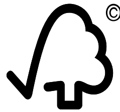
Proteinuria and function loss

in native and transplanted kidneys



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Koop, Klaas

Proteinuria and function loss in native and transplanted kidneys

Thesis, Leiden University, Leiden, The Netherlands

Cover The glomerular filtration barrier at ~48,000 times magnification.
Adapted from Pavenstadt H, Kriz W, Kretzler M: Cell biology of the glomerular podocyte.
Physiological Reviews 2003, used with permission.

Printing Gildeprint, Enschede, the Netherlands

ISBN 9789490122430

Proteinuria and function loss in native and transplanted kidneys

Proefschrift

ter verkrijging van

de graad van Doctor aan de Universiteit Leiden,

op gezag van Rector Magnificus prof. mr. P.F. van der Heijden,

volgens besluit van het College voor Promoties

te verdedigen op woensdag 2 september 2009

klokke 16:15 uur

door

Klaas Koop

geboren te 's Hertogenbosch

in 1979

Promotiecommissie

Promotor:	Prof. dr. J.A. Bruijn
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The studies described in this thesis were performed at the Department of Pathology (head: prof. dr. G.J. Fleuren), Leiden University Medical Center, Leiden, The Netherlands.

Financial support for the printing of this thesis by the J.E. Jurriaanse Stichting, Stichting het Scholten-Cordes Fonds, Bristol-Myers Squibb B.V., Genzyme B.V. and Novartis Pharma B.V. is gratefully acknowledged.

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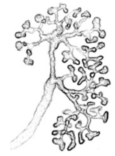

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The kidney: development, anatomy and function

Causes and consequences of proteinuria

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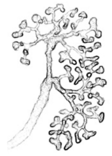
“Bones can break, muscles can atrophy, glands can loaf, even the brain can go to sleep, without immediately endangering our survival, but when the kidneys fail to manufacture the proper kind of blood neither bone, muscle, gland nor brain can carry on”.

Homer Smith, From Fish to Philosopher (1).

It is probably of limited value to ascribe the kidney a function superior to that of other organs; this quote merely indicates the importance of the kidney for the maintenance of the harmonious composition of body fluids – a function the kidney performs in a way that joins efficacy with elegance. Some of these processes will be lined out in this introductory chapter. The first section gives an overview of kidney development, anatomy and function. This is followed by a description of malfunction of the kidney and its consequences in the second part, which focuses on the development of proteinuria. Complete failure of the kidneys necessitates kidney replacement therapy or transplantation. The third section gives an overview of long-term problems that limit the success of transplantation as a treatment modality. This is followed by an outline of the thesis.



PART 1 | THE KIDNEY: DEVELOPMENT, ANATOMY AND FUNCTION

Overview of kidney anatomy and function

The kidney is a bean-shaped organ with a length of about 11 cm and a weight of approximately 150 grams. The kidneys are located in the retroperitoneal space at either side of the vertebral column, just below the diaphragm. The concave part of the kidney forms the renal sinus that contains the renal vessels, nerves, and the renal pelvis. The arterial blood supply sprouts directly from the aorta; the renal veins drain into the vena cava. Via the ureter the renal pelvis is continuous with the bladder and the outside world. The functional tissue of the kidneys surrounds the renal sinus, and is divided into cortex and medulla.

The human kidney contains about one million functional units or nephrons (30,000 in rats). A nephron consists of a filtration body – a tuft of anastomosing capillaries called glomerulus – connected to a long, tortuous tubule. The glomerular capillaries function as a microfilter that restricts passage of blood cells and proteins, but is permeable for smaller plasma components and waste products dissolved in the plasma water. Some ten percent of the blood that flows into the glomerulus is filtered, and the ultrafiltrate is delivered into the tubule (see below for a more comprehensive description of glomerular filtration). The blood that escapes filtration flows into the capillary network in the tubular compartment. Here, the tubular epithelial cells exchange substances between the fluids in the tubular lumen and the blood, thus gradually converting the ultrafiltrate into urine. Most of the exchange is from tubular lumen to the blood: the tubular epithelial cells reabsorb almost all water filtered in the glomerulus, together with salts, glucose, amino acids, vitamins, and other small molecules. But there is also active excretion of substances from the blood into the tubular lumen. When the urine via the collecting ducts flows into the renal pelvis, it is a concentrated solution of dissonants that the nephron has produced to maintain the harmonious composition of the blood.

In the next paragraphs, the microscopic anatomy of the nephron is described in some more detail, along with an overview of glomerular filtration. First, a brief overview of kidney development is provided.

Pathophysiologic processes sometimes recapitulate mechanisms that play a role in embryonic development (2). It is therefore of importance to understand some of the processes that take place during the organogenesis of the kidney. This paragraph describes the development of the kidney, with a focus on the development of the glomerulus. For more extensive overview of the developmental processes and their molecular regulation the reader is referred to several reviews on the topic (3-7).

The kidneys develop from the intermediate mesoderm of the embryo. The intermediate mesoderm is made up of two tissue compartments, the nephric duct and the nephrogenic cord, that form the different structures of the kidney. Derivates of the nephric duct form the collecting system of the kidney. The glomerular and tubular parts of the nephron arise from the nephrogenic cord.

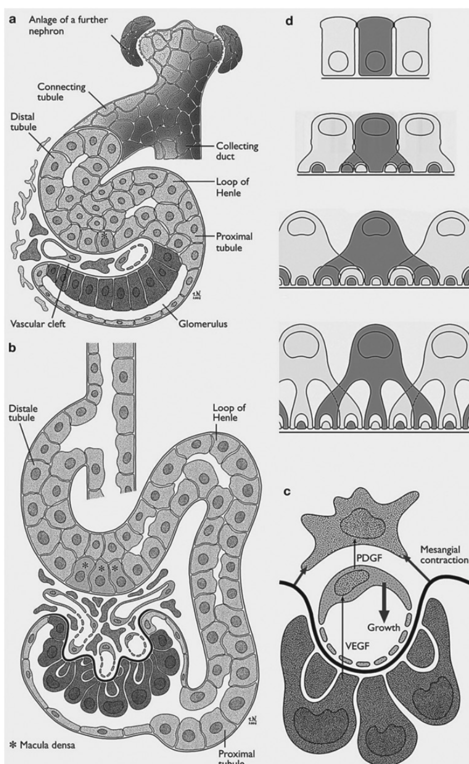


Figure 1. Embryonic development of the glomerulus. In the S-shaped body, the presumptive podocytes form a columnar epithelium that is continuous with the tubular epithelial cells. Endothelial and mesangial cells migrate into the vascular cleft (a). In the capillary loop stage, further differentiation of podocytes takes place (b). The presumptive podocytes start to form foot processes that interdigitate with those of neighboring cells (d). Differentiation of podocytes stimulates further development of endothelial and mesangial cells (c). See text for further details. Adapted from (152), with kind permission from S. Karger AG and the author.

During embryonic development, a sequential development of three more or less separate excretory systems, referred to as the pro-, meso-, and metanephros, takes place through interaction between the nephrogenic cord and the nephric duct. The pronephros is formed first and most cranial. It is a transient, non-functional structure that consists of a few tubules that connect to the nephric duct. Caudal to the pronephros the nephric duct induces the formation of the mesonephros, a structure consisting of a glomerulus and a relatively simple tubule. Though also transient, the mesonephron forms a functional excretory organ (8). The blood supply to each glomerulus sprouts directly from the aorta, while the tubules drain into the nephric duct that at this stage of development is called the mesonephric or Wolffian duct.

Subsequent to the development of the mesonephros, and again more caudally, the

development of the permanent kidney or metanephros starts. The metanephros develops through reciprocal interactions between the mesonephric duct and a condensed part of mesenchyme referred to as the metanephric blastema. The metanephric blastema induces the formation of a bud from the mesonephric duct. This ureteric bud grows into the metanephric blastema, and induces it to form a cap on the ureteric bud. The mesenchymal cap in turn induces dichotomous branching of the ureteric bud. The close apposition of the tips of the ureteric bud and the proliferating mesenchyme leads to continuous branching of the ureteric bud (a movie of ureteric bud branching will play in the upper right corner when quickly thumbing through the pages of this book from back to front. Courtesy of dr. Frank Constantini, Columbia University Medical Center). This reciprocal stimulation eventually produces an arborized system of ducts, of which each final branch is in close proximity to a mesenchymal condensate. Remodeling, ie, coalescence of the branched ducts shapes a system of collecting ducts that via the renal calyces, renal pelvis and ureter drains into the bladder; the mesenchymal condensate undergoes a sequence of events that transforms it into a functional nephron that connects to the collecting duct.

The invasion of the ureteric bud into the metanephric mesenchyme rescues the mesenchymal cells from apoptosis, and induces expression of the genes paired box 2 (PAX2) and Wilms Tumor 1 (WT1). In the presence of PAX2 and WT1 expression, and through stimulation by Wnt-4 and Bone morphogenetic protein 7 (BMP-7), mesenchymal cells acquire an epithelial phenotype, a process called mesenchymal-to-epithelial transition (MET) (9-12). After completing MET, the condensed cells start to form a vesicle. The renal vesicle then undergoes a series of morphologic changes: it first changes into a comma-shaped body that then elongates and folds back on itself to form an S-shape (figure 1a). The molecular mechanisms that regulate this process are not completely known. From this stage onward, epithelia in different parts of the S-shaped body start to differentiate, thus forming the at least 15 different epithelial cell lineages of the future nephron (5). During differentiation, expression of PAX2 is downregulated, probably through increasing expression of WT1 (13). This occurs most prominently in the proximal part of the S-shaped body, where the future podocytes show high expression of the WT1 protein, which continues to be a marker of podocytes throughout differentiation and in the mature glomerulus (14,15). The presumptive podocytes that surround the capillary tuft are first organized as a columnar epithelium, but progressively lose their lateral cell-cell adhesions and begin to form cellular extensions called foot processes. Eventually, the cell bodies of the podocytes float freely in the urinary space, and the adhesion between two adjacent podocytes is restricted to the slit diaphragm between the interdigitating foot processes (figure 1d) (16,17). Exactly which signals drive foot process formation is currently unknown, although the interaction with the GBM seems to be of crucial importance (17).

In the S-shaped stage of glomerular development, the presumptive podocytes express vascular endothelial growth factor A (VEGF-A) (18). This, either through stimulation of local angioblasts or

through incorporation of endothelial cells sprouting from nearby vessels (19,20), leads to the recruitment of endothelial cells into the cleft of the S-shaped body adjacent to the future podocytes (figure 1a,b) (14,21). In turn, endothelial cells of the capillaries express platelet-derived growth factor-B (PDGF-B), thus recruiting mesangial cells – of which the origin remains elusive (22) – that express PDGF receptor-β (figure 1c) (23). Through further branching of capillaries, differentiation of epithelial cells, and deposition of extracellular matrix, the three glomerular cell types shape the glomerulus (17,23).

The tubules elongate and differentiate and the most distal part of the S-shaped body connects with the branch of the ureteric bud that has developed into the collecting duct. By week 34 of fetal development, the formation of nephrons is complete. Further differentiation of the renal tissues continues postnatally (24).

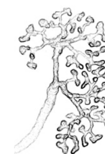
The glomerulus

As the renal artery enters the kidney, it subdivides in smaller arteries and arterioles. The last branch, the afferent arteriole, gives rise to a tuft of anastomosing capillaries called the glomerulus. Like all capillaries, the glomerular capillary lumen is lined by endothelial cells. At the inside, the tuft of capillaries is held together by mesangial cells, while the outer aspect of the capillaries is covered by the visceral epithelial cells or podocytes. The glomerulus is surrounded by Bowman's capsule and its parietal epithelial cells. Bounded by the visceral and parietal epithelial cells is the urinary space, which is continuous with the lumen of the tubuli. The different glomerular cell types will be discussed, followed by an overview of podocyte cell biology and glomerular filtration.

Glomerular cell types

The endocapillary lumen is lined with endothelial cells that are fenestrated. The cells rest on the glomerular basement membrane (GBM). With special staining techniques, a thick layer of glycocalyx has been visualized on glomerular endothelial cells, a finding that may shed light on the contribution of endothelial cells to glomerular filtration (25-27).

Mesangial cells are located between the glomerular capillaries, and reinforce the structure of the glomerulus. Contractile extensions of the mesangial cells bridge opposing portions of the GBM, and balance the outward forces of the blood pressure (28). Additionally, contraction of the mesangial cell myosin filaments provides a mechanism for control of glomerular blood flow. In this regard, mesangial cells resemble smooth muscle cells. Mesangial cells also have macrophage-like



functions, through which they are able to clear macromolecules and immune complexes from the glomerulus.

The outer aspect of the GBM is covered by visceral epithelial cells, or podocytes. These are highly differentiated cells with a complex, arborized phenotype. The podocyte cell body floats in the urinary space and gives rise to primary processes. The podocyte attaches to the GBM by means of foot processes, further cellular extensions that sprout perpendicularly from the major processes. Foot processes of two adjacent primary processes interdigitate, leaving a 40 nm space or 'slit pore' between them that is bridged by the slit diaphragm. In this way, the podocyte foot processes and the interposed slit diaphragm completely enwrap the glomerular capillaries. Recent three-dimensional ultrastructural analysis of the podocyte revealed an even further complexity. The space between the foot processes and the overlying cell body and primary processes appeared to be more restricted than previously appreciated, delineating a so-called sub-podocyte space. The finding that this space covers as much as 60 percent of the glomerular surface may bring important insights into the mechanisms of glomerular filtration (29,30).

Apart from its role in glomerular filtration, the podocyte probably also functions as a structure-stabilizing cell, providing forces to counteract capillary distension (31). Additionally, podocytes produce the extracellular matrix components that make up the GBM, and they provide growth factors like TGF-β and VEGF. The latter plays a role in the maintenance of the glomerular endothelium. The cell biology of the podocyte will be discussed in more detail in the next paragraph.

In contrast to the elaborately shaped podocytes and not reminiscent of their common embryonic origin, parietal epithelial cells (PECs) appear as a simple flat epithelium that lines Bowman's capsule. Recent reports have made clear that occasionally podocyte-like cell types can be found at parietal cell positions (32). These cells may be PECs that transdifferentiate to become podocytes (33). PECs themselves have long been regarded as relatively inert cells that, although they may show secondary reactivity in response to glomerular pathology, play no crucial role in glomerular diseases. Recent studies have changed this view (34). Several reports have indicated that PECs do play a role in animal models of focal segmental glomerulosclerosis (35,36) and HIV-associated nephropathy (37), as well as in human glomerular diseases (38,39).

Glomerular extracellular matrix

During the development of the glomerulus, endothelial cells and podocytes together produce the extracellular matrix they rest on and that separates the two cell types: the 300 nm thick GBM. In the mature glomerulus, all three glomerular cell types synthesize components of the GBM (40-42)

Ultrastructurally, the GBM consists of three layers: the laminae rara interna, densa and rara externa, but it is not known whether these different layers also represent different molecular compositions, or are a reflection of a fixation artifact (43). The molecular constituents of the GBM include

laminin (isoform $\alpha5\beta2\gamma1$), collagen IV (isoform $\alpha3\alpha4\alpha5$), heparan sulphate proteoglycans agrin, perlecan, collagen XVIII, syndecan, fibronectin, and nidogen/entactin. The collagen IV and laminin chains are crosslinked by nidogen, and form a strong, porous, network. The GBM proteoglycans are responsible for the negative charge of the GBM, thought to be instrumental in glomerular ultrafiltration (see below).

Mesangial cell extensions and mesangial ECM fibers are continuous with the GBM, and are thought to reinforce the glomerular structure (44). The mesangial matrix is composed of fibronectin, collagen IV (isoform $\alpha1\alpha1\alpha2$), laminins (isoform $\alpha5\beta1\gamma1$) and proteoglycans, with – similar to the GBM – heparansulphate as the major glycosaminoglycan (45). Many glomerular diseases result from an imbalance between mesangial matrix synthesis and degradation. For example, expansion of mesangial matrix is an important feature of diabetic nephropathy.

Cell biology of the podocyte

The molecular processes that support the podocyte's complex architecture and provide the basis for its function have received much attention in recent years. The elucidation of genetic causes of rare hereditary disorders, the generation of conditional and inducible knock-out mice, and the possibility to culture human and rodent podocytes have been instrumental in the advancement of understanding the cell biology of the podocyte.

Cytoskeleton

An elaborate cytoskeleton that combines strength with flexibility is indispensable for the correct shape and function of the podocyte. In the foot processes all membrane domains are physically linked to the cytoskeleton, and at the functional level the establishment of a connection between membrane associated molecules and the cytoskeleton has become a common theme.

The cytoskeleton of the cell body and primary processes is composed of microtubules and intermediate sized filaments such as vimentin. Foot processes lack these filament types; their cytoskeleton instead consists of actin filaments. Ichimura et al showed that there are two distinct populations of these actin filaments in the foot processes: a dense bundle that runs along the longitudinal axis of the foot processes, and a cortical actin network beneath the plasma membrane (46). At the base of the foot processes the bundle of actin filaments connects to that of the adjacent foot process as well as to the cytoskeleton of the major processes (figure 2). At the tip of the foot process the filaments are attached to the GBM via various linker molecules. Apart from actin, the foot process cytoskeleton contains several actin-associated molecules, including myosin and α-actinin-4. This suggests that the cytoskeleton has contractile capacities, and may serve to counteract the capillary pressures (14,31,47), although such contractions have until now not been observed in vivo (48).

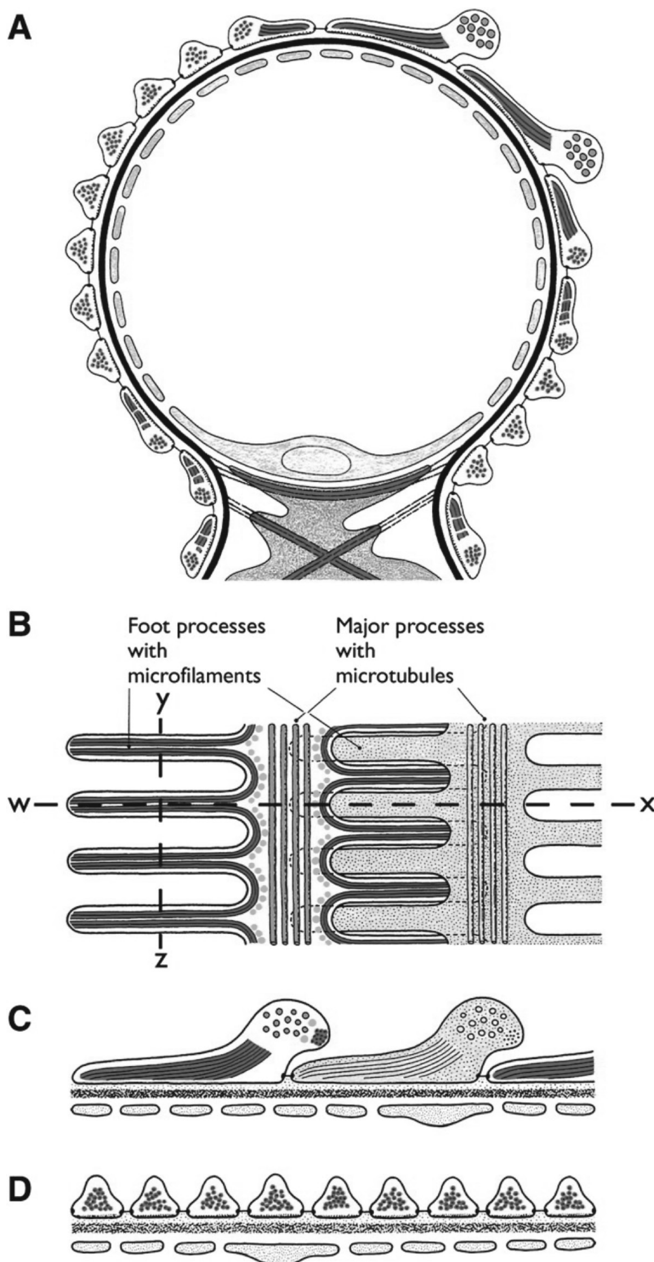


Figure 2. Organization of the podocyte cytoskeleton. A cross section through a capillary loop shows that the capillary is surrounded by podocyte foot processes. At the base of the capillary, contractile mesangial cell filaments are attached (a). A schematic view from above shows that the foot processes sprout perpendicular to the major processes. The cytoskeleton of the major processes is composed of microtubuli, to which the actin-based cytoskeleton of the foot processes is attached (b). In (c) and (d) the lateral view capillary wall is depicted, corresponding to the w-x and y-z line in (b), respectively. Adapted from (14), used with permission from The American Physiological Society and the author.

In response to damage, the podocyte ultrastructure and organization of the actin cytoskeleton are severely altered: foot processes are typically lost – a process called foot process effacement – and the highly organized actin fibers are rearranged to form a dense mat of interconnected fibers at the base of the effaced foot processes (14,49,50). This extensive and sometimes quickly reversible rearrangement of cytoskeletal elements has stimulated research into the regulation of actin and actin associated proteins in the podocyte.

Actin filament elongation is regulated by Rho GTPases such as RhoA, rac, and cdc42, and these molecules probably have an important role in the formation of the cytoskeleton of the podocyte (51). Indeed, mice that lack an inhibitor of Rho GTPases, ie, in which these proteins are more active, develop heavy proteinuria and foot process effacement (52). Others have shown that the deleterious effect that plasma

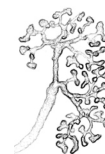
proteins have on podocytes is regulated in a RhoA dependent manner (53). Other actin regulating molecules including cortactin, Ena/VASP, and Arp2/3 are expressed in podocytes, illustrating the wide range of actin cytoskeleton activity displayed by podocytes (48).

The crucial role of the actin cytoskeleton is underscored by the fact that dysregulation of actin associated molecules results in a loss of adequate glomerular function. For example, MYH9, the gene coding for myosin heavy chain IIA, is related to the development of Fechtner syndrome that includes podocyte abnormalities (54). An upregulation of α-actinin-4 was shown to precede proteinuria and development of foot process effacement in PAN nephrosis (55). Later, mutations in ACTN4, the gene coding for α-actinin-4, were shown to be the cause of a hereditary form of late-onset focal segmental glomerulosclerosis (56). These mutations lead to an increased affinity of α-actinin-4 for actin, probably reducing cytoskeletal dynamics (56). At the same time, these mutations increase the protein degradation rate (57), and mice that lack α-actinin-4 also have glomerular disease (58), showing that both gain- and loss-of-function mutations in ACTN4 impair correct podocyte function.

Heat shock protein 27 has been reported to play a role in actin polymerization, and has been implicated in foot process effacement (59-61). Synaptopodin, also called pp44, was identified by Mundel et al to be an actin binding protein which expression is restricted to podocytes and neurons (62,63). Disruption of synaptopodin does not lead to glomerular disease, but seems to lower the threshold for development of glomerular abnormalities (64,65). This may relate to the involvement of synaptopodin in actin dynamics: synaptopodin regulates the actin bundling activity of α-actinin-4 (64) and prevents the proteasomal degradation of RhoA (66).

