Sprague-Dawley (albino), DA (brown) and PVG (hooded) rats, that were injected intraperitoneal with 4.5-5.5 mg/g
bodyweight. The levels of proteinuria and glomerular damage induced varied widely, being greatest in the male Wistar and smallest in the male Sprague-Dawley. Also in mice, strain related differences in the response to albumin overload have been reported [64]. Intraperitoneal injection of albumin (for 9 days out of 11; rising from 2 mg/g on day 1 to 10 mg/g bodyweight on day 5 until day 11) lead to proteinuria in 129S2/Sv mice with significant tubulointerstitial macrophage influx, while it did not in C57BL/6J mice. This strain difference was observed in both males and females.

Most protein-overload studies have made use of heterologous albumin, i.e. albumin derived from a different species, but the few studies that used homologous albumin [20,65], i.e. derived from the same species, reported similar renal changes and an increase of proteinuria. This indicates that immunological factors do not play a major role in the induced effects. Also, Eddy et al [20] could not find glomerular deposits of immune complexes or circulating antibodies against the heterologous albumin. However, immunological effects can not be excluded.

The protein-overload model has been applied in rodents with two kidneys, but also in rodents that had undergone a unilateral nephrectomy before the start of protein-overload. Unilateral nephrectomy has been shown to enhance the effects of protein-overload. For example Eddy et al [20] showed that rats that underwent unilateral nephrectomy before protein-overload showed approximately twice as much proteinuria as rats with two native kidneys (both were injected with the same amount of albumin), although a small subset of the uninephrectomized rats developed an unusual low degree of proteinuria. In most rat studies with two native kidneys [47,52,61,66] the daily amount of injected albumin is 2 g, while studies with a unilateral nephrectomy inject 1 g daily [20,22,60,67], thus resulting in a similar albumin load of the kidney(s). In mice, most researchers used the two kidney model and injected approximately 10 mg/g bodyweight (with a bodyweight of around 20 mg this corresponds to approximately 200-250 mg albumin daily) [51,64,68,69]. However, Donadelli et al [40] injected the same amount of albumin in unilateral nephrectomized mice. Although most studies inject the albumin daily, some studies inject only 5 days a week [40,68], but this does not alter the outcome in renal damage profoundly.

**Tubulointerstitial damage**

Irrespective of the underlying cause, most chronic renal diseases will eventually show a progressive renal function decline towards ESRD via a final common pathway of tubulointerstitial damage. Tubulointerstitial damage is characterized by inflammatory cell infiltrates, loss of peritubular capillaries, tubular atrophy, and interstitial fibrosis. Proteins in the ultrafiltrate, such as albumin, but also lipids, glucose and growth factors, may injure/activate the tubular epithelial cells that consequently will produce various proinflammatory cytokines and chemokines, such as MCP-1, RANTES, fractalkine and different interleukins [25,33,40,70,71]. These in turn can attract inflammatory cells, such as monocytes and macrophages, to the renal interstitium. Macrophages can produce several factors contributing to ongoing renal damage and have an important role in the initiation and progression of interstitial damage [72]. Activated tubular epithelium cells can
undergo apoptosis leading to tubular atrophy. Resident interstitial fibroblasts and myofibroblasts proliferate in response to macrophage-derived profibrotic cytokines. Also, under the influence of TGF-β1 tubular epithelium cells can undergo epithelial to mesenchymal transformation (EMT), and migrate into the interstitium where they transform into myofibroblasts [73]. Activated fibroblasts and myofibroblasts in the renal interstitium, which can be identified by α-smooth muscle cell expression [74], are a major source of extracellular matrix (ECM) proteins. An excessive production in parallel with reduced degradation of ECM proteins can lead to excessive ECM deposition and fibrosis [75]. The final outcome of tubulointerstitial injury depends on the capacity of tubules to regenerate and inflammation to regress, and has been shown to be (to a certain extent) reversible [76].

**Kidney Injury Molecule-1 (KIM-1) in the progression of renal disease**

Tubular cells are important players in the initiation and progression of renal damage. In response to kidney injury their phenotype changes, i.e. their cellular appearance and protein expression is altered. One of the proteins that will be expressed after renal injury is Kidney Injury Molecule-1 (KIM-1). KIM-1 is a tubular protein that was first described in 1998 by Ichimura et al [77]. KIM-1 is not detectable in normal kidneys, but its expression is markedly upregulated in post-ischemic rat kidney cells. KIM-1 expression was observed at the apical membrane of proximal tubular epithelial cells in damaged regions. High KIM-1 expression was found in the S3 segment of the proximal tubule in the outer stripe of the outer medulla, a region that is very susceptible for ischemic and toxic damage. Although the function of KIM-1 is unclear, its abundant tubular expression after damage points to a role in either tubular damage or repair.

KIM-1 is also known as hepatitis A virus cellular receptor 1 (HAVcr-1) and T cell Ig and mucin domain (TIM-1). HAVcr-1 was first identified in African green monkey [78], and later in human [79], as the receptor exploited by hepatitis A for viral entry. Tim-1 was first described in 2001 by McIntire et al [80], and belongs to the TIM family. The human TIM family consists of 3 members (TIM-1, TIM-3, and TIM-4) and is located on chromosome 5. The mouse TIM family consists of 8 members (Tim-1 till Tim-8) and is located on chromosome 11 [81]. All the TIM molecules share a common structural organization, they are type I transmembrane proteins with an extracellular V-type immunoglobulin (Ig) domain, an highly O-glycosylated mucin subdomain, a stalk with multiple N-glycosylation sites, a transmembrane domain and a relatively short cytoplasmic tail [82,83]. The TIM family members appear to have a role in the regulation of T-cell responses and are differentially expressed on subsets of immune cells. Tim-1 and Tim-2 are preferentially expressed by T helper (Th)2 cells, Tim-3 by Th1 cells, and Tim-4 by antigen-presenting cells, i.e. macrophages and dendritic cells [81]. In both human and mice the TIM family is located within the T cell and airway phenotype regulator (TAPR) locus. Allelic variation at the TAPR locus is associated with atopic immune response. In particular, variation in human TIM-1 is associated with susceptibility to atopic disease [84,85]. Accumulating functional data are supportive of this notion. For example, Th1-
prone mouse strains (i.e. C57BL/6) and Th2-prone strains (Balb/c) express different Tim-1 and Tim-3 polymorphisms [81].