Apical membrane

Mature podocytes are polarized cells, and the apical membrane of the foot processes is fundamentally different from the basal and baso-lateral parts (see figure 3 for a schematic representation of the molecular organization of the podocyte foot process). A long-known characteristic of the apical membrane is that it is highly negatively charged (67). Kerjaschki et al identified the sialomucin podocalyxin as the molecule that provides this negative charge by means of several sialic acid residues (68). It has been suggested that the extracellular domain of podocalyxin serves as a 'spacer molecule' with anti-adhesive characteristics, preventing a connection between two adjacent foot processes (69,70). In keeping with the importance of podocalyxin for the integrity of the podocyte, podocalyxin knock-out mice show an impaired kidney development, with failure of podocytes to form foot processes (71). In vivo, interference with podocalyxin, for example through infusion of the polycation protamine sulfate, leads to proteinuria and foot process effacement (72). Such a change in foot process architecture would require a reorganization of the actin cytoskeleton, suggesting that podocalyxin interacts with this structure. Indeed, Farquhar's group showed that podocalyxin is physically linked to actin via a complex including NHERF2



and ezrin, and that this linkage is disrupted in foot process effacement (72). Follow-up studies showed that podocalyxin may also directly bind ezrin and activate RhoA, showing additional actin-modulating properties (73).

Recently, the heavily glycosilated negatively charged α-subunit of dystroglycan has been suggested to perform a function similar to that of podocalyxin (74). Loss of dystroglycan from the apical membrane may result in foot process effacement, in addition to a pathogenic role that loss of dystroglycan from the basal site of the foot process may have (discussed below) (75).

The transmembrane glycoprotein podoplanin adds further negative charge to the apical membrane (76-78). Little is known about the intracellular connections of podoplanin in the podocyte, although in other cell types this protein has been shown to interact with ezrin (79,80).

GLEPP-1 (protein tyrosine phosphatase receptor type O) is a phosphatase expressed in the apical membrane of the podocyte. As demonstrated in knock-out mice, the protein has a role in the correct shaping of the actin cytoskeleton (81), but the substrates of its phosphatase function remain undefined (14).

Slit diaphragm region

The slit diaphragm that spans the space between two adjacent foot processes is inserted in the basolateral membrane of the foot process. The multitude of proteins and protein-lipid complexes that make up and support the slit diaphragm and both physically and functionally link the structure to other parts of the cell, make this a highly specialized cell compartment.

Molecular architecture of the slit diaphragm

Although the slit diaphragm and some of its morphological characteristics have been recognized since the application of electron microscopy in kidney research (82,83), its molecular constituents have long remained obscure. The tight junction protein zonula occludens-1 (ZO-1) was one of the first molecules described to be associated with the slit diaphragm. Reiser et al showed that P-cadherin and ZO-1 co-localize at the slit diaphragm region, suggesting that the slit diaphragm is a modified adherens junction (84).

In 1988, Shimizu's group showed that the injection of monoclonal antibody raised against a component of rat glomeruli (mAb 5-1-6) in rats produced massive and transient proteinuria. The epitope recognized by this antibody localized almost exclusively to the slit diaphragm (85). Proteinuria, induced through infusion of the antibody or through protamine sulfate, caused an apical dislocation and internalization of the protein (86,87).

A breakthrough in podocyte biology was the finding that mutations in NPHS1, coding for nephrin, cause the congenital nephrotic syndrome of the Finnish type (CNF) (88), a syndrome characterized by proteinuria in utero and rapid development of end-stage renal disease requiring kidney transplantation (89). Nephrin was found to have a restricted expression, and in the kidney

localized to the slit diaphragm (90-92). In retrospect, the epitope recognized by mAb 5-1-6 appeared to be identical to rat nephrin (93). Because nephrin, a member of the Ig protein superfamily, has a large extracellular domain and could form homodimers in vitro (94), it was suggested that nephrin strands from opposing foot processes may form the actual slit diaphragm through nephrin-nephrin interactions (95). This hypothesis was tested in an extensive study using electron tomography in combination with immuno-gold labeling of nephrin in glomeruli from various mammalian species, both in health and disease (96). From this study, it was concluded that nephrin, together with other proteins that were not molecularly identified, contributes to the slit diaphragm. In CNF patients the slit pore was much smaller and did not contain a slit diaphragm. Confirming these findings, different reports on nephrin knockout mice consistently showed heavy proteinuria and early death of the mice, with absence of the slit diaphragm (97-99), although foot processes were assembled normally (17). These studies established nephrin as an important structural component of the slit diaphragm.

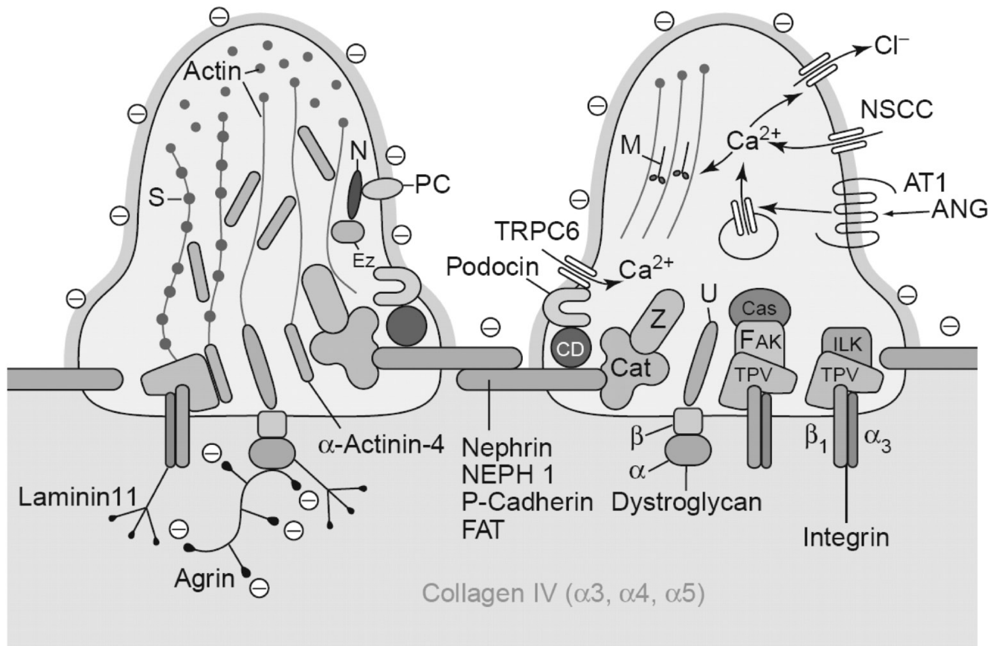


Figure 3. Molecular organization of the podocyte foot process. Two podocyte foot processes with the bridging slit diaphragm are depicted, resting on the glomerular basement membrane (GBM). The central part of the foot process is the actin cytoskeleton (indicated with grey lines and dots), which is reinforced by synaptopodin and α-actinin-4, and has contractile properties as a result of myosin fibers (M). Connected to the cytoskeleton are several molecules that reside in the negatively charged apical membrane, including podocalyxin (PC) via NHERF2 (N) and ezrin (Ez), and podoplanin (not depicted). At the basal membrane, the actin cytoskeleton is connected to the GBM via dystroglycan (linking utrophin (U) to agrin), and the integrin-complex (integrin, talin-paxillin-vinculin (TPV), integrin linked kinase (ILK), focal adhesion kinase (FAK)). The slit diaphragm is composed of nephrin, NEPH-proteins, P-cadherin, and FAT, and the slit diaphragm domain contains several molecules that play a role in the anchoring and signaling of the slit diaphragm (TRPC6, podocin, CD2AP (CD), β-catenin (cat), and ZO-1 (Z)). The podocyte has several receptors, including the angiotensin II receptor AT1. See text for further details. Adapted from (505) and (506), with permission from Elsevier, Lippincott Williams & Wilkins, and the author.

Since the discovery of nephrin several other molecular components of the slit diaphragm have been identified. The nephrin homologues NEPH1-3 were identified in the mouse. As is the case with nephrin, NEPH1 deficient mice show proteinuria and neonatal death (100). Several groups subsequently showed that NEPH1 and nephrin form homo- and heterodimers, suggesting a shared role in formation of the slit diaphragm (101,102). Injection of individually subnephritogenic doses of antibodies against nephrin and NEPH1 induced proteinuria (103), underscoring the functional link between these two molecules. NEPH3 (syn. filtrin, KIRREL2) is expressed at the slit diaphragm region, and its expression was found to be reduced in acquired proteinuric diseases, both at the protein and the mRNA level (104,105).

In a search for other cell-cell adhesion proteins that may be of relevance, Inoue et al found that the protocadherin FAT1 is expressed at the site of the slit diaphragm (106). Mice that lack FAT1 develop, among other abnormalities, proteinuria and foot process effacement (107).

Anchoring to the actin cytoskeleton

The slit diaphragm is inserted in a highly organized membrane region modified by lipid rafts. Podocin is one of the proteins that localizes to this region, and like nephrin is present in lipid rafts (108-110). The protein has a hairpin structure, with both the N- and C-terminus ending in the cytoplasm. Mutations in NPHS2, the gene encoding podocin, lead to a steroid-resistant form of nephrotic syndrome (111). Podocin interacts with several components of the podocyte foot process, including nephrin and CD2-associated protein (CD2AP). TRPC6, coding for a cation channel with a preference for Ca^{2+}, was recently found to be mutated in patients with a late-onset form of FSGS (112). Subsequent analysis showed that TRPC6 localizes to the slit diaphragm region, and associates with other components such as nephrin and podocin (113,114).

As pointed out before, the different membrane compartments of the podocyte are linked to the subcortical actin cytoskeleton. This is also true for the slit diaphragm region. FAT1, for example, was shown to recruit Ena/VASP proteins that play a role in actin polymerization (115). P-cadherin forms a multimolecular complex with α-, β-, and γ-catenin and ZO-1, both ZO-1 and β-catenin could link this complex to the actin cytoskeleton (84). Nephrin interacts with a wide array of intracellular molecules that relate to actin dynamics. One of the first intracellular linkers to be discovered was CD2AP (116), a protein that directly connects to the actin cytoskeleton (117). Studying the role of this protein in the immunological synapse, it was serendipitously discovered that absence of CD2AP in mice leads to heavy proteinuria (118), which brings functional relevance to the nephrin-CD2AP-actin interaction. Other actin-associated molecules that interact with nephrin include IQGAP1, spectrins, and α-actinin-4, while nephrin also connects to MAGUK family proteins that link it to other signaling and cell-junction molecules (119).

Signaling at the slit diaphragm

The studies mentioned so far have established numerous structural components of the slit diaphragm and their intracellular connections; a major finding was that several of the proteins located in the slit diaphragm region participate in signaling pathways. Signal transduction in cells is mostly regulated by the attachment or detachment of phosphate groups on serine, threonine, or tyrosine residues of proteins, enabling the binding of other proteins (120). Both the intracellular (C-terminal) part of nephrin and NEPH1 contain tyrosine residues that can be phosphorylated by kinases such as Fyn; the importance of this is underscored by the fact that Fyn knockout mice show foot process effacement and proteinuria (121). Nephrin tyrosine phosphorylation results in activation of AP1, an effect that is enhanced by binding of nephrin to podocin (122). Later experiments showed that in vivo, phosphorylated nephrin in conjunction with CD2AP activates PI3 kinase. This in turn initiates a series of phosphorylations that may lead to intracellular responses including cell survival, actin dynamics, proliferation, metabolism, and endocytosis. Two examples that give some insight in the importance of signaling for podocyte integrity are listed here; more in-depth reviews are provided in references (120) and (123). One specific response is the regulation of apoptosis: nephrin and CD2AP induced PI3 kinase activity may increase AKT expression, thus suppressing TGF-β signaling and preventing podocyte apoptosis (124,125). As will be discussed in a later paragraph, loss of podocytes is linked to the progression of renal disease (126). Secondly, two different groups showed that nephrin phosphorylation by Fyn is crucial for its binding to the adaptor protein nck (127-129). Nck is able to recruit a protein complex that regulates actin polymerization. This suggests that the phosphorylated nephrin – nck interaction may be of importance in states with high dynamic activity such as development and foot process effacement or rearrangement (128-130).

The different interactions between the proteins in the slit diaphragm region, both in physical attachments of proteins and in signaling, make clear that protein complexes rather than single molecules are responsible for a correct podocyte and slit diaphragm function. And not only proteins, but also lipids may be involved. The role of lipid rafts in the cell membrane has already been alluded to; in addition, recent studies by Huber et al indicate that the regulation of TRPC6 by podocin requires binding of cholesterol by podocin (114).

Interaction with the GBM

At the basal site, the 'sole' of the foot process, the podocyte attaches to the matrix they have in part themselves produced: the GBM. This interaction is accomplished by several transmembrane matrix binding proteins, including integrins and dystroglycan.

Integrins are heterodimers, consisting of an α and β subunit, that attach to extracellular matrix molecules. The podocyte's integrin is made up of the α3 and β1 integrin subunit that attach to collagen, fibronectin, and laminins in the basement membrane (131). Blocking the β1 integrin

in animal models leads to proteinuria. α3 integrin knockout mice die in the neonatal period and show defects in lung maturation and kidney development, including a disorganization of the GBM and a failure to form podocyte foot processes (132). Upon extracellular ligand binding, integrins cluster to form so-called focal adhesions. At the intracellular side these focal adhesions recruit several adapter molecules, including talin, paxillin, and vinculin, which attach the integrins to the cortical actin network. As with the slit diaphragm, these interactions serve not only a structural function, but are also involved in signaling pathways. Integrins are able to mediate both inside-out and outside-in signaling, an action in other cells frequently mediated by phosphorylation through focal adhesion kinase (Fak). In podocytes, this route has not been unequivocally demonstrated. Another candidate for mediating the signaling by integrins could be integrin-linked kinase (ILK). Using differential display analysis, the groups of Holthofer and Kretzler found an increase of – among other molecules – ILK mRNA expression in glomeruli of patients with CNF (133). Subsequent experiments showed that ILK mRNA expression was increased in several proteinuric kidney diseases in vivo (134), and that increased ILK activity was related to reduced matrix adhesion in vitro (135). Also, clustering of integrin receptors by ECM molecules leads to a decrease in ILK activity, the outside-in route. Further studies identified several molecules interacting with ILK, linking this complex to actin cytoskeleton dynamics, hypoxia signaling (via HIF-1α and VEGF), the wnt signaling pathway, and proliferation (136-138). ILK activity also induces expression of matrix metalloproteinase 9 (MMP-9) that has a role in GBM remodeling. Podocyte-specific ILK knockout mice showed a normal development, but after three weeks started to become proteinuric. The initial changes were primarily found in the GBM, indicating a possible role for ILK in integrin mediated GBM organization (139). Using a comparable mouse model, another group showed that disruption of ILK signaling also resulted in changes at the slit diaphragm (140).

Apart from integrins, dystroglycans have been implicated in the embedding of the podocyte foot processes in the GBM. Indeed, dystroglycan seems to be well-adapted to such a function: the extracellular domains bind ligands including laminin, agrin, and perlecan – all present in the GBM; the intracellular domain (the transmembrane β subunit) is linked to the actin cytoskeleton via the adapter protein utrophin (50,141). Reports on the localization of dystroglycan in the podocyte have been controversial; some studies reported an expression limited to the basal foot process membrane (142), while others also found dystroglycan expression at the apical membrane (74,141). Raats et al found utrophin expression only at the basal membrane (141), suggesting this to be the place of a dystroglycan-mediated GBM-actin association. Loss of dystroglycan expression at the basal part of the foot processes has been reported in minimal change disease (as opposed to FSGS) that was reversible after steroid treatment (142). A similar finding was reported in a patient with proteinuria but otherwise with no glomerular abnormalities (143). Raats et al instead reported an increase in basal membrane dystroglycan expression in adriamycin nephropathy (141). Later studies have added to the notion that the dystroglycan-GBM connection is of

importance for the correct function of the glomerular filtration barrier: reactive oxygen species were found to decrease the adherence of dystroglycan to agrin, possibly leading to podocyte detachment (75). In a comparable study, Kojima et al found that protamine as well as reactive oxygen species disrupted the link between dystroglycan and its ligands (144). This resulted in a disorganization of the lamina rara externa of the GBM, substantiating the hypothesis, as put forward by Kerjaschki (50,142), that via dystroglycan the actin cytoskeleton of the podocyte may function as a blueprint for the organization and spacing of GBM proteins.

Other proteins that are located at the basal membrane include megalin/gp330 (in rats) and neutral endopeptidase/CD10 (in humans). These proteins have been discovered to be the pathogenic antigens in Heymann nephritis and neonatal membranous nephropathy, respectively (145-148). Megalin may be linked to the cytoskeleton via the adaptor protein MAGI-1 (149).

Podoplanin has also been reported to be present in the basal membrane of the foot processes, its predominant localization being at the apical membrane domain. As discussed above, the intracellular linkers of podoplanin in the podocyte have not been identified, nor is the function of podoplanin at this location clear. In lymphatic endothelial cells podoplanin has been reported to play a role in the shaping of a gradient of the chemokine SLC/CCL21 (150), which is also expressed in podocytes, and which is important for mesangial function (151). It is tempting to speculate that podoplanin is important for the mediation of this cross-talk between podocytes and the mesangium.

Exocytosis of ECM molecules and matrix modifying enzymes such as metalloproteinases takes place at the basal membrane of the podocyte. Also, growth factors such as VEGF are excreted at the basal site of the podocyte, although little is known about the kinetics and precise mechanisms by which this occurs (152). But interactions at the basal membrane of the podocyte are not restricted to binding and modifying the GBM, several other interactions take place. For example, there is extensive endocytosis in this membrane compartment, as can be inferred from the widespread presence of clathrin-coated pits and vesicles (14,153). In vitro, podocytes were shown to endocytose albumin, possibly important in clearing the glomerular filter from macromolecules (154). Others have also found that the podocyte is able to perform transcytosis, yielding a transcellular rather than paracellular, ie, slit diaphragm, route between the intracapillary lumen and Bowman's space (155).

Receptors and signaling pathways

Numerous receptors and coupled signaling pathways that are involved in podocyte function have been investigated, but will not be discussed in detail here. For further details on this subject the reader is referred to an extensive review (14). The bottom-line of the different receptors is the notion that intracellular second messengers, including cyclic AMP, cyclic GMP, and Ca2+ and their related pathways in the podocyte are modifyable by a wide range of extracellular and circulating

molecules. These include vasoactive compounds as nitric oxide, atrial natriuretic peptide, hormones (156), and medication (including dexamethason (157) and cyclosporine (158)). Of some importance, also from a clinical point of view, is the fact that podocytes carry both types of the angiotensin II receptor, AT1 and AT2, suggesting podocytes have a local renin-angiotensin system (RAS) (159). It is well known that ACE inhibitors or AT1 antagonists have a beneficial effect on the kidney, which is not completely explained by their blood pressure lowering properties (152). The inhibition of angiotensin II receptor mediated effects may be the explanation for these observations. Indeed, hypertrophy in podocytes was prevented by ACE inhibition (14), and several other studies have shown a 'podoprotective' effect of RAS inhibition (160-163). Conversely, transgenic rats that overexpress the human AT1 receptor specifically in podocytes develop albuminuria, podocyte foot process effacement, and eventually FSGS (164).

Rastaldi et al found that podocytes express several molecules associated with neuronal synaptic vesicles, and showed that in podocytes these molecules also associate with vesicles (165). These findings expand the extent of similarities between neurons and podocytes. Indeed, the branched appearance, cell cycle quiescent phenotype, cytoskeletal organization (166), and gene expression pattern (167) of these cells are strikingly similar. Moreover, these findings could indicate that apart from intracellular signaling, also an intercellular communication by means of synaptic-like exocytosis of glutamate may take place in podocytes (168).

Cell cycling and transcription factors

During the capillary loop stage of glomerular development, podocytes start to differentiate, they form foot processes and express typical podocyte markers. At the same time these cells stop to proliferate. In the mature glomerulus, podocytes are considered to be terminally differentiated, post-mitotic cells. Proliferation, or progression through the cell cycle, is regulated by a complex set of stimulatory and inhibitory proteins. Cyclins and their respective cyclin dependent kinases (CDKs) promote proliferation, while CDK inhibitors such as p21, p27, and p57 inhibit proliferation. In podocytes, an upregulation of CDK inhibitors is seen in the capillary loop stage, promoting a quiescent podocyte phenotype. Also in response to injury, podocytes, in contrast to other glomerular cells such as mesangial cells, do not divide, although they do show hypertrophy and sometimes multi-nucleation. Petermann et al found that in response to injury, podocytes do enter the cell cycle: they show (limited) DNA amplification and upregulate proteins that mark the start of the cell cycle. However, there was no proliferation of podocytes, suggesting that they do not have the ability to complete the cell cycle and perform cytokinesis (169). Others have suggested that the complex cellular architecture of podocytes prohibits cytokinesis (14). Consequently, there must be cell cycle inhibitory molecules that prevent cell division. Loss of such inhibitory regulators, as exemplified by the p21 and p27 knockout mice, results in podocyte proliferation in response to damage (170,171). While loss of podocytes is considered to be the initial step towards

nephron loss, also certain glomerular diseases have been associated with a proliferating podocyte phenotype. Almost all of these diseases show a detrimental course when left untreated. Moeller et al studied transgenic mice with tagged podocytes and showed that podocytes contribute to cellular crescents, seen in some forms of glomerulonephritis (172). In HIV-associated nephropathy podocytes are presumed to be able to escape their cell-cycle control and re-proliferate, leading to FSGS of the collapsing type with a rapidly progressive clinical course (173-175). It has been difficult to prove that these cells are really podocytes – the cells are dedifferentiated, they have lost their typical markers and they are allegedly podocytes merely on the basis of their localization in the glomerulus. Studies by Dijkman and Smeets et al brought evidence for the role of parietal epithelial cells in proliferative glomerular diseases (34,39).

Transcription factors that regulate podocyte development and maintenance include PAX2, pod1, Kreisler, Lmx1b, and WT1 (176). WT1 has been linked to the expression of several podocyte markers, including nephrin and podocalyxin (177-180). The crucial role for WT1 is underscored by the fact that mutations in the gene cause syndromes (Denys-Drash, Frasier, and WAGR syndrome, OMIM 607102) that frequently involve podocyte and glomerular abnormalities.

Mutations in the gene that encodes the transcription factor Lmx1b cause nail-patella syndrome, characterized by the absence of the patella and nails and by the occurrence of nephropathy. Two groups of investigators studied Lmx1b knockout mice and found that this transcription factor is important for the expression of collagen α3(IV) and α4(IV), and the slit diaphragm associated proteins podocin and CD2AP (181,182), indicating a role for this transcription factor in both GBM formation and slit diaphragm function.

Glomerular filtration: characteristics and theoretical models

How exactly the kidneys produce urine has been investigated for over 150 years and remains unresolved. Initially, the question was whether the glomerulus takes at all part in formation of urine. At that time, tubular secretion probably was a more plausible option, as secretive epithelia in salivary, gastrointestinal, lactating glands etc. had just been extensively studied. The notion that urine is formed by glomerular filtration and tubular resorption and excretion was only proven through micropuncture studies in the 1920-60s (183-185). Still, the molecular mechanism of glomerular filtration remains incompletely understood.

In this paragraph, the characteristics of the glomerular filtration barrier are described: the amount and concentration of fluids, small solutes, and macromolecules involved, the forces that drive filtration, and the biochemical and biophysical properties of the filter. This is followed by a description of several theories on how glomerular permselectivity is accomplished at subcellular and molecular levels.

Characteristics of glomerular filtration

Amounts and concentration of fluids

The kidney receives about 20 percent of the cardiac output, ~1.2 liters of blood per minute. Some ten percent of this total volume is filtered in the glomerulus, and enters the tubular system as pre-urine; the total volume of pre-urine thus amounts to 180 liters per day. Most of the pre-urine is reabsorbed in the tubules, leaving an average of 1.5 liters of urine each day for excretion (range 0.8 – 20 liters). The ultrafiltrate has almost the same composition as plasma-water, it is acellular and contains a low amount of protein. It is generally agreed upon that the concentration of albumin in the pre-urine is about 25 μg/ml, compared to a plasma albumin concentration of 45mg/ml. This indicates that the glomerular filter is restrictive for proteins, a feature referred to as glomerular permselectivity. The extent of restriction differs for each protein (see below), and is expressed as the glomerular sieving coefficient (Bowman's space-to-plasma concentration ratio) θ, theta.

Forces that drive filtration

There are different forces that drive transport of fluids through the glomerular capillary wall. These so-called Starling forces include the hydrostatic and colloid osmotic pressure, determined by the fluid-pressures and the colloid osmotic value of the fluids, respectively. These two forces work in opposite directions: the hydrostatic pressure in the glomerular capillaries is higher than that in Bowman's space, providing an outward force. Instead, the colloid osmotic pressure within the capillaries exceeds that in Bowman's space, and this will drive transport of fluids inwards. In the upstream part of the glomerular capillaries, the hydrostatic outward force is higher than the colloid osmotic inward force, resulting in filtration. Since the filtration barrier is restrictive to proteins, the extraction of fluids from inside the capillaries will increase the intracapillary protein concentration, and thus the colloid osmotic pressures. Eventually, in the downstream part of the glomerular capillaries this will lead to an osmotic inward force that equals the hydrostatic outward force, the so-called filtration pressure equilibrium. Downstream of the point where the filtration pressure equilibrium is reached, there is no filtration.

The amount of filtration is further influenced by the characteristics of the filter, represented in the ultrafiltration coefficient K_f, the constant that indicates the resistance to fluid flow through a barrier. Higher levels of K_f indicate a higher permeability. Both the GBM and the cellular constituents of the filter contribute to the resistance to flow.

Small molecules are mainly transported by convection, and thus hold pace with the transport of the fluids over the capillary wall, while macromolecular transport is determined by both convection and diffusion.

Biochemical and biophysical properties of the glomerular filter

The general view is that the glomerular filter restricts passage of macromolecules on the basis of their size, shape, and charge. The influence of these factors determines the sieving coefficient θ

for each macromolecule. Because macromolecular transport is passive, θ varies between 1 (free passage through the filtration barrier) and 0 (complete restriction). The different determinants of glomerular permeability will be discussed here.

Size selectivity – The size of a molecule, commonly indicated by its Stokes-Einstein radius (SE radius), is influenced by the compactness of the molecule and its molecular weight. For albumin (molecular weight 3000 kDa, SE radius 36 Å) the sieving coefficient θ is about $6 \cdot 10^{-4}$ (25 µg/ml, the albumin concentration in Bowmans' space divided by 45 mg/ml, the plasma albumin concentration). The θ for smaller proteins is considerably larger, and proteins smaller than 14 Å have a θ that is 1, indicating that these molecules are not restricted by the glomerular filtration barrier. For larger proteins such as IgG (molecular radius 55 Å) or IgM (molecular radius 120 Å), the θ is considerably smaller; these molecules may even be completely absent from the ultrafiltrate in Bowman's space. The exact size selectivity of the glomerulus has been difficult to determine: direct measurement of proteins should be performed in the glomerular ultrafiltrate, before reabsorption in tubules may occur. This requires micropuncture techniques that have been criticized because they may measure proteins released as a result of the tubular damage associated with the measurement itself, or by contamination of peritubular capillary blood proteins. The most reliable direct measurements of protein concentrations are probably experiments by Tojo and Endou (186), who used sophisticated techniques to circumvent the problems associated with micropuncture techniques. These studies have established the before mentioned θ for albumin of $6 \cdot 10^{-4}$. Furthermore, patients with Fanconi syndrome have been used for estimations of the glomerular filtration selectivity. These patients have an impaired tubular protein uptake, and the protein concentration in urine is thus a reflection of their glomerular protein filtration. In these studies, the θ for albumin was found to be $\sim 8 \cdot 10^{-5}$, which is even lower than that of rodents (187). Probes of different sizes, such as the polysaccharides dextran and Ficoll as well as different proteins, have been used to get insight in the exact size characteristics of the filter. Results from these experiments have been used in models of glomerular size selectivity, in which the glomerular filtration barrier is considered to be perforated by pores with a certain diameter. In most models, there are two 'pore-populations': a large number of restrictive small pores with a radius between 37 and 55 Å, and far less frequent unrestrictive large pores or 'shunts', with a radius of 80 – 100 Å (43,188-191).

Macromolecular shape – Uncharged probes with a similar SE radius may show a divergent filtration behavior. For example, the polysaccharide dextran has a θ that is about 7-fold higher than that of an uncharged protein of the same size, and is also larger than Ficoll with the same radius. This is attributed to the fact that dextran may change its conformation from a sphere to a more elongated molecule, and thus pass through the capillary wall more easily. Also for Ficoll, although more spherical than dextran, the glomerular filtration barrier was found to be to some extent

hyperpermeable, probably due to the compressibility of Ficoll (188). This indicates that the form, globular or linear, and deformability of a molecule is of importance for its sieving characteristics.

Charge selectivity – Several studies have demonstrated that the capillary wall restriction for anionic proteins is higher than that for neutral or cationic proteins, indicating the presence of charge selectivity (192-194). The extent of this charge selectivity has, however, been difficult to quantify. This relates in part to the use of tracers that may not truly mimic the behavior of proteins in the capillary wall: negatively charged sulfated dextrans may more rapidly interact with the components of the glomerular filter, thus retarding their passage (188). As pointed out before, the capillary wall seems to be hyperpermeable for Ficoll in comparison to albumin of the same size. This has led to the suggestion that the negative charge of albumin accounts for the apparent restriction, which would fit with an important charge barrier (195), although the divergent behavior may in fact be related to the compressibility of Ficoll. Furthermore, comparison of tracers such as sulfated dextrans and carboxymethyl Ficoll (tracers that have been rendered negatively charged) with their neutral counterparts even showed an increased permeability of negatively charged tracers (196). Removal of molecular constituents of the negative charge in the GBM – using enzymatic and genetic methods – failed to induce proteinuria, further questioning the role of the GBM in charge selectivity (197-201). It has been suggested that the actual charge barrier may reside in the endothelium rather than in the GBM (see below). Also, a charge effect could be built up by negatively charged components of the blood, such as albumin, which accumulate during filtration or interact with the endothelial cell glycocalyx (202). An indirect proof for this is the observation by Ryan and Karnovsky, that a continuous blood flow is needed to maintain a normal filtration barrier (203). Direct proof for this mechanism is lacking. Thus, although most authors acknowledge the presence of charge selectivity, at least with regard to the filtration of proteins, its relative contribution to permselectivity remains controversial.

Different explanations for permselectivity

The description of the functional characteristics of glomerular filtration directly relates to the question how filtration is accomplished on a structural level. In the literature, the different structural components of the glomerular filter have all been given attention, with a recent skewing towards the contribution of the podocyte. An overview of the different explanations and hypothesis is lined out below.

The GBM as the main filter for plasma proteins

In 1975, Farquhar in a review on glomerular filtration concluded that 'the bulk of the evidence available at present favors the basement membrane as the primary filtration barrier in the glomerulus' (204). Indeed, in most tracer studies, an accumulation of tracer molecules at the subendothelial rather than at the subepithelial side has been observed. On the molecular level, the GBM contains proteins that permit the formation of sieve-like structures, for example through

cross-linked collagen IV networks, and removal of some essential components leads to the development of proteinuria. Localization of the charge restriction in the GBM has been inferred from the observation of regularly spaced anionic sites demonstrated using for example polyethyleneimine, and the corresponding molecules have been identified as the glycosaminoglycan sidechains of proteoglycans such as agrin, perlecan, and collagen XVIII. In conclusion, charge and size selectivity can both be explained by the structural and molecular properties of the GBM. Yet, this view leaves several observations unexplained. For example, while IgG is mostly absent from the glomerular ultrafiltrate, injection of IgG directed against nephrin and megalin binds these antigens expressed on the podocyte membrane (85,145), indicating that these IgGs have passed through the GBM. The contribution of GBM proteoglycans to the charge selectivity has been criticized by investigators pointing out that in other tissues such as cartilaginous tumors albumin is found in the stroma, which is even more rich in proteoglycans than the GBM (205,206).

Furthermore, fitting data obtained from experiments with isolated GBM into a theoretical model of glomerular filtration, Deen et al concluded that the contribution of the GBM to permselectivity is relatively small in comparison to that of the cellular components of the filter (26,207,208). These could then either be the endothelial or the epithelial cells. Over the last decade, the contribution of the podocyte to the permselectivity of the glomerular filter has received most attention.

The podocyte slit diaphragm is the main barrier for plasma proteins

Tryggvason and coworkers, after the discovery of nephrin, argued that the podocytes and especially the slit diaphragm would be the main site of ultrafiltration (95,209-211). This hypothesis was supported by observations by Rodewald en Karnovsky in the 1970's, who observed that the slit diaphragm had a zipper-like ultrastructure and suggested that this could explain the glomerular size selectivity (83). Using electron tomography, Wartiovaara et al visualized nephrin strands spanning the slit pore and leaving lateral pores of about the size of an albumin molecule (96). The fact that absence or abnormal function of many other slit diaphragm-associated proteins leads to proteinuria brings additional evidence for the importance of the slit diaphragm in glomerular permselectivity, and possibly for its dominant role in ultrafiltration.

If, however, the most selective barrier is indeed localized downstream in the filter, one would expect that proteins that pass through the upstream layers would 'pile up' in the sub-epithelial part of the GBM, a phenomenon called concentration polarization (202). In other words, the filter would clog (152,202,211,212). Several mechanisms that prevent such a clogging of the filter have been put forward. For example, the characteristics of the previously mentioned subpodocyte space theoretically leave open the possibility of an inversion of the direction of the fluid flow in the capillary wall, and could thus play a role in the unclogging of the filter (29,30). Another recently pursued hypothesis is that the proteins that accumulate at the basal membrane of the podocyte are transported to Bowman's space by transcytosis (155). Indeed, coated pits

are frequently observed at the basal membrane, and in vitro podocytes have been shown to be capable of large scale endocytosis (154).

Alternatively, the restrictive properties of the glomerulus could be localized to the other cellular component, the endothelium.

The endothelial cells restrict passage of proteins

Deen showed that theoretically the endothelial layer is able to contribute notably to the glomerular filtration barrier (208). Early studies of glomerular permselectivity had ruled out a contribution of the glomerular endothelium, as it was recognized that the fenestrations would be too large to restrict passage of macromolecules (204). Later studies, however, have changed this view. Rostgaard and Qvortrup used special fixation and staining techniques that allowed them to study the ultrastructural organization of the glomerular capillary wall in the absence of changes that would be caused by lower blood pressure or anoxia. They found that the endothelial cells are covered with a 300nm thick glycocalyx, and that the fenestrae of the endothelial cells were bridged by filaments (25). In a later study, the same authors described the presence of a surface coat, presumably made up of proteoglycans on the endothelial cells, which extended into and filled up the endothelial fenestrations (213). This led them to hypothesize that these 'sieve plugs' or 'fascinae fenestrae' would be the actual basis for the glomerular permselectivity (213). Others have shown that the glomerular endothelial cells produce negatively charged proteoglycans (214). Taken together, the glomerular endothelial cells may indeed play a more important role in glomerular filtration than has been generally acknowledged (43,202,208,211).

Integrative views of glomerular filtration

Some explanations bring a more integrative view of the filter. These include the view of the glomerular filration barrier as size and charge barriers in series, the 'Electrokinetic glomerulus theory' by Douglas Somers, and Oliver Smithies' 'Permeation diffusion hypothesis'.

Size and charge barriers in series

In the classic view of the glomerular filtration barrier, the GBM and the podocyte slit diaphragm are two size and charge selective barriers that are placed in series. The GBM functions as a coarse filter for the larger molecules, while the slit diaphragm is the fine filter (204,215). If this view of the glomerular filtration barrier were correct, this would lead to a concentration polarization, ie, a clogging of the filter. Thus, this view of glomerular filtration seems to suffer from the combined inconsistencies mentioned in the discussion of the individual components of the filter.

Electrokinetic glomerulus theory

In Douglas Somers' 'Electrokinetic glomerulus theory' (Somers D, J Am Soc Nephrol 2005 (16): 109A), the central tenet is that anion transport over the GBM occurs more easily than cation transport, because the latter will continuously interact with the fixed negative charges of basement membrane components. This transport imbalance will result in the accumulation of

negative charge at the urinary space side of the GBM. In steady-state conditions, this will lead to a charge gradient with the highest negative charge downstream in the GBM. The increasing negative charge provides the restriction to negatively charged molecules such as albumin and, according to this model, explains the glomerular filtration characteristics. The observation that the concentration of molecular tracers was higher in the subendothelial than the subepithelial part of the GBM (193,216) has been explained to support this view of an increasingly restrictive barrier, although other explanations are possible for this finding.

Because ion transport takes place by convection, the formation of the charge gradient is dependent on flow. Flow, as described above, is dependent on the filtration pressure characteristics in the glomerulus: the combined pressure and osmotic forces that drive the fluids out of the capillaries minus those that would drive fluids in. Halfway the glomerulus the in- and outward forces are similar ("raising the question why we have an efferent capillary in the first place"), resulting in a filtration pressure equilibrium. Consequently, in the glomerular capillaries downstream of the filtration pressure equilibrium, there will be no flow and thus no negative charge will build up. This would result in a loss of albumin by simple diffusion through the GBM. To explain the fact that this does not seem to occur, Somers suggests that the podocyte, by crossing the space between different capillaries, may transport the negative charge built up in afferent capillaries to efferent capillaries. This is compatible with the negatively charged surface of the podocyte's major and foot processes that would prevent a loss of charge through an 'electric shortcut'.

One of the predictions of this model is that proteinuria will occur in situations where GFR is decreased, because the subepithelial negative charge will not build up completely. Indeed, Rippe et al recently found that the sieving coefficient of Ficoll is increased (meaning that Ficoll is less effectively retained by the glomerular barrier) in low GFR conditions (217).

Permeation/diffusion hypothesis

In the Permeation/diffusion hypothesis (212), the size-selective properties of the glomerular filtration barrier are thought to reside in the GBM. Smithies assumes that the GBM can be viewed as a gel, and argues that – as in all gels – only a limited fraction of gel space is available for macromolecules, determined by factors such as concentration of the gel and radius of the macromolecules. The small amounts of macromolecules that can permeate into the gel are subsequently transported across the GBM by either flow or by diffusion. With regard to the GBM-gel, the fraction available for albumin is about 0.02. Smithies calculates that the main means of macromolecular transport in the glomerulus is diffusion. In contrast, transport of water and small solutes depends on flow. Changes in flow have no effect on the diffusion, and will thus have only a minimal effect on the amount of albumin in Bowman's space. However, the concentration of albumin is greatly influenced by flow. A decrease in glomerular flow (GFR) will lead to an increase in albumin concentration, saturating the tubular albumin reabsorption mechanisms and thus leading to

albuminuria. In other words, the transport of albumin into Bowman's space is fairly constant, but the absence of the diluent is responsible for proteinuria. In this view, albuminuria is not necessarily the result of a pathologic change in the glomerular filtration barrier, but may be the physiologic result of a decrease in GFR. On the other hand, changes in the glomerulus do induce albuminuria if they increase the hydrodynamic resistance of the filtration barrier, as for example in podocyte foot process effacement (218), or if they change the properties of the GBM (219). The experiments by Rippe et al, showing increased sieving coefficients for Ficoll in low-GFR conditions, are in line with this theory of glomerular filtration.

In general, rodents have a relatively higher urinary protein excretion than humans. This could possibly be explained to be in line with the permeation diffusion theory: if the protein concentration is determined by the diffusion of albumin and the filtration of the diluent, the factors that determine these two variables are, respectively, the surface available for diffusion, and the amount of filtrated fluids. Human glomeruli are larger than those of rodents, and thus the diffusion surface and fluid content of human glomeruli is also larger than that of rodents. But diffusion surface increases with a factor to the power 2, while fluid content increases with a factor to the power 3. Thus more diluent is available, suggesting that larger glomeruli would elaborate a more dilute pre-urine.

The permeation-diffusion theory does not readily explain the proteinuria seen in situations with a normal GFR and normal foot process morphology, as has been observed in animal models and sporadic human cases (85,103,143,220).

Absence of glomerular permselectivity

The most extreme view of glomerular filtration is that there is no, or at least very limited glomerular permselectivity, a hypothesis that has been fueled by several studies of Comper and coworkers. Their hypothesis explains the inconsistencies that remain in the other theories concerning glomerular selectivity, but brings up another problem: if the glomerulus would filter 'nephrotic levels of albumin' (221), this would result in an albumin excretion of about 600 g per day (202). To explain the fact that only limited amounts of this reach the urine, Comper et al have hypothesized that there is a high capacity albumin retrieval pathway in the proximal tubulus. Over the years, Comper et al have brought evidence for the absence of glomerular size and charge selectivity (205), the presence of tubular retrieval pathways for example through tubular transcytosis (221), and have raised a hypothesis to explain proteinuria seen in glomerular diseases (222). Although the potential interest of their findings is acknowledged (223), the methods of the experiments have been criticized (14,43,202,223,224) and their results could not be reproduced (190).

Synthesis

Most reviews on the subject of permselectivity move towards the recognition that the three layers of the glomerular filtration barrier are functionally interdependent, making it difficult to attribute

a more important role to one of the three (225). Indeed, damage to any of the three layers can be associated with the development of proteinuria (see below). Although intuitively true – why else would the glomerular capillary wall be endowed with its special properties – this view leaves the question as to how exactly the glomerulus elaborates its low-protein ultrafiltrate unanswered.

From Bowman's space, the glomerular ultrafiltrate empties into the tubular lumen, where its composition is modified on its way to the collecting ducts. The tubular system will be discussed next.

The tubular system

Each glomerulus is connected to a ~5 cm long renal tubule that is built of a proximal, intermediate, and distal segment. The proximal segment starts as a contortuous tube, containing cells with a prominent brushborder, and connects to a straight part. This part of the proximal tubule forms the transition to the intermediate segment that loops through the medulla as Henle's loop. Ascending from the medulla, Henle's loop connects to the distal convoluted tubule that on its path to the collecting duct makes contact with the glomerular arterioles at the macula densa (226).

The different segments of the tubule contain cells with a different morphology and accordingly different functions. The main function of proximal tubular cells is reabsorption of water and the dissolved salts and small molecules of the ultrafiltrate. To this end, the cells are equipped with microvilli that provide a ~40 times increase in apical surface area. At the beginning of Henle's loop, the amount of ultrafiltrate has been reduced to about 70 percent. The cells in Henle's loop further reabsorb water and salts, with a predominance of salts, thereby increasing the medullary salt concentration. This forms the basis for the concentration of urine that is eventually performed in the collecting ducts. The distal collecting duct further reabsorbs NaCl as well as calcium. Water is less efficiently reabsorbed in this segment, resulting in a hypoosmotic fluid that is delivered into the collecting duct.

The reabsorption and excretion mechanisms are extensively regulated throughout the course of the tubules by hormonal factors. Of further interest for the regulation is the juxtaglomerular apparatus, in which the macula densa of the distal segment takes part. This apparatus is – via incompletely elucidated mechanisms – responsible for the so-called tubulo-glomerular feedback that regulates the glomerular filtration in relation to the distal tubular output. The exact transport and regulation mechanisms at play in the tubular system will not be further dealt with in this

introductory chapter. In the interest of a discussion on the effects of filtered proteins on tubular epithelial cells, the mechanisms of tubular protein resorption will be discussed in some more detail.

Proteins are mainly reabsorbed in the proximal tubules through receptor-mediated endocytosis. The main receptors for albumin in the proximal tubule are cubulin and megalin. Both are multi-ligand endocytic receptors that are able to bind albumin. Binding of albumin to these receptors is followed by endocytosis and transport of the endocytic vesicles to the lysosome. Here albumin is degraded into amino acids that are subsequently excreted in the PTCs, while the receptors are recycled to the apical membrane (227,228). One research group has found that other ways of albumin transport may exist, namely through transcytosis (221). The endocytosed albumin is transported through the cell and delivered undegraded into the circulation. This way of albumin transport awaits further confirmation.

The tubular epithelial cells manufacture and rest on a basement membrane (TBM) of collagen IV (isoforms $\alpha1\alpha1\alpha2$), laminins (111 and 511), and HSPGs (mainly perlecan).

Interstitium and the extracellular matrix

Between tubuli and glomeruli is the interstitium, which contains the blood vessels, peritubular capillaries (PTCs) as well as lymphatics. These vascular structures are embedded in a loose extra-cellular matrix, produced by the interstitial fibroblasts. Other interstitial cell types include macro-phages and – more recently described – dendritic cells that form a network surrounding the tubuli and glomeruli (229,230).

The normal interstitial matrix contains collagens type I, III, V, VII, fibronectin, and tenascin. These molecules are present only in small amounts, for in the normal kidney cortex the interstitial space is limited: tubuli are normally positioned side by side, and the intervening PTCs fill most of the remaining space, often fusing their basement membranes with the TBM, at least optically.

Lymphatics accompany the larger arteries of the kidney, but are otherwise not found in the normal interstitium.

PART 2 CAUSES AND CONSEQUENCES OF PROTEINURIA

Proteinuria, the presence of abnormally high amounts of plasma proteins in the urine, is a symptom associated with many different kidney diseases. Although the causes of proteinuria are diverse, the consequence of proteinuria in different diseases is similar, in that it confers an increased risk of loss of kidney function, in association with an increased risk of cardiovascular complications. This, together with the fact that the development of proteinuria may be modifiable, makes it an important risk factor in both native and transplant renal disease. In this paragraph, a brief overview of the epidemiology of proteinuria and the kidney diseases in which proteinuria is seen is provided. The paragraph focuses on the causes and consequences of proteinuria on the histopathological and molecular levels. The paragraph ends with a more general discussion of the mechanisms implicated in the progression of renal disease.