The mouse Tim-1 gene encodes a 305-amino acid membrane protein that has 78% identity with rat KIM-1 and 42% identity with human HAVcr-1 [80]. TIM-1 expression is not only restricted to T cells, but TIM-1 transcripts were also broadly expressed in liver, small intestine, colon, spleen, kidney and testis [79].

In this thesis we will solely focus on TIM-1, and the renal expression of this molecule, and therefore call it from now on KIM-1. After the original discovery of KIM-1 induction after ischemic injury to the kidney, KIM-1 was also found upregulated after nephrotoxicant-induced renal injury [83], and in a murine model of polycystic kidney disease [86]. Also, in human renal carcinoma KIM-1 was shown to be expressed [87]. All these studies revealed KIM-1 expression in dedifferentiated tubular epithelium cells. Mukherjea et al [88] demonstrated that KIM-1 expression is also induced in the rat cochlea (inner ear) after cisplatin administration.

Bally et al [89] were the first to show in cultured kidney cell lines that the extracellular domain of KIM-1 can be cleaved and shed into the culture media. The release of soluble KIM-1 could be blocked with 2 different broad range matrix metalloproteinase (MMP) inhibitors, indicating that shedding is dependent on MMP’s. Subsequently, shedding of the KIM-1 ectodomain was also shown in the urine of patients with acute kidney injury [82].

Also, after nephrotoxic-induced renal injury in rats the shedded KIM-1 ectodomain was present in the urine [83]. The early upregulation of renal KIM-1 expression after renal injury, and the reported ectodomain shedding of KIM-1 tempted different researchers to speculate about use of shedded KIM-1 as a biomarker. Vaidya et al [90] demonstrated that shedding of KIM-1 was increased already 1 day after ischemia-reperfusion injury in rats, before a rise in proteinuria or plasma creatinine could be observed, indicating that KIM-1 is a very early biomarker of acute kidney injury. However, from the above mentioned studies it is unclear whether KIM-1 is also induced in proteinuric renal diseases, i.e. primarily non-toxic and non-ischemic injury, and whether urinary KIM-1 levels are a good reflection of the renal tissue damage. Also, it is unclear in which state of epithelial dedifferentiation KIM-1 is expressed. These issues will be addressed in this thesis.

Outline of the thesis

The aim of this thesis is to gain more insight in the effects of albumin-bound fatty acids on tubular cells and their possible contribution to the inflammatory and fibrotic renal response to proteinuria. The specific response of these tubular cells to injury was evaluated by determining the renal expression and urinary excretion of the tubular protein Kidney Injury Molecule-1 (KIM-1). Furthermore, we investigated whether KIM-1 can be used as a biomarker and predictor of renal damage and renal function loss in human renal disease and renal transplantation.

To study the effects of albumin-bound fatty acids in comparison to delipidated albumin on renal inflammation and fibrosis in rats we used the experimental protein-overload model (Chapter 2).
After evaluating the short-term (3 weeks) effects of albumin-bound fatty acids, we also investigated the chronic, long-term, effects of albumin-bound fatty acids on renal damage. Therefore, we extended the protein-overload model from 3 to 12 weeks (Chapter 3). To ensure the delivery of albumin-bound fatty acids to the proximal tubules and overcome the possible disturbing effects of passage through the circulation that may alter the albumin properties before it actually reaches the renal tubules, we also studied the effects of albumin-bound fatty acids in the Axolotl. The Axolotl has a unique kidney anatomy with closed and open nephrons. In closed nephrons tubules and glomerulus form a closed unit, while open nephrons drain the peritoneal cavity via a funnel. Injection of protein into the peritoneal cavity causes selective uptake and storage of proteins in tubular epithelium cells of nephrons with funnels. Therefore, in the Axolotl kidney it is possible to study the direct effects of albumin-bound fatty acids on tubular epithelium cells without passage through the circulation (Chapter 3).

To evaluate the specific response of tubular cells to injury we studied KIM-1 expression in a non-toxic model of acute proteinuria, protein-overload nephropathy. As the KIM-1 ectodomain can be cleaved and the cleaved ectodomain is quantified in the urine as a biomarker for acute kidney injury, we also investigated whether urinary KIM-1 levels reflect renal tissue damage (Chapter 4).

After studying KIM-1 expression and quantifying KIM-1 ectodomain shedding in a proteinuric animal model, we also studied KIM-1 expression and its ectodomain shedding in different proteinuric and fibrotic human renal diseases. Therefore, we determined KIM-1 expression in renal biopsies and also urinary KIM-1 excretion at the time of biopsy. This enabled us to explore whether urinary KIM-1 excretion could be of use as a biomarker (Chapter 5).

After investigating the usability of KIM-1 as biomarker of renal damage, we studied whether urinary KIM-1 excretion is predictive of renal function decline. Therefore, we prospectively followed renal transplant recipients for occurrence of graft loss after measuring urinary KIM-1 excretion at baseline (Chapter 6).

In the final chapter a summary of the results and conclusions of the studies described in this thesis is provided along with some of the perspectives arising from this research (Chapter 7).

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