Epidemiology

Several large population screenings have established the epidemiological characteristics of proteinuria. In the district of Okinawa, Japan, more than 100,000 individuals were screened for proteinuria and follow-up was recorded for more than 20 years (231). From these studies it became clear that proteinuria is an important independent predictor of the development of end stage renal disease. In Groningen, The Netherlands, a large population study, the PREVEND study, was performed in which about 40,000 persons were screened for albuminuria (232,233) (www.prevend.org). These studies made clear that in 6.6 percent of the otherwise healthy population microalbuminuria was present. Furthermore, not only was microalbuminuria found to be associated with the progression of renal disease, it also was an important predictor of cardiovascular diseases (232,233). Several other epidemiologic studies have found a strong correlation between proteinuria and the progression of renal disease (234-236). Based on this association, in 2006 the Dutch Kidney Foundation started the 'Niercheck', a national campaign to detect kidney damage in an early, preclinical stage on the basis of macroalbuminuria measurements (www.nierstichting.nl).

Causes of proteinuria

Proteinuria can either be caused by a failure of tubular reabsorption of the small amounts of filtered proteins, or by a loss of glomerular permselectivity, leading to larger amount of proteins in the pre-urine that saturate the tubular retrieval systems.

Examples of the first include the Fanconi syndrome, in which there is a dysfunctional proximal tubular reabsorption mechanism (187). Such 'tubular proteinuria' usually results in only mild increases in the protein content of the urine. Instead, glomerular proteinuria can be associated with large increases of albumin and other proteins in the urine, and may lead to the development of a nephrotic syndrome (see below).

Glomerular proteinuria can be associated with damage to all different levels of the glomerular filtration barrier, the endothelium, the GBM, and the podocyte.

For example, preecclampsia is associated with endothelial cell damage and profound proteinuria. In the glomerulus, swelling of endothelial cells (endotheliosis) is seen. Pathogenetically, the development of preecclampsia has been linked to the excess production of a soluble VEGF receptor (soluble Fms-like tyrosine kinase, sFlt), that binds and inactivates VEGF. In the glomerulus, VEGF-A produced by podocytes may therefore not reach the endothelial compartment in sufficient amounts, leading to a loss of endothelial viability (21,237,238). Enzymatic breakdown of the endothelial glycocalyx resulted in an increased permeability for proteins that was mainly due to a loss of charge selectivity (239).

The correct molecular make-up of the GBM is crucial for a normal filtration barrier; impaired assembly of the GBM components leads to proteinuria or an otherwise more permeable GBM. This is most clearly underscored by hereditary syndromes that have been associated with mutations in genes coding for GBM components. Patients with Alports syndrome carry a mutation in one of the collagen chains of the mature GBM. Alports syndrome is characterized by hematuria, proteinuria, and loss of renal function (240). Similarly, in nail-patella syndrome, a malfunction of the transcription factor Lmx1b leads to an aberrant collagen IV assemblage, followed by nephrotic syndrome (241). Also, incorrect assembly of the GBM matrix components as seen in the ILK knockout mice (139), is related to the development of proteinuria.

A laminin β2 chain knockout mouse was shown to develop severe proteinuria, despite normal glomerular development and continued expression of the fetal laminin β1 chain (242). The laminin 521 composition thus appears crucial for the correct function of the GBM. In retrospect, the laminin β2 knockout mouse appeared to accurately mimic the characteristics of Pierson's syndrome, a hereditary nephrotic syndrome that was described by Pierson et al in 1963 (243).

Recently, it was recognized that the cause of Pierson's syndrome is a mutation in the gene coding for laminin β2 (244,245).

Strikingly, mice that in a podocyte-specific fashion lack expression of perlecan (200) or large parts of agrin (197,199) – the major heparin sulphate proteoglycans of the GBM – show no proteinuria, although perlecan null mice seem to be more susceptible to glomerular damage (246). Likewise, mice that lack Ext, the protein responsible for heparan sulphate biosynthesis, in podocytes are not proteinuric (201). Also, in vivo degradation of heparan sulphates in the GBM does not cause proteinuria (198). As discussed before, this indicates that the charge barrier of the GBM may be of less importance for the correct function of glomerular permselectivity than previously thought.

Damage of podocytes is almost always accompanied by proteinuria. There are several ways in which podocytes can be damaged, including through genetic, inflammatory, and infectious diseases, through growth factors, cytokines, medication, and mechanical stress. Several of the hereditary forms of proteinuria have already been discussed, as they have been closely related to the elucidation of the cell biology of the podocyte. The currently known forms of hereditary proteinuria are listed in table 1. Other forms of podocyte damage will be illustrated in the description of proteinuria that occurs in various kidney diseases.

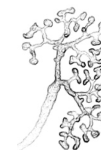
Causes of proteinuria in various kidney diseases

Proteinuria is a symptom of many kidney diseases and is especially seen in diseases characterized by a glomerular involvement. These comprise among others minimal change disease, focal segmental glomerulosclerosis, diabetic nephropathy, membranous nephropathy, membranoproliferative nephropathy, and lupus nephritis. In these diseases, proteinuria can occur as a component of the nephrotic syndrome, characterized by a loss of more than 3,5 gram of protein per 24 hours (nephrotic range proteinuria), edema, and hypoalbuminemia.

How does the development of proteinuria in these different diseases relate to the different processes discussed above?

Minimal change disease

Minimal changes disease (MCD), also termed minimal lesion or lipoid nephrosis, is the main cause of proteinuria in children, and the third most common cause of nephrotic syndrome in adults (247). The presentation is that of a nephrotic syndrome, not infrequently following infections or vaccinations. The condition is generally responsive to treatments with steroids, although



patients may show relapses, and some may even be steroid resistant. In such cases FSGS should be suspected.

The histopathology of MCD, as the name implies, does not show gross abnormalities on light microscopy. On electron microscopic evaluation, extensive effacement of podocyte foot processes is seen. The pathogenesis of MCD remains unknown, although several mechanisms have been hypothesized (247). A T-cell derived permeability factor has been suggested to induce the development of MCD (248), although definitive proof for this is lacking. Several cytokines have been shown to be upregulated in MCD, and these could act via cytokine receptors that have been found to be expressed by podocytes (249). Van den Berg et al (250) found that through the action of IL-4 and IL-13 on podocytes, the activity of the proteases cathepsin L and heparanase in the GBM is increased. This may subsequently lead to a degradation of heparan sulphate in the GBM, leading to proteinuria. Indeed, alterations of the heparan sulphate moieties in the GBM have been repeatedly found in MCD (251).

The group of Bakker and coworkers has postulated that hemopexin might be the circulating factor that is responsible for the development of MCD. Injection of hemopexin in rats resulted in a disorganization of the anionic sites at the subendothelial side of the GBM, and transient proteinuria (252).

Table 1. Hereditary forms of proteinuria

Gene	Disease	Protein	Glomerular morphology	References
NPHS1	Congenital Nephrotic syndrome of the Finnish type	Nephrin	FSGS	(88)
NPHS2	Steroid resistant nephrotic syndrome	Podocin	FSGS	(111)
ACTN4	FSGS1	α-actinin-4	FSGS	(56)
TRPC6	FSGS2	TRPC6	FSGS	(112,113)
CD2AP	FSGS3	CD2-associated protein	FSGS	(502)
PLCE1	Nephrotic syndrome	Phospholipase Cε1	DMS	(503)
LMX1B	Nail-patella syndrome	Lmx1b	DMS, FSGS	(181,182,241)
WT1	Isolated DMS, Denys Drash syndrome, Frasier syndrome, WAGR	Wilms tumor protein 1	DMS/FSGS	(504)
LAMB2	Pierson's syndrome	Laminin β2	DMS	(245)

FSGS – focal segmental glomerulosclerosis; DMS – diffuse mesangial sclerosis; WAGR – Wilms' tumor, aniridia, genitourinary malformations, mental retardation.

Others have attributed a more direct role to the podocyte in the development of MCD. Patrakka et al found that the slit diaphragm is often lacking in patients with MCD (253). Regele et al (142) found that the expression of dystroglycan was decreased in minimal change disease in comparison to FSGS. Recognizing the role of podocyte-associated proteins in hereditary proteinuric syndromes, several investigators have studied the expression of these proteins in MCD (254-257). The results varied, but seem to be most compatible with a secondary change rather than primary involvement in the development of proteinuria in MCD (258,259). In a scenario in which the podocyte is the key player in the development of MCD, the fact that this disease is responsive to steroids could be explained by the steroid responsiveness of podocytes (157).

Focal segmental glomerulosclerosis (FSGS).

FSGS is also characterized by nephrotic range proteinuria, often in the setting of a nephrotic syndrome. In contrast to MCD, the proteinuria is mostly refractive to steroid treatment, and comes with a progressive decline in renal function that eventually may necessitate transplantation. The histopathology of FSGS is characterized by scarring of some of the glomeruli (focal), in a way that involves only limited parts of a glomerulus (segmental). The lesions that are seen in FSGS are diverse, and recently a classification has been made to describe the different forms of FSGS (260). In this classification, five patterns of FSGS are distinguished on the basis of their morphologic features, namely the collapsing, tip lesion, cellular, perihilar, and not otherwise specified variants of FSGS. Diagnosis of one of the variants requires exclusion of the previous variants in the order listed here. These different variants of FSGS may coexist in a single renal specimen (38), and it is unclear whether the classification on morphologic basis relates to different pathophysiologic mechanisms. The first studies that evaluate the clinical implications of the classification do show differences between the variants; for example, the collapsing variant is associated with a population that differs in demographics from that of the other variants, and clearly carries a worse prognosis (261).

FSGS should not be regarded as a disease in itself, but rather as the stereotypic histomorphological representation of different specific diseases. These include hereditary diseases, obesity, hypertension, viral infection, medication, and mechanical stress. A substantial percentage of cases of FSGS, however, are of unknown origin, the so-called primary or idiopathic forms of FSGS.

In diseases that lead to FSGS, the secondary forms of FSGS and hereditary syndromes, podocyte injury has been shown to be a central step in development of FSGS and the associated proteinuria (262). In hereditary cases, the mutated genes often code for proteins with a more or less specific expression in podocytes, as discussed before. In animal models, direct damage to podocytes, for example through the injection of puromycin aminonucleoside, leads to proteinuria and subsequently development of FSGS. Medication like cyclosporine and pamidronate may damage the

podocyte; the latter gives rise to a collapsing variant of FSGS with high proteinuria (263). Podocytes have been shown to be a target of infection by HIV (264), which may lead to HIV-associated nephropathy, a disease characterized by rapid decline in renal function, high proteinuria, and FSGS of the collapsing type (265). FSGS can be seen in hypertensive patients. In these cases, the mechanical stress put on podocytes through the increased intraglomerular pressure may lead to damage of podocytes. Indeed, cultured podocytes have been found to be stress-responsive (266). Protein overload also leads to the development of FSGS (267,268). Consequently, not only damage to the podocyte, but also other mechanisms that cause increased passage of proteins through the glomerular filtration barrier may, via podocyte injury, lead to FSGS.

Despite this progress in the elucidation of the secondary FSGS, the etiology of the primary forms of FSGS remains elusive. Much research has been done to identify a putative humoral permeability factor. Such a factor would explain the high rate of FSGS recurrence in patients that receive a renal transplant because of FSGS. Also, a permeability factor would explain the transmission of a proteinuric condition from mother to her unborn child (269). Several groups, most notably that of Savin and coworkers, identified characteristics of this permeability factor, but the ultimate structure and origin remain unknown (262,270). Others have supposed that not the presence of a permeability factor, but the absence of crucial plasma factors lead to the development of proteinuria and FSGS. The hypotheses about the pathophysiologic mechanisms again incorporate the podocyte as a crucial target. Coward et al tested the influence of plasma of normal and nephrotic patients on the distribution and signaling of slit diaphragm proteins in cultured podocytes, and found that the effect of nephrotic plasma could be abrogated by adding normal plasma (271). Wei et al found that the plasma concentration of soluble urokinase receptor (uPAR) is elevated in serum of patients with recurrent FSGS (Wei et al, J Am Soc Nephrol 2008(19):103A). In mouse models, uPAR signaling in podocytes has been shown to cause foot process effacement and proteinuria (272), but mechanistical evidence for a pathogenic role of soluble uPAR in human FSGS is lacking.

Irrespective of the cause of proteinuria, podocyte injury is a crucial step in the further development of FSGS. Using combined observations from different animal models, Kriz (273) has described a sequence of events that explains the development of FSGS: podocyte injury leads to loss of podocytes, which results in denuded parts of the GBM and hypertrophy of the remaining podocytes. This will increase the possibilities for the formation of adhesions of the podocytes or the GBM to the parietal epithelial cells. Once such an adhesion has been formed, this leads to an encroachment of the parietal cells on the capillaries, and to 'misdirected filtration', the delivery of glomerular filtrate to the space between Bowman's capsule and the overlying parietal epithelial cells.

Despite the initial pivotal role of the podocyte, the further development of the FSGS lesions does involve other glomerular cell types. Kihara et al and Nagata et al suggested that in the collapsing and cellular variants of FSGS, the proliferating cells are of parietal origin (274,275). In a series of studies, Smeets et al and Dijkmans et al have recently brought more evidence for the involvement of parietal epithelial cells in the development of collapsing FSGS in animal models (35), human idiopathic FSGS (38), as well as HIV and pamidronate associated collapsing FSGS (39).

Taken together, the pathogenetic mechanisms in secondary FSGS all converge on podocyte damage as a central initiating step of development of proteinuria. In primary causes of FSGS, the initial pathogenetic mechanisms of proteinuria remain unclear, although a contribution of the podocyte is to be expected, and certainly this cell is at play in the later development of FSGS.

Diabetic nephropathy

Diabetic nephropathy is a complication of both type I and type II diabetes, and is currently the main cause of end stage renal diseases. Clinically, diabetic nephropathy is characterized by a decline in renal function that is preceded or accompanied by the development of proteinuria. Microalbuminuria is an important risk factor for the progression of the disease, and is currently used to screen patients at risk for development of diabetic nephropathy (276).

The histopathologic features of diabetic nephropathy comprise glomerular hypertrophy, thickening of the GBM and mesangial expansion in early stages of the disease. Later phases are characterized by the presence of glomerulosclerosis, sometimes showing the pathognomonic Kimmelstiel-Wilson nodules, together with interstitial fibrosis.

Mesangial cells as well as podocytes have been implicated in development of glomerulosclerosis in diabetic nephropathy, and especially cytokines and growth factors, such as TGF-β, connective tissue growth factor (CTGF), and insuline like growth factor have been extensively studied. With regard to the proteinuria in diabetic nephropathy, different components of the glomerular filtration barrier have received attention, and all seem to be affected by the diabetic condition. The thickening of the GBM may relate to a dysregulated assembly of matrix components, which translates into dysfunction. The endothelial cells are damaged in the diabetic milieu, through both metabolic and hemodynamic factors (277). In recent years, most attention has been given to the role of podocyte injury and podocyte loss in diabetic nephropathy. The attention for the role of the podocyte has been fueled by the seminal observation by Pagtalunan et al (278), that in Pima Indians with type II diabetes glomeruli show a marked decrease in podocyte number, a feature now called ‘podocytopenia’. Podocyte damage in diabetic nephropathy can be induced by different factors, including high glucose per se (279), oxidative stress, the altered glomerular hemodynamic conditions (280), advanced glycation end products (281), growth factors such as TGF-β (279), and an increased activity of the local renin-angiotensin system (282,283). The

podocytes may subsequently be lost through different mechanisms. For example, increased TGF-β signaling has been linked to podocyte apoptosis (124). Also, the adhesion of podocytes to the GBM may be diminished as a result of a decreased integrin expression (284). These loosely attached podocytes may shed into the urine, and podocytes, as well as podocyte proteins, can be recovered from urine of diabetic patients (285,286). Furthermore, the podocyte may show functional deficits, as is exemplified by a decreased nephrin expression and foot process effacement. Many of the deleterious factors in the diabetic milieu affect the podocyte through stimulation of local angiotensin II production. Stimulating podocytes with angiotensin II in vitro leads to upregulation of TGF-β, providing a link to podocyte apoptosis (287). The effect of angiotensin II on the expression of nephrin and other slit diaphragm-proteins has been studied. A decreased immunostaining for these proteins in the presence of elevated angiotensin II levels has been described (283,288,289). These findings also explain the notion that in diabetic nephropathy angiotensin converting enzymes and angiotensin II inhibitors have a beneficial effect that cannot be explained solely by systemic blood pressure normalization.

Through podocyte loss, mechanisms are set in motion that further aggravate the proteinuria and glomerular damage. For example, Baelde et al (126) suggested podocyte loss as the cause of decreased glomerular VEGF expression, and hypothesized that this may infringe on the endothelial maintenance.

Membranous nephropathy

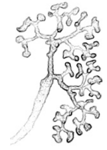
Membranous nephropathy is characterized by its histopathologic presentation, namely deposition of subepithelial immune complexes along the GBM in a granular fashion, without inflammation. In response to this, the podocyte forms and deposits GBM components between these immune complexes, visible as 'spikes' in light microscopy. Proteinuria, usually in the nephrotic range, is the most frequent symptom. The clinical course is related to the severity of the proteinuria, with frequent progression to end-stage renal disease in high-proteinuric individuals. Although several diseases, including malignancies, are associated with the development of membranous nephropathy, most cases are idiopathic.

Most of the knowledge about the pathogenetic mechanisms of proteinuria in membranous nephropathy has been derived from studies in an animal model that closely mimics this disease, Heymann nephritis (290). In this model, antibodies directed against the rat podocyte protein megalin (see above) give rise to the formation of immune complexes at the podocyte basal membrane that are subsequently shed and deposited in the lamina rara externa of the GBM. Here, these immune complexes activate complement, and the C5b-9 complex causes damage to the podocyte. Apart from causing direct damage, the C5b-9 complex also activates podocytes, resulting in the formation and release of proteases and reactive oxygen species (146,291). These components

damage the GBM as well as the podocyte itself through lipid and protein peroxidation. Furthermore, there are several intracellular consequences that result from the damage of podocytes by complement, including DNA damage (292) and cytoskeletal reorganization (293). The latter also influences the expression and distribution of nephrin and podocin (294,295), and presumably alters the function of the slit diaphragm. The activated podocytes also increase their production of GBM components, but it has been suggested that the assembly of this extracellular matrix is abnormal (171,296). Consequently, the proteinuria that is seen in the Heymann nephritis model has been explained by 1) changes in the GBM through abnormal assembly, increased breakdown, and damage through reactive oxygen species; and 2) damage to the podocyte, diminishing its adhesive properties to the GBM, disrupting the organization of the slit diaphragm, increasing podocyte DNA damage and apoptosis.

It is likely that in human membranous nephropathy, similar pathogenetic mechanisms are at play. Indeed, the role of reactive oxygen species has been suggested by a small clinical study that showed beneficial effects of the antioxidant probucol in patients with membranous nephropathy (297). However, in most cases, the antigen responsible for the development of this sequence of events is still elusive. The one exception is the antigen that is responsible for a rare congenital form of the disease, described by the group of Ronco in 2002 (148). They found that antenatal membranous nephropathy is caused by antibodies directed against neutral endopeptidase (NEP), an enzyme expressed at the podocyte membrane but also in the placenta. Anti-NEP antibodies developed in mothers that were NEP-deficient due to a mutation in the MME gene, but were exposed to the protein of fetal origin during pregnancy. In a subsequent pregnancy, the antibodies could cross the placenta and induce the disease (147,148). Recently, a preliminary report suggested that the phospholipase A2 receptor could be the target antigen in a subset of patients with membranous nephropathy (Beck et al, J Am Soc Nephrol 2008(19):104A).

If the pathogenetic mechanisms that have been revealed in animal models would also apply to humans, this could be of particular interest in patients that have developed membranous nephropathy secondary to a malignancy. These patients have apparently developed antibodies directed against components of the tumor that also react with normal components of the glomerulus. In the glomerulus, these antibodies are able to activate complement, thus it could be suspected that the same antibodies could have anti-tumor effects. It would be of interest to evaluate the clinical course of patients that have developed membranous nephropathy in comparison to those with the same type of cancer, but without membranous nephropathy development.



Consequences of proteinuria

Effects of proteinuria on the glomerulus

The effects of proteinuria on the glomerulus are often difficult to distinguish from causes of proteinuria. Injury to podocytes may, as discussed above, lead to proteinuria, but also one might envision harmful effects of increased protein passage on podocytes. Adding to this difficulty is the notion that factors that give rise to proteinuria may in addition lead to histopathologic changes, but without a causal relation between the two. A disturbed GBM make-up, for example, may lead to proteinuria and podocyte changes independently.

Cause and effect discussions aside, there are some clear histopathologic observations in proteinuria, the most consistent being that proteinuria is almost always accompanied by profound changes in podocyte morphology. The highly organized cellular podocyte architecture is lost, transforming the podocyte into a flattened, simplified epithelial cell. Effacement of foot processes takes place through widening and shortening, and at the base of the flattened foot processes, a dense band of actin filaments is seen (298). Podocyte hypertrophy is often observed, together with microvillous transformation and pseudocyst formation. Also, protein reabsorption droplets that contain plasma proteins are seen in the podocyte cytoplasm. There is often an increased or renewed expression of mesenchymal – and thus for podocytes embryonic – markers, and according to Kerjaschki it may be concluded that in proteinuria 'podocytes recapitulate their development in reverse' (50). There is some discussion as to whether the extent of foot process effacement is related to the amount of proteinuria, with some studies indicating a positive relation (259,298), while others did not find such a correlation (299). Foot process effacement influences the ultrafiltration coefficient, and there is a correlation between the amount of foot process effacement and the GFR (218). Consequently, according to Smithies, the decrease in GFR may result in proteinuria (212). In most cases, however, podocyte foot process effacement seems to accompany the development of proteinuria, sometimes lagging behind.

Abbate et al studied the changes in podocytes in a rat model of renal mass reduction, and described that the changes in the podocytes related to the amount of protein accumulation in podocytes in vivo, and that anti-proteinuric therapies proved to be helpful. In vitro, stimulation of podocytes with albumin led to an increases production of TGF-β (267). In a follow-up study, the same group found that protein overload led to the upregulation of the noxious molecule endothelin-1 via dysregulation of the podocyte cytoskeleton (53). In this way, proteinuria per se may be the podocyte-damaging factor that leads to loss of podocytes, and to a sequence of changes as described before under focal and segmental glomerulosclerosis (300).

Effect of proteinuria on the tubulointerstitial compartment

The pathophysiology of the consequences of proteinuria for the tubulointerstitial compartment has been more clearly delineated. Again, it has been difficult to indicate to what extent the amount of proteinuria is a marker of the damage, instead of the actual cause of it (301). This question has been addressed using the amphibian axolotl as a model system. Some nephrons in the kidneys of these amphibians are connected to the peritoneal cavity, while others are closed. Gross et al found that upon injection of fetal bovine serum in the peritoneal cavity of these animals, only the tubular epithelial cells of the nephrons with a connection to the peritoneal cavity were activated and showed interstitial fibrosis (302). This suggests a causal role for increased protein passage in tubulointerstitium injury, and supports hypotheses based on more correlative evidence.

Several mechanisms may be responsible for the toxic effect of proteinuria on tubules. For example, protein cast formation may lead to obstruction of tubuli (301), filtered proteins such as complement factors may be toxic to the tubular epithelial cells, and cytokines may directly activate the tubular epithelium (303). Most attention has been given to uptake of proteins by tubular epithelial cells. During proteinuria, multiple macromolecular components reach the apical side of the tubules. Which one of these is harmful is a matter of discussion. Below is a description of the factors that are held responsible for the toxic effects of proteinuria on the tubulointerstitial compartment, and the molecular pathways through which such an effect is mediated.

The toxicity of albumin has received a lot of attention. In vitro, incubation of tubular epithelial cells with albumin has a profound effect: the endocytosed albumin, either directly or via the protein kinase C-dependent formation of reactive oxygen species (304), activates several signaling proteins, including mitogen-activated protein kinases (305), nuclear factor κB (306,307), and signal transducer and activator of transcription proteins (308). This activation of tubular epithelial cells results in the modulation of cytokine production and ECM regulation: albumin upregulates the tubular expression of interleukin-8 (307), macrophage chemoattractant protein-1 (306), RANTES (309), and endothelin-1 (310); it increases the expression of different collagens (311), profibrotic cytokines such as TGF-β (312), and tissue inhibitors of metalloproteinases (TIMPs) (313). Also tubular production of angiotensin II is increased (314). These changes lead to an interstitial inflammatory response, and promote the development of interstitial fibrosis. Furthermore, protein overload has been shown to directly lead to increased tubular apoptosis (315).

However, there are reports that failed to show a direct effect of albumin on tubular epithelial cells (316). For example, Burton et al found that although plasma had a clear effect on cytokine production and ECM regulation, purified albumin failed to induce this effect (317,318). This suggested that the substances bound to albumin, but not albumin itself, promotes the aforemen-

tioned cellular changes. Indeed, several experiments have shown an effect of albumin-bound free fatty acids (319,320).

Proteinuric Nagase analbuminaemic rats do develop tubulointerstitial fibrosis in the absence of albumin (321). This, together with other observations, has suggested that other proteins may also be responsible for the toxic effect of proteinuria on the tubular epithelial cells. These include transferrin, immunoglobulins, cytokines, growth factors, and complement (301). For example in membranous nephropathy, the C5b-9 complex may bind to the tubular apical surface and damage these cells (293). Also, filtered complement components may be activated locally via the alternative pathway, and tubular epithelial cells also synthesize complement in response to protein overload (322).

Most of the insight into the consequences of exposure of tubular epithelial cells to increased amounts of plasma proteins has been derived from in vitro studies. These studies have been criticized because they use protein concentrations that are outside the physiologic range. However, as stressed by Remuzzi (323), the protein concentration in vivo may be low in Bowman's space, but increases as the pre-urine passes along the nephron and water is absorbed. Also the duration of the in vivo exposure is usually longer than in cultured cells.

Taken together, several components that are present in excessive amounts during proteinuria seem to act on the tubular epithelial cells, thereby creating a pro-inflammatory and pro-fibrotic milieu. Together with tubular atrophy and apoptosis, interstitial inflammation and fibrosis form the histopathological representation of progressive renal diseases, for which mechanisms are discussed in a later paragraph.

Cardiovascular risk

The nephrotic syndrome comes with changes in the blood lipid profile (hyperlipidemia and hypercholesterolemia). Such changes are related to the development of atherosclerosis. However, also low levels of albuminuria, even in the high normal range, increase the risk for cardiovascular disease. How can the effect of microalbuminuria on the cardiovascular system, most notably atherosclerosis, be explained at a pathophysiological level? This is a question that remains largely unanswered. In a review on the different possibilities to explain the clear epidemiologic link between the two, Stehouwer and Smulders (324) propose that atherosclerosis and microalbuminuria may be the result of a common pathophysiologic process, such as endothelial dysfunction. This hypothesis, modified from the Steno hypothesis that places more emphasis on systemic changes in basement membrane composition (325), is of interest in the context of an increasing appreciation of the importance of the endothelium in the permselectivity of the glomerulus. Another pathophysiological explanation is that mutations in genes that cause proteinuria also affect the heart, as may be the case with NPHS2 (326). However, it is unlikely that this explains

the cardiovascular risk in the majority of patients that do not have similar mutations. Furthermore, a systemic proinflammatory state associated with nephrotic syndrome may provide a link to cardiovascular changes (327).

Progression of renal disease

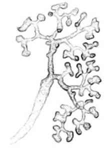
The kidney has a stereotypic reaction to injury

Regardless of the initial cause of renal disease, the histopathologic lesions that parallel the decline in renal function include glomerulosclerosis, tubular atrophy, interstitial fibrosis, and interstitial inflammation. This has given rise to the widely accepted notion that the progression of renal damage involves a common mechanism. Ideas about the nature of this mechanism and, accordingly, the temporal relationship of the histopathological lesions (e.g., does the inflammation precede or follow the development of fibrosis) vary, as discussed below in more detail.

Next to common histopathological features, the different renal diseases, both in native and transplant nephropathies, share risk factors for progression. These include proteinuria and hypertension. Proteinuria as a risk factor for native kidney diseases has already been discussed. Proteinuria has a comparable detrimental effect in the transplantation setting. Several groups found that even low grade proteinuria in renal transplant patients is an independent predictor of graft loss, and anti-proteinuric therapies have a beneficial effect on graft survival (328-330). Zayas et al measured dextran sieving coefficients in allografts and found that even in well-functioning grafts, there is some loss of glomerular permselectivity (331), and suggested a link between this glomerular leakiness and progression of transplant pathology.

Hypertension represents another risk factor for progression of both native and transplant renal disease (332). Haroun et al found a relationship between the severity of hypertension and the risk to develop end stage renal disease (333). This is also true in the transplantation setting (334). Proteinuria and hypertension seem to amplify each others effect (330,332). The pathophysiologic explanation for this interaction will be outlined below.

Of all the histopathological characteristics of progressive kidney disease, the lesions that correlate most closely with renal function loss are those found in the interstitium (335,336). Interestingly, a substantial portion of renal disease primarily affects glomeruli. The link between the damage in these two tissue compartments and the role of the mentioned risk factors therein will be described in the following paragraphs.



How does glomerular injury lead to tubular damage

There are several theories about the mechanisms that connect glomerular damage to tubular injury and interstitial fibrosis (303,336,337).

1) In a previous paragraph, the hypothesis that proteinuria has a central role in the connection between glomerular and tubular injury has been discussed. This so-called Remuzzi theory attributes a toxic effect of the increased amount of proteins filtered by the glomerulus on the tubular epithelial cells, resulting in a cellular reaction that promotes influx of inflammatory cells and fibrogenesis (234,235,322).

2) In a series of studies, Kriz has lined out another sequence of events that connects glomerular damage to tubular injury and nephron loss (303,338-340). The starting point is damage to or loss of podocytes that predisposes for the formation of an adhesion of the naked GBM to Bowman's capsule. Capillaries in these adhesions may still be patent, at least for some time, leading to filtration of plasma products into the space between the parietal epithelial cell and their basement membrane – so-called misdirected filtration. This leads to the formation of a proteinaceous crescent that, if misdirected filtration persists, spreads over a large part of the glomerular circumference and continuously into the tubular compartment, separating the tubular epithelial cells from their basement membrane (338). This leads to atrophy of the proximal part of the tubule or even complete obstruction of the tubular lumen, resulting in collapse of the tubule and further atrophy downstream. Tubular atrophy is followed by apoptosis of tubular epithelial cells, removal by the infiltrating mononuclear cells, and scar formation, ie, interstitial fibrosis.

Damage remains confined to the individual nephron involved in the process of misdirected filtration – proximal tubular atrophy and obstruction – tubular decomposition and scar formation. This is in seeming contradiction with the notion that loss of renal function is related to tubulointerstitial changes, and not to glomerular injury. Kriz and co-workers explain this by pointing out that the nephron disappears completely, leaving a fibrotic nephron recognized as a tubulointerstitial scar, but including a sclerotic glomerulus (338).

3) The glomerular cytokine theory (341) is yet another explanation of the link between tubular injury and glomerular damage. The theory applies most directly to inflammatory glomerular diseases. Three phases are distinghuished: i. delivery of cytokines and growth factors expressed in the glomerulus via diverse routes to the tubulointerstitial compartment, stimulating local interstitial and tubular cells; ii. stimulated cells express further chemotactic factors that attract mononuclear cells; iii. these cells release growth factors that mediate further injury to the tubules, and promote interstitial fibrosis.

4) Another concept of the etiology of chronic kidney disease focuses on the role of vascular changes and ischemia (342). Indeed, areas of tubular atrophy and interstitial fibrosis show a

decreased density (rarefaction) of peritubular capillaries. In these areas, there seems to be an impaired angiogenic balance, with a predominance of antiangiogenic factors such as thrombospondin-1 and a decrease of angiogenic factors such as nitric oxide and VEGF (342,343). The resulting ischemia drives the development of interstitial fibrosis, which in turn results in an even more impaired delivery of nutrients to the tubular epithelial cells, thus perpetuating the process. Conversily, injection of pro-angiogenic compounds such as VEGF slow the development of interstitial fibrosis (344). Also, hypoxia promotes inflammation, resulting in further tissue damage and fibrosis.

While these observations clearly point at the importance of the microvasculature in chronic renal diseases, the way in which glomerular damage leads to ischemia and tubulointerstitial microvascular changes is less clear (303). One might envision an impaired blood flow as a result of glomerular capillary damage (345). Furthermore, glomerular production of angiotensin II may cause vasoconstriction in the efferent arterioles, resulting in a diminished blood flow into the peritubular capillaries. Also, pro- and anti-angiogenic factors expressed in the glomerulus may reach the tubular compartment via the normal blood flow; a disturbance in the glomerular expression pattern of pro- and anti-angiogenic factors may thus translate to a shift in the tubulointerstitial angiogenic balance. In this regard, it is of interest that Baelde et al (126) found a negative correlation between glomerular VEGF production and interstitial fibrosis. However, the exact nature of the mechanisms that link glomerular changes to interstitial vasculature deserves further study.

5) Related to the previous mechanism of interstitial fibrosis is the hypothesis that, as nephrons are lost during disease progression, the remaining nephrons start to hyperfunction, thereby increasing their metabolic demands (336). This will impact on their oxygen consumption and even in the presence of a normal microvasculature this may lead to hypoxia (345). Another consequence of the increased workload of tubular epithelial cells may be the increased formation of ammonia, which in turn can activate complement via the alternative pathway, favoring an inflammatory response (346,347).

These pathways are not mutually exclusive, and several pathways may be at play simultaneously and in an additive fashion. For example, interstitial fibrosis as a result of tubular activation will aggravate tubular epithelial hypoxia; tubulotoxic effects of proteinuria induce expression of vasoconstrictive molecules and evoke inflammatory responses that suppress angiogenic factors, leading to further ischemia.

Kriz' misdirected filtration theory is the most dissimilar of other pathways, although misdirected filtration does not rule out a concomitant role for excess protein trafficking. The misdirected filtration theory is especially applicable in diseases that have a glomerular cause, while the other pathways listed also apply to situations in which the tubulointerstitium is primarily targeted, for example in the case of transplant rejection. The most crucial difference between the misdirected

filtration theory and the other theories listed relates to the role that interstitial fibrosis is assumed to have. From a teleological point of view, Kriz et al (303) argue that the inflammation clears the remnants of an already irreversibly damaged nephron, which is subsequently replaced by fibrotic material. This limits the damage to a single nephron and at the same time retains the architecture of the remaining tissue. A common theme in the other pathways is the attraction of inflammatory cells to functional, albeit damaged, nephrons that orchestrate the development of interstitial fibrosis. In this scenario interstitial fibrosis is assumed to be a harmful event leading to further interstitial damage and loss of functional tissue. The molecular mechanisms that underlie development of such inflammation and fibrosis will be discussed next.

Mechanisms of interstitial fibrosis

Inflammation

As a result of the injuring mechanisms lined out above, tubular epithelial cells become activated and express pro-inflammatory molecules. MHC class II expression on tubular epithelial cells is upregulated, adhesion molecules including osteopontin and VCAM are expressed, and cytokines and chemokines including MCP1, RANTES, fractalkine, endothelin 1, and IL-6 and IL-8 are released (348,349). This is generally seen as the starting point of further damage: The pro-inflammatory milieu attracts leukocytes, primarily monocytes/macrophages and T-lymphocytes. These in turn secrete factors such as IL-1, interferon γ, and TNF-α which further activate tubular epithelial cells, thus initiating a self-perpetuating process of injury and inflammation.

Cells that contribute to fibrosis; EMT

Injury, inflammation, and ischemia are at the basis of fibrosis: this milieu promotes fibrogenesis by different cells. Tubular epithelial cells may increase their production of ECM, and also macrophages are thought to contribute to the interstitial fibrosis, although they additionally regulate matrix degradation. The cells that are mainly responsible for the deposition of the ECM are fibroblasts. There is some controversy as to what the main source of these fibroblasts is. Increase in the number of fibroblasts in the tissue results from the proliferation of local fibroblasts. A small percentage probably originates from the circulation. Furthermore, fibroblasts may derive from the tubular epithelial cells through a process of epithelial-mesenchymal transformation (EMT), a process that has received much attention in recent years. During EMT, epithelial cells lose typical epithelial characteristics (intracellular adhesions, cellular polarity, differentiation markers), gain features of mesenchymal cells (cell motility, expression of fibroblast markers such as FSP1/S100A4), and migrate to the interstitium. In mouse models of progressive fibrosis, EMT was an important source of interstitial fibroblasts, responsible for at least 36 percent of these cells (350). Although such a cellular plasticity may seem striking at first, it should be remembered that tubular epithelial cells derive from mesenchymal cells, making EMT a known pathway traveled in the opposite direction

(351). Others have disputed the importance of EMT, pointing at the fact that tubular epithelial cells in transit have up until now never been observed (352). Irrespective of the source of fibroblasts, the inflammatory milieu in the interstitium promotes their proliferation, activation, and ECM production. Activated fibroblasts are characterized by the expression of α-smooth muscle actin, although not all fibroblasts that contribute to fibrosis express this marker (353).

The signaling pathways that promote fibrogenesis have been investigated, but will not be extensively covered here. One of the best studied proteins in this respect is TGF-β, a multifunctional cytokine that has a central role in interstitial fibrosis. Indeed, TGF-β increases the production of ECM components by tubular epithelial cells and fibroblasts. Also, TGF-β, together with other cytokines such as basic fibroblast growth factor 2 (FGF-2), is an important inducer of EMT (2).

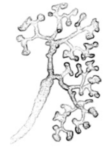
ECM composition and regulation

As described in paragraph, under normal conditions collagen IV is present in the tubular basement membrane. During interstitial fibrosis tubular epithelial cells also express other types of ECM molecules such as fibronectin, and collagens I and III. Fibroblasts express these ECM molecules, and also laminin and tenascin.

Accumulation of ECM is the result of an imbalance between synthesis and repair. The factors that determine this balance have been mapped in some detail. Matrix metalloproteinases degrade ECM components, and may thus contribute to the resolution of fibrosis. The activity of MMPs is regulated by, among other factors, tissue inhibitors of metalloproteinases (TIMPs), indicating that the synthesis and degradation-balance is regulated at multiple levels. Adding further complexity to the system is the fact that the role these molecules play is in part dependent on their spatial expression pattern. For example, MMP2 may have a beneficial effect in degradation of interstitial fibrotic material, but at the other hand may promote the process of EMT by disruption of the tubular basement membrane (2,353).

Damage is progressive

After loss of a critical amount of nephrons loss of kidney function is progressive. The widely tested and accepted mechanism for this progression is explained by the hyperfiltration hypothesis, as put forward by Brenner and Hostetter and co-workers in the 1980s (354,355). This hypothesis states that as nephrons are lost, an adaptive process is initiated that leads to increased intraglomerular pressure and flow across the glomerular capillary wall. These two factors increase the single nephron glomerular filtration rate. While these adaptations limit the total loss of renal function (as determined by the GFR), they are eventually detrimental. In rat models a severe reduction in renal mass brought about hemodynamic and histopathologic changes within one week (355).



The mechanisms that regulate the initial adaptive hemodynamic changes are incompletely understood. Also, several factors may be held responsible for the further damage of the glomerulus, including mechanical stress due to increased intraglomerular pressure that disrupts vascular integrity or leads to podocyte damage. Increased protein trafficking has also been suggested to directly damage podocytes. These uncertainties at the molecular level aside, it is clear that at the tissue level the adaptive response to a loss of nephrons has an adverse effect that potentially results in glomerular damage, leading to further nephron loss along pathways lined out before, thus resulting in a self-perpetuating loss of renal function.

As a result of this progressive course of kidney diseases, patients suffering from these diseases will eventually need renal replacement therapy. One of these therapies, kidney transplantation, will be discussed in the next paragraphs.

PART 3 | LONG-TERM DYSFUNCTION OF KIDNEY TRANSPLANTS

In the last 40 years, transplantation has become the treatment of choice for end-stage kidney failure. In 1954, the first successful kidney transplantation was performed by dr. Joseph Murray, who was later awarded the Nobel Prize for this achievement. In the absence of rejection – the transplanted kidney was donated by the twin brother of the recipient – this operation showed the technical feasibility of transplantation. Developments in immunosuppression, starting with prednisone and azathioprine, made non-HLA identical transplantation possible, although rejection severely limited graft survival. In these early days of kidney transplantation the first year graft survival rate was around 50 percent (356). The application of the newly discovered immunosuppressive drug Cyclosporine A in 1978 (357) proved to be a breakthrough in transplantation. This immunosuppressive drug, together with improvements in donor matching and storage condition of the graft, provided the basis for the current successful use of kidney transplantation as a treatment of end-stage renal disease. Nowadays, the factors that limit the success of transplantation have shifted from the acute to the chronic phase after transplantation. Long-term failure of kidney transplants, chronic allograft dysfunction, is the main reason of allograft loss.

This paragraph describes the clinical and histopathological characteristics, pathophysiologic mechanisms, and diagnostic molecular markers of chronic allograft dysfunction.

General description and definitions

Limited improvement in long-term survival

The introduction of current immunosuppression including calcineurin inhibitors and mycophenolate mophetil fostered an increase in short-term graft survival. Currently, the first year renal transplant survival rate is about 90 to 95 percent. In contrast, long-term graft survival has not made similar progress. The long-term attrition rate has even remained fairly constant over the past 25 years, with a half-life of cadaveric transplants of about 8 years in the United States (358-360). Thus, for patients with a kidney transplant the most threatening problems are those that arise on the long-term, with chronic allograft dysfunction and death with a functioning graft being the two leading causes of graft loss (359,361,362). Intriguingly, diverse and seemingly opposed processes may contribute to this long-term attrition of allografts, including ongoing immunologi-

cal activity or 'chronic rejection', as well as toxicity of immunosuppressive medication, meant to prevent chronic rejection.

Definitions of long-term graft failure

Over the years ideas about the pathophysiological mechanisms leading to long-term allograft failure have changed, and description of the clinical and histopathological presentation has varied. This has led to some unclarity in definition of the subject, with terms as chronic allograft/ transplant dysfunction, chronic allograft nephropathy, and chronic rejection used interchangeably to describe the same problem. In this paragraph, the following definitions are used:

Chronic allograft dysfunction describes the clinical aspects of long-term renal allograft failure. The clinical features are not specific for one diagnosis, and chronic allograft dysfunction should thus be regarded as a clinical syndrome that can be brought about by different and mixed pathophysiological processes (363). Its pleomorphic etiology is in part reflected by the elaborate list of risk factors.

Chronic allograft nephropathy (CAN) is the term used to describe the histopathological changes seen in biopsies from patients with chronic allograft dysfunction. Again, these histopathological changes are not specific for one diagnosis, and in this definition CAN is the histopathological representation of the spectrum of causes that lead to chronic allograft dysfunction. In biopsies with features compatible with only one diagnosis the term CAN should be avoided (364). Chronic rejection, for example, is a distinct diagnosis. It denotes a central role for immunological processes as the cause of chronic allograft dysfunction, and can be differentiated from other causes of allograft functions by certain discriminatory features in the renal biopsy (365).

This paragraph will describe chronic allograft dysfunction and its risk factors, chronic allograft nephropathy, and the pathophysiological processes involved in these two syndromes, with a special focus on chronic rejection and chronic calcineurin inhibitor toxicity.

Chronic allograft dysfunction

Clinical manifestations

Chronic allograft dysfunction is characterized by a slow but progressive decline in renal function starting after the first three months post transplantion. This is often combined with aggravation or de novo development of hypertension and proteinuria (366,367). The time to eventual complete loss of renal function depends on the initial graft function (intercept) and rate of progression

(361). The decline in GFR in patients that have survived the first year post transplantation varies between 1.2 and 2.5 ml/min per year (368).

Scope of the problem

Once chronic allograft dysfunction has started to develop, the process will invariably progress to end stage allograft failure. Chronic allograft dysfunction is the main cause of returning to dialysis after transplantation (369). Currently, patients waiting for a repeat transplant make up about 15 to 20 percent of the total number of patients on the waiting list for renal transplantation (www.transplantatiestichting.nl, www.optn.org). Retransplantation carries an additional risk to develop chronic allograft dysfunction (370).

Risk factors

The development of chronic allograft dysfunction is influenced by a plethora of factors, both immunological and non-immunological, and related to donor and recipient. The intuitive bottom-line, as stated by Paul in a review on the subject, is that chronic allograft dysfunction 'seems to develop in kidneys from older donors or kidneys that have acquired damage later on' (371). The most important risk factors are listed below, grouped as immunological and non-immunological factors. It should be noted that grouping risk factors is artificial, as it is their combined and often synergistic effects that modulate graft survival. Indeed, different forms of primarily non-immunological damage directly affect graft function, and in addition modulate the immunogenicity of the tissue (372).

Immunological risk factors

Histoincompatibility

Evaluation of large series of renal transplants in the US (373), Europe (374), and the UK (375) has made clear that human leukocyte antigen (HLA) mismatches have a detrimental effect on long-term allograft survival. In a US series, the 10 year graft survival was 52 percent in HLA-matched and 37 percent in HLA-mismatched transplants (373). Mismatches in the different HLA loci (HLA-A, B, DR) seem to have a comparable impact on graft function, at least on the long-term (376). The importance of HLA matching for long-term allograft survival may be declining (377,378). Indeed, in comparison to risk factors such as donor age, the effect of HLA mismatches is nowadays relatively small (368).

Sensitization and Panel Reactive Antibodies

Blood transfusions, pregnancies, and previous transplantations may give rise to the formation of anti-HLA antibodies. The presence of such antibodies is tested by evaluating the reactivity of the serum of a potential transplant patient with a 'panel' of lymphocytes obtained from selected

blood donors. The number of donor samples in the panel with which the patients' serum reacts is an indication of the patients' extent of presensitization. The amount of 'panel reactive antibodies' (PRA) present before transplantation and the formation of such antibodies after transplantation is related to the development of chronic allograft dysfunction (363,379-381).

Immunosuppression and non-compliance

Improvements in immunosuppression have been instrumental in reducing the number of acute rejections but not the long-term complications. Non-compliance to immunosuppressive drugs may be one of the factors explaining this discrepancy (361,382). In a meta-analysis the odds ratio of graft loss in non-compliant versus compliant patients was 7.1 (382).

Acute rejection

HLA-mismatches, presensitization, and non-compliance all relate to what is the most important immunological risk factor of chronic allograft dysfunction: acute rejection (363,383). Progress in graft survival has been less pronounced in patients that have experienced acute rejection episodes (362). The effect on long-term allograft function varies with the number, severity, timing, type, and response to treatment of acute rejection (369,384,385). When adequately treated, early acute rejection (within 3 months after transplantation) does not (386,387), or to a lesser extent (388,389), affect long-term outcome when compared to late acute rejections. The effect of vascular rejection is worse than that of interstitial rejection (390).

At the same time, improvements in treatment of acute rejection have not translated into better survival on the long-term (360). This may indicate that only the less harmful acute rejections are treated sufficiently, and also points towards the influence of other risk factors that may coexist or interfere (386). For example, it has been suggested that while cyclosporine accurately prevents acute rejection, it also inhibits development of tolerance (391,392). Additionally, nephrotoxic adverse effects of immunosuppressive medication such as cyclosporine may explain the lack of enhanced long-term graft survival over the years.

Non-immunological risk factors

Elegant studies by Gourishankar et al showed that the function of a renal allograft could be predicted by the function of its 'mate graft' – the other graft from the same donor (393). Even though rejection was not influenced by this so-called 'mate effect', long-term outcome was paired even up to 8 years after transplantation (368). This suggests that donor-related factors have an important role in transplantation.

Donor age and renal mass

Donor age is an important risk factor for long-term graft loss (383,394-396), and explains 30 percent of the variation in transplant outcome after five years (366,397). The influence of donor age on graft survival can in part be explained by immunological mechanisms. Donor age modulates the occurrence of rejection and subsequent tissue repair: older kidneys seem to be

more immunogenic and have impaired repair mechanisms (398). However, it is unlikely that this increased immunogenicity completely explains the effect of donor age, as donor age also influences survival of zero HLA-mismatch transplantations (394).

A risk factor related to donor age is renal mass: small kidney size in relation to the recipient is related to adverse outcome (399), although this may not be true in a pediatric setting (400). Likewise, male donor gender seems to convey a benefit in long-term graft survival, which is probably also related to renal mass (368).

Recipient age and sex also have a clear influence on allograft survival, explaining 10-25 percent of the variation of the long-term outcome (397), with female sex and younger age having a better prognosis (401). In the long-term, the effects of donor and recipient age are synergistic (402).

Peritransplant injuries

The donation and transplantation procedures have influence on the long-term function of renal allografts. Risk factors include brain death, preservation of the allograft, and ischemia/reperfusion injury. Terasaki et al (403) studied the three-year survival rates of cadaveric donors, living related donor, and unrelated donor grafts (e.g. spouses). They found that survival rates were similar between living related and living unrelated donors, despite a higher number of HLA mismatches in the latter. Both living donor groups showed a higher survival than cadaveric donors (403). This shows the importance of brain death, preservation, and ischemia/reperfusion injury in determining survival on the long-term. Through which mechanisms these factors influence graft function is not completely known. Again, an interaction between non-immunological and immunological mechanisms is to be expected. Koo et al demonstrated an increased expression of tubular antigens and inflammatory molecules in biopsies from cadaveric donor kidneys in comparison to living donor kidneys. This was associated with the occurrence of acute rejection episodes (404).

Delayed graft function

Delayed graft function is a form of acute renal failure, mostly defined as the requirement of dialysis early after transplantation. According to some studies delayed graft function is an independent predictor of late allograft function (405-407). Others attribute the effects of delayed graft function to the influences on the occurrence of acute rejections, for example through an increased immunogenicity of the graft (367). Indeed, acute rejection in the setting of delayed graft function has an additive adverse effect on graft function (405,408). In itself, delayed graft function is the result of a number of risk factors, including some of those mentioned above (368,406,409).

Post transplant injuries

After transplantation, complications may arise that have a detrimental influence on long-term graft survival:

Proteinuria conveys an increased risk of renal allograft loss (328,329,383,386,398,410-413). Twenty to 28 percent of the patients with chronic allograft dysfunction have proteinuria, compared to six to eight percent of patients without this condition (386,414). In a study by Hohage et al, early development of proteinuria that lasted longer than 6 months was noted in a quarter of patients. The proteinuric patients had a 5 year survival rate of 59 percent, compared to 86 percent in the non-proteinuric patients (329).

Hypertension is also associated with increased chances of late graft failure (334,415), although the effect is less pronounced than that of proteinuria (412). Development of proteinuria seems to be linked to development of hypertension (330), and the presence of both conditions has an additional risk for allograft loss (410).

Immunosuppressive treatments contribute importantly to the development of chronic allograft dysfunction. The nephrotoxic effects of calcineurin inhibitors such as cyclosporine A and tacrolimus are well-recognized, but also corticosteroids have been found to contribute to chronic allograft dysfunction (366). The extent of their contribution is more difficult to estimate (369). The histopathologic changes that are related to calcineurin toxicity will be discussed in more detail in the following sections.

The pathology of chronic allograft dysfunction

Changing definitions

As pointed out before, concepts about pathophysiological mechanisms that lead to chronic renal allograft dysfunction have changed over the years. In the Banff 91 classification (see below), the term chronic allograft nephropathy was introduced to describe the histological changes seen in biopsies from patients with chronic allograft dysfunction that could not be related to one specific cause. These changes had previously been described as chronic rejection, but as this term implied a predominantly immunological mechanism of damage, the more neutral terms chronic allograft nephropathy (CAN) was chosen (364-366). Since then, definitions of CAN in the literature have been different, ranging from strict histopathological entities to loose descriptions of clinicopathological syndromes. It was increasingly noticed that the indiscriminate use of the term CAN would limit the possibilities of establishing an etiologic diagnosis, and would result in a term with 'little value other than to hide our ignorance' (364). Indeed, treatments for the different causes of CAN, once they could be distinguished, are likely to be fundamentally different (416). In order to

prevent further indiscriminate use of CAN, the latest iteration of the Banff classification has now moved towards elimation of the term CAN, replacing it by the category 'Interstitial fibrosis and tubular atrophy, no evidence of any specific etiology'.

Because most of the studies described herein have used the term CAN to denote the changes seen in biopsies, we will also use it in the following paragraph. Here, CAN is defined as the histopathological changes seen in biopsies from patients with chronic allograft dysfunction without a recognizable single cause or diagnosis. In this definition, CAN is the histopathological representation of the spectrum of causes that lead to chronic allograft dysfunction. From a clinical point of view the most important differential diagnoses of CAN are chronic rejection, chronic calcineurin inhibitor toxicity (414), obstruction, and infection (365). In this paragraph, the histopathology of CAN, chronic rejection, and chronic calcineurin inhibitor toxicity will be described.

Causes and course of CAN

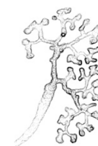
The factors that contribute to the development of CAN in allograft kidneys include ischemia, hyperfiltration, proteinuria, hypertension, de novo or recurrent glomerular disease, infection, drug toxicity, and (chronic) allo-immune injury (417). These different factors damage all compartments of the kidney (vasculature, tubules, interstitium, and glomeruli), though not always simultaneously or to the same extent. This often limits the possibilities to distinguish the contribution of different causes to the development of the lesions seen in CAN.

At 1 year post transplantation CAN is already widely present in renal allografts. In protocol biopsies the number of patients affected by CAN ranges from 50 to 94 percent (418-420). The largest of these per-protocol series, a study of 120 simultaneous kidney-pancreas transplants with over 950 biopsies, found that the development that CAN could be divided in two phases. The initial phase was characterized by interstitial damage and rapid development of tubular atrophy and interstitial fibrosis. CAN developed earlier in allografts that showed signs of peritransplant injury (acute tubular necrosis) and acute rejection (420). Also, subclinical rejection was related to earlier development of CAN, but this was not found in later studies specifically addressing this relation (421). The later phase of CAN was characterized by a more severe involvement of the vasculature and glomerulosclerosis (420).

Histopathologic features

The three main features of CAN are fibrous intimal thickening, interstitial fibrosis, and tubular atrophy.

Fibrous intimal thickening indicates the changes seen in renal arteries of allografts, and affects mostly the larger (arcuate) arteries. The normal intima consists of a single layer of endothelial cells, based on the internal lamina elastica. In CAN, the renal arteries show a concentric expansion



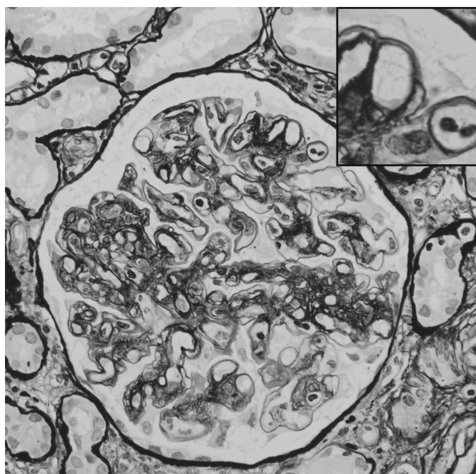


Figure 4. Transplant glomerulopathy. Transplant glomerulopathy is seen in chronic rejection. This glomerular lesion is characterized by a duplication of the glomerular basement membrane (inset).

of the intima, narrowing the arterial lumen. Although this is comparable to the changes found in normal atherosclerosis (369), the concentric nature of the lesion is somewhat specific for CAN. The presence of inflammatory cells in the neo-intima, as well as disruption of the internal elastica seems to be more specifically related to chronic rejection (see below). Smaller vessels and arterioles may also be affected in CAN, showing for example arteriolar hyalinosis. This lesion is often indicative of calcineurin inhibitor toxicity, and will be discussed below. Multilayering of the basement membrane of peritubular capillaries is another vascular feature of CAN. Severe multilayering is associated with chronic rejection (366).

Interstitial fibrosis and tubular atrophy often occur simultaneously. Atrophic tubules show a thick tubular basement membrane and small tubular epithelial cells. An aspecific inflammatory reaction is often found in conjunction with the atrophic tubules and interstitial fibrosis. The molecular composition of the interstitial fibrotic lesions will be further discussed below and in chapter 6 of this thesis.

Glomerular changes may also be seen in CAN, and include focal segmental as well as global glomerulosclerosis, and mesangial matrix expansion. Other lesions affecting the glomeruli include mesangiolysis and duplication of the GBM. This may again point to a specific etiology discussed below.

Since 1991, the Banff Working Classification of Renal Allograft Pathology ('Banff classification') has been used for standardization and grading of renal allograft biopsy interpretation. The Banff classification provides a grading for the individual CAN lesions (tubular atrophy, interstitial fibrosis, fibrous intimal thickening, mesangial changes, glomerulopathy). Because tubular atrophy and interstitial fibrosis are in general most accurately sampled, the extent of these changes is used for grading the severity of CAN (in grades I to III, corresponding to mild/moderate/severe) (422). The most recent iteration of the Banff classification has maintained this grading system, but now with respect to the category 'Interstitial fibrosis and tubular atrophy, no evidence of any specific etiology' (365).

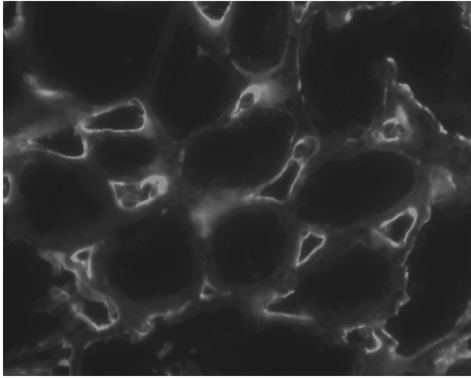


Figure 5. C4d deposition. In chronic humoral rejection, the complement split product C4d remains covalently bound to endothelial cells. This picture shows widespread deposition of C4d in peritubular capillaries.

Lesions related to specific etiology

As mentioned before, the mixture of pathophysiologic processes operating simultaneously leads to a histopathological picture that does not allow diagnosis of a single factor as the cause of the allograft dysfunction. In some cases, however, a single cause may be of predominant importance, and can be recognized by certain clinical and histological features. Two of these disease entities are described below: chronic rejection and chronic calcineurin inhibitor toxicity.

Chronic rejection

Chronic rejection is defined as injury caused by alloreactivity directed against the graft. Signs of both cellular and humoral forms of chronic rejection can be present in the renal allograft biopsy. The Banff classification denotes transplant vasculopathy, ie, disruption of the internal elastica, and infiltration of inflammatory cells in the thickened intima of the renal vasculature, as a sign of chronic rejection (422). At the level of the peritubular capillaries, extensive duplication of the peritubular basement membrane, as seen with electron microscopy, is associated with chronic rejection (423). Furthermore, characteristic lesions in the glomerulus (transplant glomerulopathy), characterized by duplication of the GBM (figure 4) also points to the involvement of chronic rejection. In recent years, the role of chronic humoral rejection (or chronic antibody mediated rejection) as a cause of chronic allograft dysfunction has received renewed attention. During anti-donor HLA antibody mediated rejection, a component of the complement system is found to remain covalently bound to the surface of endothelial cells. This component, C4d, can be visualized by immunohistochemistry, and indicates an ongoing or previous antibody mediated rejection (424,425) (figure 5). In 88 percent of patients that showed widespread C4d positivity in peritubular capillaries circulating anti-donor HLA antibodies were found, compared to none of the C4d negative patients (416), indicating an ongoing humoral rejection.

Calcineurin inhibitor toxicity

Calcineurin inhibitors (CNIs), such as cyclosporine A and tacrolimus are very potent immunosuppressive drugs that are widely used in transplantation. By inhibiting the enzyme calcineurin these compounds suppress IL-2 production and thus prevent maturation of T-cells. Already in the first report on cyclosporine A a nephrotoxic side effect was mentioned (357). In subsequent years, the histopathology of the chronic form of CNI toxicity – although structurally unrelated, cyclosporine A and tacrolimus cause identical histopathologic lesions (426) – has been described, mostly

through studies by Mihatsch and coworkers (427-429). Studies in non-kidney transplantation in which cyclosporine A was used have been instrumental, since the renal effects of cyclosporine can be studied in the absence of other types of damage to the kidney (430).

Chronic CNI toxicity develops after months to years of CNI use, and in large series it is related to the dose. Since the bioavailability is highly variable in individual patients, dosage of CNIs is not a good correlate of CNI exposure. Sophisticated drug-monitoring and dosing may help overcome this problem.

The scope of the problem seems to be considerable. In the Leiden University Medical Center, 20 percent of a cohort of patients that switched from once daily Sandimmune to twice-daily Neoral (two different formulations of cyclosporine A) developed chronic cyclosporine A toxicity, even though they received their medication according to nationwide guidelines (431). In this study, cyclosporine toxicity was defined by functional and histological criteria, ie, a decline in renal function temporally related to the switch in cyclosporine A formulation that could not be explained by other features in the renal biopsy. The number of patients that show histological signs of CNI toxicity after long-term treatment with CNIs may be even higher. In the aforementioned study by Nankivell et al CNI toxicity became 'virtually universal' by 10 years after transplantation (420).

Similar to the chronic lesions seen in CAN, the histopathology of chronic CNI toxicity is characterized by tubular atrophy and interstitial fibrosis, representing a nonspecific response to damage. The fibrosis has been reported to affect rays of cortex and medulla, leading to so-called 'striped fibrosis'. This band-pattern of fibrosis is thought to be caused by a watershed infarction, ischemia in the debit of a larger artery affected by cyclosporine. The lesion is typical but not specific for CNI toxicity (432), and its value is debated.

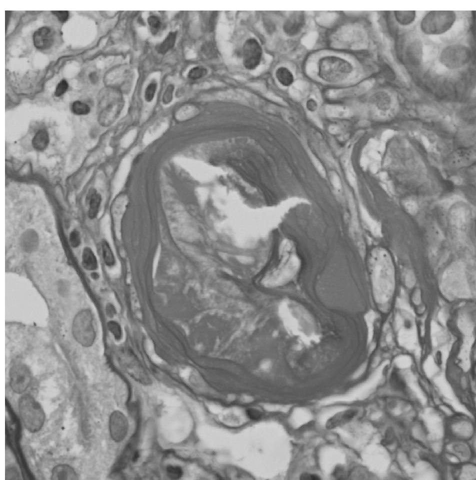


Figure 6. Arteriolar hyalinosis. Nodular depositions of hyaline material are seen in the media of arterioles, indicating the presence of chronic CNI toxicity.

Glomerular lesions are also common in chronic CNI toxicity and include glomerular hypertrophy, mesangial matrix expansion, and focal segmental glomerular sclerosis. Again, these changes are of limited specificity (433). The most specific lesion of chronic CNI toxicity is arteriolar hyalinosis, in which nodular depositions of hyaline material are seen in the media of arterioles (figure 6). Pathogenetically, CNIs are thought to damage arteriolar smooth muscle cells that are subsequently replaced by hyaline material from the circulation (427,433). In advanced cases, the hyalinosis may be circumferential (434), and severely narrow the vascular lumen.

Nevertheless, use of arteriolar hyalinosis as a marker of chronic CNI toxicity has certain drawbacks. For example, lesions that are characteristic for CNI toxicity appear focally in the tissue and thus carry the risk of sampling error (435). Also, the interobserver consistency of scoring renal biopsies has been low (436,437). Attempts have been made to increase the reproducibility of arteriolar hyalinosis scoring by introducing a different classification (434). This new classification system seemed to outperform the previous one, but it is questionable whether this is due to a real improvement or just to a reduction of categories in the new scoring system (438). Another complicating factor in the use of arteriolar hyalinosis as a marker of CNI toxicity is that this lesion is also seen in association with other diseases such as diabetic nephropathy, hypertension, and hemolytic uremic syndrome / thrombotic microangiopathy (439).

Although chronic rejection and chronic CNI toxicity are both individual causes of chronic allograft dysfunction, they can coexist and contribute to the histopathological picture of CAN.

Pathophysiologic processes in chronic allograft dysfunction

There are several non-mutually exclusive pathophysiologic processes that damage the kidney and lead to development of chronic allograft dysfunction and CAN, often in a way that is similar to the progression of renal disease in native kidneys. Indeed, the kidney responds to injury in a relatively stereotypic manner, eventually culminating in replacement of functional nephrons by sclerosed glomeruli, atrophic tubuli, and interstitial fibrosis. The shared features and final common pathway of renal disease progression is highlighted in a previous paragraph. Those of special interest in the setting of transplantation will be discussed here and include alloimmunity, immunosuppressive drug toxicity, and accelerated senescence.

Alloimmunity

Shishido et al found a relation between subclinical acute rejection and extent of CAN (387). This implies that cellular infiltrates continue to inflict damage on the allograft on the long term. This continuing inflammation will lead to a destruction of the tissue, and may in addition lead to an enhanced allorecognition due to an increase in MHC expression (369), resulting in a self-perpetuating cycle of injury and inflammation. At the same time, not all cellular infiltrates are associated with the development of CAN (421), suggesting that cellular infiltrates differ in their destructive properties.

An interesting process contributing to the immunological cause of chronic allograft nephropathy was recently described in a series of studies by Kerjaschki. Using relatively novel markers for lymphatic endothelial cells, including the glycoprotein podoplanin, they found that the number of lymphatic vessels was increased over 50-fold in allografts that showed nodular infiltrates (150). In a follow-up paper they showed that these newly formed lymphatics are in part derived from recipient lymphatic progenitor cells, possibly macrophages (440,441). The lymphatics were co-localized with a nodular infiltrate that contained active and dividing T and B lymphocytes, macrophages and dendritic cells, resembling an intragraft lymphoid organ. Moreover, the lymphatic endothelial cells were shown to produce cytokines that would enable them to recruit lympocytes and thus initiate or sustain an anti-allograft immune response. Not only could this response be of a cellular nature, this study and several other studies also found evidence for the involvement of B-cells in rejection (442).

Indeed, humoral allograft rejection has received renewed attention in the last decade since the discovery of C4d as a marker of chronic rejection coinciding with the presence of circulating antidonor antibodies (see above). The pathogenic processes will only be described briefly, and have been reviewed in detail by several authors (367,424). Antidonor antibodies are generally directed against donor HLA. Dendritic cells of the donor (the direct pathway) or the recipient (the indirect pathway) present donor HLA antigens to recipient T-cells. This activates the T-cells that, through production of a series of cytokines and co-stimulatory molecules such as CD28 and CD40, activate the B-cell (turning them into plasma cells) and thus initiate formation of antidonor antibodies. These antibodies bind to donor tissue, predominantly endothelial cells, and cause complement fixation, leading to formation of the membrane attack complex, attraction of other inflammatory cells, and activation of endothelial cells. This either leads to apoptosis of endothelial cells, or, if the cells survive, production of inflammation promoting factors such as intracellular adhesion molecule 1 (ICAM), vascular cell adhesion molecule 1 (VCAM) and E-selectin, and other chemotactic cytokines, providing a link to cellular immunity. Also, the cytokines produced by activated endothelial cells may be harmful to the kidney.

Circulating antibodies may also be directed against non-HLA antigens. Joosten et al described the presence of antibodies directed against the GBM proteoglycan agrin in patients with transplant glomerulopathy (443).

Immunosuppressive drug toxicity

Immunosuppressive drugs, in particular CNIs, may promote allograft damage through a variety of processes, including vasoconstriction, direct cellular toxicity, and modulation of fibrosis-related gene transcription. CNIs are known to increase vascular resistance and promote vasoconstriction. This may result from an inhibition of nitric oxide production or an increased release of renin from

the juxtaglomerular apparatus, activating the renin-angiotensin system (RAS) (414,444). Also, necrosis of arteriolar smooth muscle cells and replacement by hyaline material may contribute to the obliteration of the vascular lumen (432). The resulting impaired blood flow may lead to ischemia, and result in glomerular and tubulointerstitial fibrosis. Also, CNIs may have a direct cytotoxic effect on renal cells, including podocytes (158), but results have been controversial with regard to tubular epithelial cells (444).

Furthermore, CNIs increase the expression of profibrotic and proinflammatory cytokines, as well as interstitial matrix molecules, either directly or via stimulation of the RAS (445). Among the cytokines influenced by CNIs are TGF-β (446-448), osteopontin (448), ET-1, MCP-1, and RANTES (449). Cyclosporine A was shown to stimulate the promoter of the collagen III gene in monkey fibroblast cells (450), and increased the expression of collagen I in human and experimental renal cortical tissue (448,451). Recently, it was described that cyclosporine A may induce epithelial-to-mesenchymal transition (EMT) of tubular epithelial cells, and thus contribute to interstitial fibrosis (452). The mechanism of EMT is discussed in more detail in a previous paragraph.

Another aspect of immunosuppressive medication is that it increases the chance of viral infections of the allograft. For example, cytomegalo virus infection and BK virus nephropathy (BK virus is named after the patient in whom the infection was first described (453)) have been related to the development of chronic allograft dysfunction. This may take place through stimulation of immunological pathways leading to chronic inflammation (365,454) as well as direct damage of renal epithelial cells (455).

Accelerated senescence

As mentioned previously, donor age is an important risk factor for the development of chronic allograft dysfunction. One pathophysiologic concept that has been raised to explain this association is the so-called accelerated senescence of renal allografts (369). In vitro, after a limited number of cell cycles, cells become quiescent and usually die. This 'cellular senescence' is characterized by a number of factors. The length of the telomeres of chromosomes, for example, decreases with each replication, and in senescent cells, cell cycle inhibitory molecules such as p16 and p21 are upregulated. Halloran and co-workers noted that the histopathological characteristics of ageing overlap with that of CAN. They studied the expression of p16, and found that this protein was expressed in increased amounts in allografts with inferior function and with signs of CAN (456). Similarly, Ferlicot et al found that the severity of CAN was correlated with the extent of telomere shortening (457). These data support the hypothesis, as put forward by Halloran (369), that the multiple stresses that the allograft encounters would put replicative stress on the allograft kidney cells, thereby accelerating cellular senescence. Indeed, in an animal model of chronic rejection studied by Joosten et al, stressors such as warm ischemia time correlated with the expression of

p16 and p21 as well as with telomere shortening in the tubular epithelial cells, although this was not exclusively associated with chronic rejection (458). Together, these data suggest that replicative senescence may be a pathophysiologic pathway of progression in both native and allograft kidney diseases: once the continuously stressed cells have reached their replicative limit, they are unable to effectively keep up their normal response to damage, leading to atrophy and fibrosis rather than reepithelialization and ECM remodeling.

Molecular diagnostics in chronic allograft dysfunction

The clinical course of long-term graft dysfunction is aspecific, and often does not provide a firm basis for therapeutic decision making. As pointed out in the previous paragraphs, histopathological evaluation of renal biopsies may help in defining the cause of renal function loss, but also has limitations.

This has urged researchers to search for markers that might complement or even replace routine histopathologic evaluation. Such studies have been performed using hypothesis-driven approaches, in which parts of the pathogenesis of renal transplant pathology were studied. Others have utilized broader approaches, for example through application of microarray and proteomics. A summary of these studies is given in the following paragraphs, with a focus on studies on chronic allograft dysfunction in humans. For a broader scope on the subject, see more thorough reviews of the use of molecular markers in native kidney diseases (459-462), and acute rejection (463,464).

Diagnostic markers at the tissue level

The extent of interstitial fibrosis is the most important histological predictor of progression of renal disease in both native and allograft kidneys. However, semi-quantitative scales as provided by the Banff classification impair the reliable measurement of the extent of interstitial fibrosis in renal allografts (436,437). To circumvent this problem, several researchers have used a semi-automated approach using digital image analysis. Nicholson et al studied the predictive properties of quantitative immunohistochemistry in protocol transplant biopsies. They found that the collagen type III positive area at 6 months was correlated with renal function at 12 and 24 months after transplantation (465). Others have used Sirius Red, a red dye that intercalates with the interstitial collagens I and III, to stain the interstitial compartment (466). These studies showed that computerized measurement of the Sirius Red positive material in biopsies taken early after

transplantation is correlated with the long term decline in renal function and may outperform the semi-quantitative scoring method (467,468). Quantification of interstitial fibrosis using Sirius Red has also proven to be useful as a surrogate marker of CAN, for example to compare fibrogenic effects of different types of CNIs (418) and long term effects of subclinical acute rejection (421).

One of the reasons to use interstitial fibrosis and tubular atrophy for grading of chronic allograft nephropathy in the Banff criteria is that these changes are fairly common in renal allograft biopsies and therefore less subject to sampling error (422). Studies in animal models of glomerulosclerosis made clear that the composition of glomerular extracellular matrix harbors disease specific features (469). Similarly, it is conceivable that the composition of the interstitial fibrotic lesions varies with the cause of the disease (470). Thus, studying composition of the interstitial fibrosis may provide valuable markers and overcome the sampling error limitations of the abovementioned histopathological markers.

Following this approach, Abrass et al (471) studied the composition of the extracellular matrix of renal allograft biopsies by staining for different interstitial collagens (I, III, and IV), laminins, fibronectin, and thrombospondin. In addition, they studied particular collagen IV and laminin chains. In a pilot study, they identified three different patterns of ECM composition in renal allograft biopsies. Pattern 1 showed no change in comparison to normal interstitium, pattern 2 showed an generalized accumulation of collagens I and III, while pattern 3 showed a de novo expression of collagen α3(IV) and laminin β2 in the proximal tubular basement membrane. Subsequently, they studied the presence of these patterns in biopsies from patients with either chronic rejection or chronic cyclosporine A toxicity. Chronic rejection was exclusively associated with the third pattern, indicating that indeed ECM patterns could help distinguish the two causes of late allograft dysfunction (471).

Other molecular markers at the tissue level have proven to be useful in the diagnosis of specific causes of chronic allograft dysfunction. For example, as discussed above, the complement split product C4d has become integrated in routine diagnostics as a marker of humoral rejection.

In conclusion, studying changes at the tissue level – using conventional histologic as well as immunohistochemical approaches – can help predict the clinical course and may help distinguish different causes of allograft dysfunction. The downside of these techniques is that the damage has to be present, at least to some extent, to make the diagnosis. This probably limits the success of subsequent treatments (472). In this context, analysis of mRNA expression levels could be promising. This will be lined out in the following section.

Genomics

Single gene measurements

In the early 1990s, studies in experimental renal disease showed that the mRNA expression levels of ECM components such as collagen I and IV correlated with the severity of glomerulosclerosis (473,474). Moreover, the changes in mRNA levels preceded the morphological changes (475). These studies raised the concept, as reviewed by Striker (476), that studying mRNA expression levels could be of predictive value with regard to both occurrence and severity of renal disease.

This concept has been elaborately tested in the setting of transplantation. Measurements of mRNA expression levels of immune activation genes in renal allografts proved to be a tool to diagnose the presence of acute rejection (477-479). Furthermore, such measurements can be performed non-invasively using urinary cells. The group of Suthanthiran showed that urinary mRNA expression levels of transcripts related to rejection (granzyme B and perforin) and regulatory T-cells (FOXP3) predict the presence of acute rejection (480) and reversal of acute rejection (481), respectively. Although these results remain to be confirmed in larger patient groups (482), and although studying the urine may neglect valuable information that can exclusively be found in the renal biopsy (483), it clearly shows the strength of molecular analysis.

Acute rejection episodes are associated with profound and quick alterations in the cellular composition of the graft, and it is in this regard not surprising that this is reflected in differences in mRNA expression. Can molecular biological techniques be applied to the more indolent development of chronic allograft dysfunction? Because CAN is characterized by interstitial fibrosis, researches have initially focused on ECM components and profibrogenic cytokines. Sharma et al were the first to describe a relation between elevated mRNA expression levels of the profibrogenic cytokine TGF-β and the presence of CAN (484), a finding later also reported in protocol biopsies (419). Expression levels of TGF-β mRNA in the urinary cells of patient with CAN also proved to be increased (485). Suthanthiran also found that TGF-β mRNA levels were related to 'chronic rejection' and interstitial fibrosis, but found no relation to acute rejection (479). This may be explained by the fact that besides its profibrogenic properties, TGF-β has a strong immunosuppressive effect. Eikmans et al (486) found that an upregulation of TGF-β mRNA in biopsies from patients with early acute rejection was associated with the absence of the development of chronic allograft dysfunction, underscoring the dual action of this cytokine. Scherer et al found similar results using a microarray approach (487). Plasminogen activator inhibitor 1 (PAI-1) is another profibrotic cytokine that has been implicated in the development of interstitial fibrosis. Delarue et al found that increased levels of PAI-1 mRNA correlated with a decline in renal function over 5 years of follow-up (488). Increased PAI-1 expression has also been observed at the protein level in CAN (489).

Not only fibrogenesis, but also an impaired ECM turnover may result in interstitial fibrosis. Indeed increased transcripts of tissue inhibitors of metalloproteinases – proteins that inhibit matrix degrading enzymes – are related to the extent of interstitial fibrosis (490).

In keeping with the multifactorial etiology of chronic allograft dysfunction, others found that the expression of immunologic transcripts was related to chronic allograft nephropathy (491-493), although this has not been a consistent finding (484).

The problem of prediction

Most of the studies listed so far describe associations between mRNA transcripts and development of chronic allograft dysfunction. Demonstration of various mRNA transcripts in chronically deteriorating kidneys may help unravel the pathogenesis of CAN. Also, comparable to the use of quantitative measurements of interstitial fibrosis as a surrogate marker for CAN, mRNA expression analysis has been instrumental in determining the fibrotic response to different immunosuppressive treatment modalities (494). Still, the ultimate goal of mRNA expression analysis would be to find markers that predict development of CAN. Few studies have actually tested the predictive value of investigated markers (495). In a protocol biopsy study, Baboolal et al found that, although TGF-β, thrombospondin and fibronectin were upregulated in biopsies that showed CAN, none of these markers alone could reliably indicate renal injury, and acute rejection and interstitial fibrosis continued to have the most profound impact on prognosis (419).

Microarray studies

The problem, that many genes are associated with CAN but few predict its development, has become more apparent after a landmark study by Sarwal et al (442). This microarray study on 52 pediatric renal transplant patients, the largest microarray study performed in transplantation so far, has raised a number of intriguing concepts. Clustering of the samples showed considerable heterogeneity in biopsies with acute rejection. One of the acute rejection clusters contained a large number of B-cell markers, again suggesting an important role for humoral rejection (see above) and opening new directions for therapy. However, such a heterogeneity was not found in the samples that were on histological criteria diagnosed as CAN (442). In a review on the use of microarray in transplantation, Sarwal et al state that the late sampling of CAN biopsies impairs the identification of specific causes of CAN. The differential regulation of genes compared to stable allografts at the late stage only reflects the ongoing damage mechanisms (495). This is exemplified by a study by Hotchkiss and co-workers, who studied 16 transplant biopsies with CAN with and without arteriolar hyalinosis in comparison to 6 normal transplant biopsies using microarray (496). They found that the CAN biopsies were clearly different from the controls, but – similar to Sarwals report – did not find differences between CAN subgroups. They did find genes known to be involved in CAN pathogenesis, but concluded that for more insight in CAN development, earlier biopsies should be studied. Donauer et al compared 13 transplants with

chronic transplant failure with normal controls and end stage renal disease (497). They found that the end-stage renal disease kidneys, either transplants or native kidneys, clustered together, suggesting a shared pathway to chronic renal failure. Moreover, the chronically rejected kidneys showed two distinct subsets at the transcriptional level, but these could not be traced back to clinical or histological differences.

In conclusion, microarray studies in the field of chronic allograft dysfunction have shown that it is difficult to obtain information on different pathogenetic pathways or markers of disease progression once CAN has started to develop. Instead, strategies should aim at defining such markers early in the course after transplantation.

A number of microarray studies have used this approach to more specifically address the predictive value of gene expression measurements. Scherer et al (487) defined two groups that showed a divergent course of allograft function between 6 and 12 months after transplantation. Using microarray, they selected and validated a set of 10 genes (eight up- and two downregulated) that could differentiate between the two groups. Eikmans et al defined two groups of patients that both experienced acute rejection but diverged in their long-term follow up. Biopsies of the two groups, designated progressors and non-progressors, were studied using microarray, and a number of differentially regulated genes were further analyzed with respect to their predictive properties in a separate group of patients (498). Surfactant protein C showed the best predictive value of long-term outcome (498), while the combination of several array-selected markers in addition to conventional histological and clinical parameters had an even superior predictive performance (M. Eikmans, personal communication).

Proteomics, metabolomics, urinomics

It is imaginable that the large scale-analysis of proteins would provide extra tools for monitoring renal transplant function and identification of complications. The validity of this concept has been scarcely tested in transplantation, but there are some examples of recognition of acute rejection using a proteomic approach with sensitivity and specificity comparable to that of mRNA analysis (499,500). Whether this also holds true for chronic allograft dysfunction remains to be established, but it should be kept in mind that the proteomics approach may meet the same caveats as discussed in relation to genomics. Metabolomics is even more in its infancy (with reviews on the subject outnumbering original research publications) but may hold promise for the development of novel and – if measured in urine – non-invasive biomarkers for transplant monitoring (501).

Outline of the thesis

Two relatively distinct topics are covered in this thesis: the molecular mechanisms related to the development of proteinuria, and those related to kidney transplant failure. Our interest in the development of proteinuria was triggered by the discovery of several genes mutated in hereditary nephrotic syndromes. Following these findings, it was suggested that the genes and respective proteins might likewise be involved in the development of acquired proteinuric diseases. This was the subject of the study described in chapter 2, in which we compared the regulation of several podocyte-associated genes and proteins in relation to podocyte morphology in patients with and without proteinuria. Studying human material often restricts the evaluation of changes in time, which was the reason to use a rat model for follow-up studies on the same subject: the regulation of podocyte molecules during the development of proteinuria. In chapter 3, the results of a time-course study of the spontaneously proteinuric Dahl salt-sensitive rat in comparison to the non-proteinuric spontaneously hypertensive rat are described. This is a largely descriptive study that focuses on the glomerular changes in the early phase of proteinuria development. In an attempt to get more insight in the cause of proteinuria in this rat model, we performed a microarray study using the same rat model, of which the findings are described in chapter 4.

Proteinuria is an important risk factor for the progression of renal disease, both in setting native and transplant diseases. Upon initial injury, kidneys succumb to a progressive course of renal function loss that is histologically characterized by the replacement of functional tissue by scar tissue, a process referred to as interstitial fibrosis. This process seems to be uncoupled from the initial cause of injury, although this injury may still persist in the progressively fibrotic kidney. This makes it difficult to install treatments directed at the etiology of the disease. This is especially true in the transplantation setting, in which different kinds of injury give rise to similar histopathological presentations. We were mainly interested in two distinct causes of kidney transplant injury that both operate in the late phase after transplantation: the toxic effects of the immunosuppressant cyclosporine A, versus the injurious effects of chronic immunologic activity. Although the clinical and histopathological presentation of these two causes is relatively similar, the modes of treatment obviously differ. We tried to dissect the role of the different causes by focusing on the differences in mRNA expression in two well-defined groups of patients suffering from either disease; the results of this study are described in chapter 5. In a similar patient group, we studied the protein composition of the interstitial fibrosis, which is the subject of chapter 6.

In chapter 7 these studies are summarized and placed in a more general perspective.

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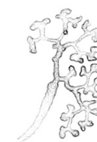

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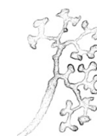

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Expression of Podocyte-Associated Molecules in Acquired Human Kidney Diseases

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J Am Soc Nephrol. 2003 Aug;14(8):2063-71

Abstract

Proteinuria is a poorly understood feature of many acquired renal diseases. Recent studies concerning congenital nephrotic syndromes and findings in genetically modified mice have demonstrated that podocyte molecules make a pivotal contribution to the maintenance of the selective filtration barrier of the normal glomerulus. However, it is unclear what role podocyte molecules play in proteinuria of acquired renal diseases. This study investigated the mRNA and protein expression of several podocyte-associated molecules in acquired renal diseases. Forty-eight patients with various renal diseases were studied, including minimal change nephropathy, focal segmental glomerulosclerosis, IgA nephropathy, lupus nephritis, and diabetic nephropathy, together with 13 kidneys with normal glomerular function. Protein levels of nephrin, podocin, CD2-associated protein, and podocalyxin were investigated using quantitative immunohistochemical assays. Real-time PCR was used to determine the mRNA levels of nephrin, podocin, and podoplanin in microdissected glomeruli. The obtained molecular data were related to electron microscopic ultrastructural changes, in particular foot process width, and to clinical parameters. In most acquired renal diseases, except in IgA nephropathy, a marked reduction was observed at the protein levels of nephrin, podocin, and podocalyxin, whereas an increase of the glomerular mRNA levels of nephrin, podocin, and podoplanin was found, compared with controls. The mean width of the podocyte foot processes was inversely correlated with the protein levels of nephrin ($r = -0.443$, $P < 0.05$), whereas it was positively correlated with podoplanin mRNA levels ($r = 0.468$, $P < 0.05$) and proteinuria ($r = 0.585$, $P = 0.001$). In the diseases studied, the decrease of slit diaphragm proteins was related to the effacement of foot processes and coincided with a rise of the levels of the corresponding mRNA transcripts. This suggests that the alterations in the expression of podocyte-associated molecules represent a compensatory reaction of the podocyte that results from damage associated with proteinuria.

Introduction

Proteinuria is an important risk factor for the progression of renal disease, but the pathophysiologic mechanisms underlying its development are unclear (1). Ultrafiltration in the glomerulus is accomplished by a fenestrated endothelial layer, the glomerular basement membrane (GBM), and the overlying podocytes, highly specialized cells with primary and secondary interdigitating branches (foot processes) that cover the external surface of the GBM (2). On the basis of its structural arrangement and isoporous substructure, the slit diaphragm, bridging the space between adjacent foot processes, was assumed to make an important contribution to the molecular sieve for glomerular filtration (3,4). However, this assumption remained a matter of debate (5). Only recently, increased insight in the molecular makeup of the podocyte foot processes and slit diaphragm led to the support for the pivotal role of these structures in the maintenance of permselectivity (6-8).

In kidney diseases accompanied by a nephrotic syndrome, the spatial organization of the podocyte is simplified, characterized by effacement of the foot processes and sporadic detachment of the podocyte from the GBM (9). In the development of the congenital nephrotic syndrome, several molecules have been pointed out to play an important role, including those that assemble and stabilize the slit diaphragm and those that anchor the foot process to the GBM. Mutations in NPHS1 and NPHS2, the genes encoding the slit diaphragm–associated proteins nephrin and podocin, respectively, lead to the development of a nephrotic syndrome (10,11). Similarly, injection of monoclonal antibody 5-1-6, directed against the extracellular part of nephrin (12), causes heavy proteinuria in rats (13). Absence of CD2-associated protein (CD2AP), normally linking nephrin and podocin to the actin cytoskeleton (14,15), also evokes proteinuria (16). Podoplanin is one of the molecules that possibly serve in the connection between foot process and GBM, and injection of antipodoplanin antibodies in rats gives rise to proteinuria and foot process effacement (17). The sialoprotein podocalyxin is normally located at the apical part of the foot process. Absence of this protein or changes in its spatial organization also has a detrimental effect on the glomerular filtration function (18,19). Thus, disruption of the normal gene regulation and protein distribution in the podocyte is related to effacement of foot processes and proteinuria. The study of molecules associated with defective glomerular ultrafiltration in acquired renal diseases might lead to a better understanding of its pathogenesis.

In the current study, we sought to examine whether glomerular expression levels of several podocyte-associated molecules differ between groups of patients who have various acquired renal diseases. We also investigated whether the expression levels were related to clinical parameters and ultrastructural changes. For this, we simultaneously quantified the expression of

several podocyte-associated molecules at the protein and the mRNA levels and combined this with clinical parameters and morphometric analysis of ultrastructural changes of the podocyte.

Materials and Methods

Patients and Controls

Forty-eight patients who had acquired renal diseases were included. From each patient, two core biopsies were taken on clinical indication. A small piece of cortex was removed from fresh biopsies, and glomeruli were microdissected for RNA extraction. One of the biopsies was snap-frozen and stored at -80°C, and the other was formalin fixed and embedded in paraffin. From 29 patients, an additional piece of cortex was available for electron microscopic analysis. Diagnostic groups were made on the basis of routine pathologic examinations. We defined the following groups: minimal change disease (MCD); focal segmental glomerulosclerosis (FSGS); IgA nephropathy; lupus nephritis; diabetic nephropathy; light-chain excretion nephropathy (LCEN); nephrotic syndrome as a result of other causes (membranoproliferative glomerulonephritis [n = 2] and membranous glomerulopathy [n = 1]); and nephritic syndrome as a result of other causes (pauci-immune glomerulonephritis [n = 3] and postinfectious glomerulonephritis [n = 1]). For controls, renal tissue was derived from cadaver donor kidneys unsuitable for transplantation for technical reasons (n = 5), autopsy kidneys (n = 1), tumor nephrectomy samples (n = 2), and biopsies without glomerular lesions (n = 5). In the last group, two showed interstitial nephritis; the biopsies of three other patients were indecisive concerning the diagnosis. However, in all biopsies,

Table 1. Patient characteristics

Diagnostic group	N	EM (b)	age (years ± SD)	gender (female)	proteinuria (g/24 h ± SD)	serum creatinine (μmol/l ± SD)
Controls (a)	13	3	51 ± 19	7 (53%)	0.4 ± 0.3	150 ± 104
MCD	10	8	31 ± 24	4 (40%)	4.4 ± 3.8	76 ± 28
FSGS	5	3	36 ± 8	1 (20%)	5.1 ± 2.1	113 ± 67
IgA nephropathy	10	6	37 ± 23	1 (10%)	2.4 ± 2.8	166 ± 150
Lupus nephritis	7	3	30 ± 12	7 (100%)	2.7 ± 2.0	147 ± 94
Diabetic nephropathy	6	1	50 ± 21	3 (50%)	1.1 ± 0.9	141 ± 85
LCEN	3	1	64 ± 6	1 (33%)	1.8 ± 1.3	354 ± 254
Nephrotic Syndrome – other causes	3	1	44 ± 8	2 (67%)	6.7 ± 3.9	92 ± 33
Nephritic syndrome – other causes	4	3	54 ± 22	2 (50%)	2.0 ± 1.4	214 ± 99
Total	61	29	44 ± 21	28 (46%)	2.7 ± 2.8	146 ± 118

a) The control group consisted of one autopsy kidney, five cadaveric donor kidneys, two tumor nephrectomy samples (taken from a location remote from the tumor), and five biopsy samples without glomerular lesions. b) Number of samples studied with electron microscopy.

the glomeruli were unaffected, (ie, they were normocellular, had a normally developed capillary network, and had a normal GBM and mesangium). The control samples were processed in the same way as the patient biopsies. Clinical characteristics, including age, gender, serum creatinine levels, and proteinuria, of the patients and controls were collected. The patient groups and their clinical characteristics are listed in Table 1.

Antibodies

The polyclonal antibody against human nephrin was generated in rabbits immunized with 1 mg of human nephrin peptide (ERDTQSSTVSTTEAEPYYRSLC, located in the cytoplasmic region) conjugated with KLH three times with an interval of 2 wk. Human podocalyxin-like protein was prepared with a WGA column according to the same method for the purification of rat podocalyxin described by Kerjaschki et al (20). Briefly, isolated glomeruli from normal human kidneys were extracted with 0.2% Triton X-100 in PBS containing protease inhibitors. The extract was then incubated with WGA-Sepharose 4B at 4°C overnight, and unbound material was removed by washing with PBS. The sialic-acid–rich material that bound to the WGA column was released with 120 mM N-acetyl-b-glucopyranoside in PBS. Rabbits were immunized with the sialic-acid–rich material three times with an interval of 2 wk. The rabbits were killed and bled 2 wk after the last immunization. Rabbit anti-human podocin antibody p35 raised against the C-terminal part of human podocin (21) was supplied by Dr. C. Antignac (Hôpital Necker, Paris, France). Rabbit anti-CD2AP was obtained from Santa Cruz Biotechnology (SC9137; Santa Cruz, CA). Horseradish peroxidase–conjugated anti-rabbit Envision was obtained from DAKO (Glostrup, Denmark). FITC-conjugated goat anti-rabbit IgG antibody was obtained from Sigma (St. Louis, MO).

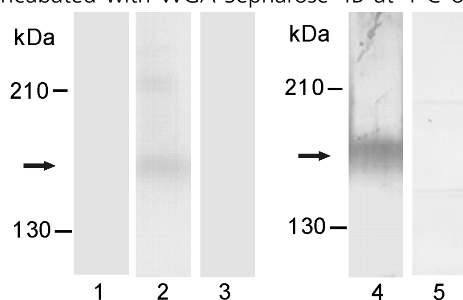

Figure 1. Characterization of the anti-nephrin and anti-podocalyxin antibodies. Western blot for anti-human nephrin antibody (lanes 1, 2 and 3) and anti-human podocalyxin antibody (lanes 4 and 5). A glomerular extract was incubated with preimmune rabbit serum (lanes 1 and 5), the anti-human nephrin antibody produced in rabbits immunized with a peptide of 21 amino acids of human nephrin sequence (lane 2), with the anti-human nephrin antibody preabsorbed with the peptide used for immunization (lane 3), or the anti-human podocalyxin antibody produced in rabbits immunized with a WGA-column purified glomerular lysate (lane 4). A clear band of approximately 180 kD was seen in lanes 2 and 4. No bands were observed in lanes 1, 3 or 5.

Western Blot Analysis

Western blot analysis of the anti-human nephrin antibody was performed with glomeruli isolated from normal human kidney by a standard sieving method with PBS containing protease inhibitors (PBS-PI; 1 mM each antipain, benzamidine, di-isopropylfluorophosphate, leupeptin, pepstatin A, and PMSF). The glomeruli were solubilized with RIPA buffer (consisting of 0.1% SDS, 1% Triton X-100, 150 mM NaCl, and 10 mM EDTA in 25 mM Tris-HCl [pH 7.2]) with protease inhibitors

described above. For Western blot analysis of the anti-human podocalyxin antibody, human glomerular lysate solubilized with 0.2% Triton X-100 in PBS-Pis was used. Insoluble material was removed by centrifugation at 15,000 x g for 10 min. Solubilized material was subjected to SDS-PAGE with 7.5% acrylamide gel according to the method of Laemmli (22) and transferred to a nitrocellulose membrane (Bio-Rad, Hercules, CA) by electrophoretic transblotting for 30 min using Trans-Blot SD (Bio-Rad). After blocking with BSA, strips of membranes were exposed to preimmune rabbit serum, anti-human nephrin, or anti-human nephrin preabsorbed with the peptide used for immunization, and anti-human podocalyxin or preimmune rabbit serum. The strips were then washed and incubated with alkaline phosphatase-conjugated anti-rabbit IgG (Bio Source International, Tago Immunologicals, Camarillo, CA). The reaction was developed with an alkaline phosphatase chromogen kit (Biomedica, Foster City, CA).

Immunohistochemistry and Immunofluorescence

Three-micrometer cryostat sections were cut, transferred to Starfrost slides, air dried, and stored at -20°C until use. For immunohistochemistry, the slides were washed in PBS and incubated for 1 h at room temperature with the primary antibody diluted in 1% BSA in PBS (rabbit anti-nephrin 1:1000; rabbit anti-podocalyxin 1:2000; rabbit anti-podocin 1:2000). The slides were then washed in PBS and incubated for 30 min with horseradish peroxidase–conjugated anti-rabbit Envision (1:1). The slides were again washed in PBS, and the staining was developed with diaminobenzidine. The color was enhanced by rinsing the slides in 0.5% $CuSO_4$ solution for 5 min. After counterstaining with hematoxylin, the slides were dehydrated and mounted.

For immunofluorescence, the slides were thawed in PBS, fixed in a mixture of 50% alcohol and 50% acetone for 5 min and subsequently in 100% alcohol for 10 min, and washed in PBS. The slides were then incubated overnight with the primary antibody at room temperature (rabbit anti-CD2AP 1:500) and thereafter washed in PBS. The slides were incubated with the FITC-conjugated anti-rabbit IgG antibody (1:200) for 30 min, washed in PBS, and covered with Vecta shield (Vector Laboratories, Burlingame, CA). For each antibody, all samples were stained in one session.

Digital Image Analysis

Of the immunohistochemically stained samples, images of all of the glomeruli in the section were taken at a x400 magnification using a Zeiss Axioplan microscope equipped with a Sony DXC-950P 3CCD color camera (Sony Corporation, Tokyo, Japan) and further analyzed using KS-400 image analysis software (Windows version 3.0; Carl Zeiss Vision, Oberkochen, Germany). The glomerular area stained was calculated by drawing a region of interest around the glomerulus in which the amount of staining within a color spectrum specific for the diaminobenzidine staining and above a fixed intensity threshold was determined, as described before (23,24).

Slides stained by immunofluorescent methods were evaluated with a Zeiss Axioplan 2 microscope, equipped with an AxioCam CCD color camera, connected to a computer equipped with AxioVision 3.0 software (Carl Zeiss Vision). In each section, five images of individual glomeruli were recorded at x400 magnification. The intensity of the staining was determined by drawing a region of interest around the glomerulus and measuring the mean luminosity value of the region with the histogram function of ImageJ 1.26t software (National Institutes of Health, rsb.info.nih.gov/ij), as described in detail elsewhere (25). Recording and analysis of the digital images were performed with fixed settings.

mRNA Isolation, cDNA Synthesis and Real-Time PCR

Total RNA was extracted from microdissected glomeruli using the Trizol-method and used for cDNA synthesis with the aid of the sensiscript-RT kit (Qiagen, Westburg BV, Leusden, The Netherlands), as described previously (26). For nephrin, podocin, podoplanin, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), β2 microglobulin (B2M), TATA box binding protein (TBP), hydroxymethyl-bilane synthase (HMBS), and hypoxanthine phosphoribosyltransferase 1 (HPRT1), forward and reverse primers (Isogen Bioscience BV, Maarsen, The Netherlands) and probes (Eurogentec Nederland BV, Maastricht, The Netherlands) were designed using Primer Express 1.5 software (PE Applied Biosystems, Foster City, CA). The sequences of the primers and probes are shown in Table 2. Real-time PCR was performed using the ABI PRISM 7700 sequence detector and software (PE Applied Biosystems). All measurements were performed in duplicate. Amplification cycles were 95°C for 10 min, followed by 50 cycles at 95°C for 30 s and at 60°C for 60 s. To correct for the amount of tissue used for RNA extraction and the efficiency of cDNA synthesis, we used the ratio between the mRNA levels of nephrin, podocin, and podocalyxin and the mRNA level of GAPDH, a constitutively expressed gene. The suitability of GAPDH as a housekeeping

Table 2. Primer and probe sequences

Molecule	Forward primer	Reverse primer	Probe
Nephrin	AGGACCGAGTCAGGAACGAAT	CTGTGAAACCTCGGGAATAAGACA	TCAGAGCTCCACGGTCAGCACAACAG
Podocin	GGCTGTGGAGGCTGAAGC	CTCAGAAGCAGCCTTTTCCG	CAGCAATCATCCGCACTTTGGCTTG
Podoplanin	CCAGGAACCAGCGAAGACC	GCGTGGACTGTGCTTTCTGA	TTGACACTTGTTGCCACCAGAGTTGTCAA
GAPDH	TGGTCACCAGGGCTGCTT	AGCTTCCCGTTCTCAGCCTT	TCAACTACATGGTTTACATGTTCCAATAT-GATTCCACCAA
B2M	TGCCGTGTGAACCATGTGA	CCAAATGCGGCATCTTCAA	TGATGCTGCTTACATGTCTCGATCCCACT
TBP	CACGAACCACGGCACTGATT	TTTTCTTGCTGCCAGTCTGGAC	TGTGCACAGGAGCCAAGAGTGAAGA
HMBS	CTGGTAACGGCAATGCGGCT	GCAGATGGCTCCGATGGTGA	CGAATCACTCTCATCTTTGGGCT
HPRT1	TGACACTGGCAAAACAATGCA	GGTCCTTTTCACCAGCAAGCT	CTTGACCATCTTTGGATTATACTGCCT-GACCA

GAPDH, glyceraldehyde-3-phosphate dehydrogenase; B2M, β2 microglobulin; TBP, TATA box binding protein; HMBS, hydroxymethyl-bilane synthase; HPRT1, hypoxanthine phosphoribosyl-transferase 1. Primers and probes are located within 1500 bp from the 3’ end of the mRNA. All 5’ ends of the probes were 6-carboxy-4,7,2’,7’-tetrachloro-fluorescein (TET)-labeled, except those for GAPDH and TBP, which were 6-carboxy fluorescein (FAM)-labeled. The quencher dye at the 3’ ends of the probes was 6-carboxy-tetramethyl-rhodamine (TAMRA).

gene for standardization of mRNA levels was confirmed by testing correlations between GAPDH mRNA for all samples (n = 61) and each of the other four housekeeping genes (B2M, TBP, HMBS, and HPRT1) measured.

Transmission Electron Microscopy and Morphometry

Small pieces of cortex were fixed in 1.5% glutaraldehyde and 1% paraformaldehyde, dehydrated, and embedded in Spurr. In semithin sections stained with toluidine blue, nonsclerosed glomeruli were localized. Ultrathin sections were made of one or two glomeruli per tissue specimen and stained with lead citrate for transmission electron microscopy. Four to ten photographs, covering one or two glomerular cross-sections, were made with a Philips CM10 transmission electron microscope (Philips, Eindhoven, the Netherlands). A calibration grid with 2160 lines/mm was photographed to determine the exact magnification. Negatives were digitized, and images with a final magnification of approximately x17,500 were obtained. With the use of ImageJ 1.26t software (National Institutes of Health, rsb.info.nih.gov/ij), the length of the peripheral GBM was measured and the number of slit pores overlying this GBM length was counted. The arithmetic mean of the foot process width (FPW) was calculated as follows: $\overline{W}_{FP} = \frac{\pi}{4} \cdot \frac{\sum \text{GBM length}}{\sum \text{slits}}$ where slits is the total number of slits counted, GBM length is the total GBM length measured in one glomerulus, and the correction factor $\pi/4$ serves to correct for the random orientation in which the foot processes are sectioned (27,28). A mean GBM length of 276 µm was evaluated in each glomerulus.

Statistical Analyses

Data are presented as means ± SD. One-way ANOVA combined with a least significant difference post hoc correction was used to test differences between groups. Correlations were calculated using Pearson correlation test. $P < 0.05$ was considered statistically significant.

Figure 2. Staining pattern of nephrin, podocin, podocalyxin, and CD2-associated protein (CD2AP) in normal and diseased human kidney sections. Nephrin (A), podocin (B), and podocalyxin (C) show a podocyte-like staining pattern in normal glomeruli as visualized by an immunohistochemical diaminobenzidine staining. The staining pattern of nephrin is more dispersed than that of podocin and podocalyxin, which show a fine glomerular basement membrane (GBM)-like line along the capillary loops of the glomerulus. CD2AP, visualized with immunofluorescence, shows a GBM-like staining pattern (D). In diseased situations, the staining for nephrin (E; minimal change disease), podocin (F; focal segmental glomerulosclerosis [FSGS]), and podocalyxin (G; FSGS) is less intense, and nephrin and podocin stainings show a more granular staining pattern. CD2AP staining shows no clear differences between control (D) and diseased tissue (H; diabetic nephropathy). Magnification x 400.

Characterization of Antibodies

The results of the Western blot assays of nephrin and podocalyxin are shown in Figure 1. For both antibodies, the Western blot assay showed a clear band of approximately 180 kD in the lane incubated with the anti-human nephrin or anti-human podocalyxin antibodies, whereas no bands were seen in the lanes incubated with preimmune rabbit serum or with antibody preabsorbed with the peptide used for immunization. This confirmed the specificity of the anti-nephrin and anti-podocalyxin antibodies.

Immunohistochemistry and Immunofluorescence

In normal glomeruli, nephrin, podocin, and podocalyxin showed an intense epithelial staining along the peripheral capillary loops of the glomeruli. Nephrin showed a more dispersed pattern than podocin and podocalyxin. The GBM-like pattern for CD2AP was very subtle, and the staining, therefore, was analyzed using immunofluorescence. In glomeruli of diseased kidneys,

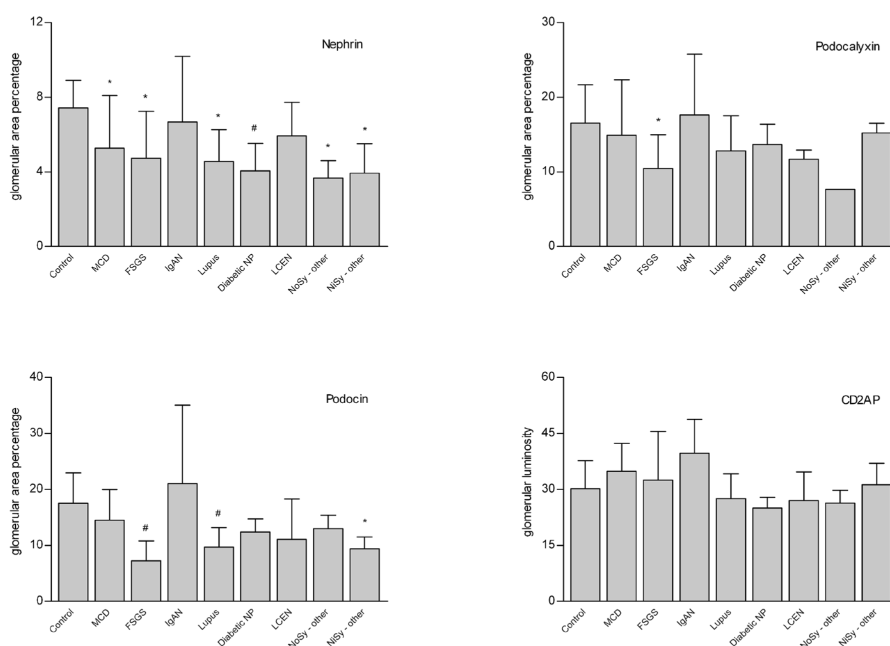

Figure 3. Glomerular expression of podocyte-associated proteins. Nephrin, podocin, and podocalyxin stainings were performed immunohistochemically. Images of the glomeruli in the sections were recorded and analyzed using digital image analysis as described in the Materials and Methods section. Nephrin, podocin, and podocalyxin show a downregulation in many disease categories compared with controls. CD2AP protein levels, as determined by measuring the mean luminosity in glomeruli stained with anti-CD2AP antibody, showed no significant differences between patient groups and controls. NoSy, other, nephrotic syndrome as a result of other causes; NiSy, other, nephritic syndromes as a result of other causes. *$P < 0.05$; ‡$P < 0.001$.

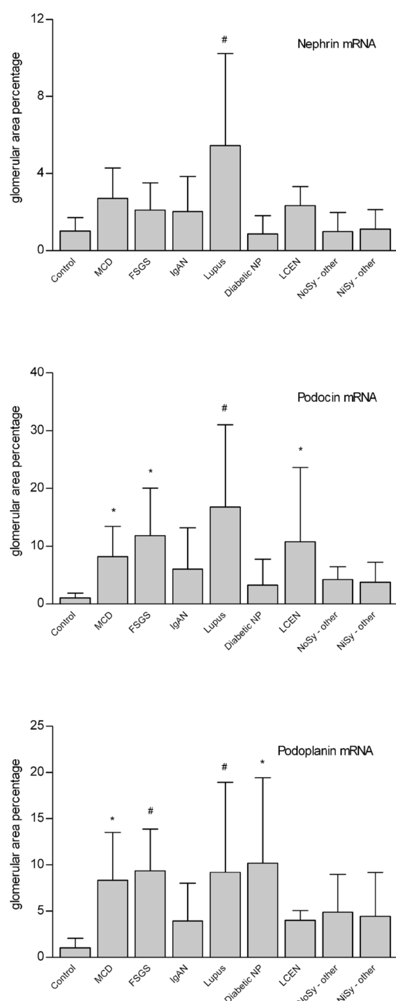

Figure 4. Glomerular mRNA levels of nephrin, podocin, and podoplanin. mRNA levels of nephrin, podocin, and podoplanin were measured in microdissected glomeruli using real-time PCR. Podocin and podoplanin mRNA levels are most prominently altered in diseased states. *P < 0.05; ‡P < 0.001.

the staining of nephrin, podocin, and podocalyxin was weaker, and sometimes the staining showed a more granular appearance. Figure 2 gives a graphical overview of the staining patterns for the different molecules in control and diseased kidneys. Quantification of the staining using digital image analysis showed that the stained glomerular surface for nephrin and podocin was significantly diminished in several disease categories, including FSGS, lupus nephritis, and the group of nephritic syndrome as a result of other causes. Podocalyxin also showed a decrease in stained glomerular surface, although in MCD and diabetic nephropathy, this was less prominent. Protein levels of all molecules were not significantly altered in IgA nephropathy compared with controls (Figure 3). The glomerular fluorescence intensity of the CD2AP staining did not differ significantly between controls and disease groups (Figure 3).

There was a strong correlation between protein levels of nephrin and podocin ($r = 0.576$, $P < 0.001$), nephrin and podocalyxin ($r = 0.477$, $P = 0.001$), and podocin and podocalyxin ($r = 0.693$, $P < 0.001$). There was no correlation between serum creatinine levels and protein levels of nephrin, podocin, or podocalyxin.

mRNA Quantification

mRNA levels for nephrin, podocin, and podoplanin were measured and corrected for the mRNA level of GAPDH. In all of the 61 samples used in this study, significant correlations ($P < 0.001$) were found between GAPDH mRNA and mRNA for each of the four other housekeeping genes, namely B2M ($r = 0.81$), TBP ($r = 0.78$), HMBS ($r = 0.82$), and HPRT1 ($r = 0.80$). This confirmed the suitability of GAPDH as a housekeeper gene in this case.

The corrected mRNA levels for the various molecules are depicted in Figure 4. In general, mRNA levels of nephrin, podocin, and podoplanin in isolated glomeruli were elevated in most disease

categories compared with controls. The mRNA levels of nephrin showed an upregulation in most disease categories, reaching a significant difference in lupus nephritis. The nephrin mRNA levels of patients who had diabetic nephropathy were slightly lower than in controls, although not significantly. The mRNA levels of podoplanin and podocin were most prominently upregulated, reaching significant difference in MCD, FSGS, and lupus nephritis for both podocin and podoplanin.

Electron Microscopy and Morphometry

Assessment of the mean FPW revealed various degrees of foot process effacement between patients, being most outspoken in patients who had MCD and FSGS (ie, up to 1900 nm compared with 640 nm in controls). The mean FPW correlated with proteinuria ($r = 0.585$, $P = 0.001$), protein levels of nephrin ($r = -0.443$, $P < 0.05$), and podoplanin mRNA levels ($r = 0.468$, $P < 0.05$). Correlation plots are shown in Figure 5.

Discussion

Resulting from the identification of the important role that podocyte-associated molecules play in maintaining the glomerular filtration barrier, the hypothesis emerged that these molecules might also be involved in acquired nephrotic syndromes (6,12). We investigated whether glomerular expression of podocyte-associated molecules is different between various groups of patients with acquired renal diseases and whether these expression levels are related to clinical parameters and ultrastructural changes.

The basolateral region of the podocyte foot process is the insertion place of the slit diaphragm (8). Nephrin and podocin are two crucial proteins in the complex of molecules that assemble and reinforce the slit diaphragm. These molecules are bound to the actin cytoskeleton via CD2AP, a general adapter molecule (14,15). The basal aspect of the podocyte foot processes is firmly attached to the GBM by several linkage proteins, including $\alpha3\beta1$-integrin and α- and β-dystroglycan (2,29). Podoplanin, a 43-kD molecule expressed in the glomerulus and in lymphatic tissue, is thought to have a similar function (8) and controls the shape of the podocyte foot processes (30). Proteins located at the apical part of the podocyte foot process play an important role in ultrafiltration, mainly by maintenance of the negative charge of the membrane domain. The sialoprotein podocalyxin is the most important of these molecules (20) and plays a crucial role in the maintenance of the normal morphology of the podocyte foot processes (19).

At the protein level, we observed in most patient groups a decrease of the examined podocyte-associated molecules compared with controls, except for CD2AP. We found that the glomerular amount of nephrin was significantly decreased in most proteinuric kidney diseases. Several studies concerning nephrin expression in human acquired renal diseases have recently been performed, but the various observations do not firmly support each other. Patrakka et al (31) did not find significant changes in the expression of nephrin at the protein level, whereas Doublier et al (32) and Wang et al (33) both reported a downregulation of nephrin protein expression in MCD, FSGS, and membranous glomerulonephritis. Our observations are in line with the latter findings. In a variety of experimental models, nephrin protein levels have been shown to decrease, suggesting a crucial role for nephrin in the development of proteinuria (25,34-36).

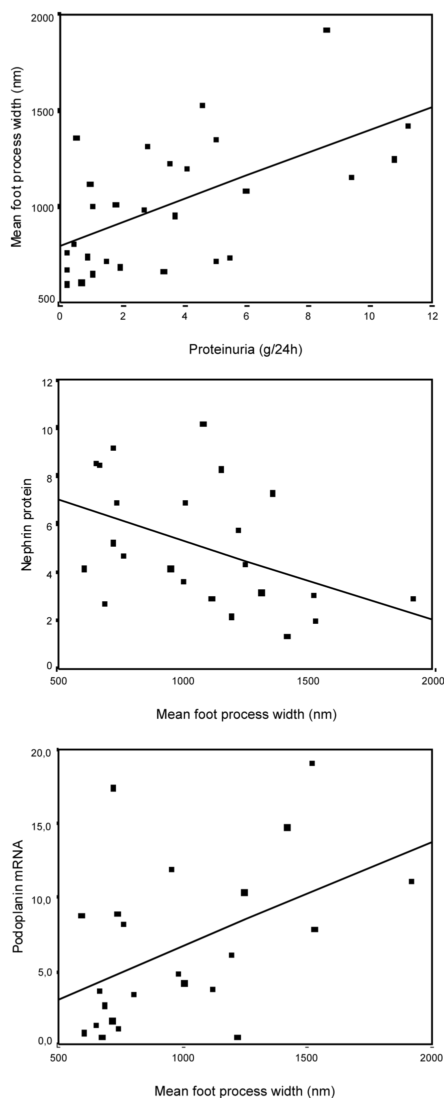

Figure 5. Correlation plots. (A) The amount of proteinuria was related to the mean foot process width (FPW; r = 0.585, P = 0.001). (B) The mean FPW of all patients was significantly correlated with the glomerular area percentage of nephrin (r = 0.443, P = 0.034). (C) Podoplanin mRNA levels were also correlated to the mean FPW (r = 0.430, P = 0.036).

Expression of podocin in human acquired proteinuric diseases has until now not been described. In our study, we found that the podocin protein levels were significantly decreased in most disease categories compared with controls. The expression patterns of podocin protein resemble those described for puromycin aminonucleoside (PAN) nephrosis in the rat, a model for MCD, which is characterized by heavy proteinuria (37).

In contrast to nephrin and podocin, the protein expression of CD2AP did not show major differences between patient groups and controls in our study. The same pattern has been described for the passive Heyman nephritis model in the rat, in which an overt decrease of nephrin protein expression was observed, whereas CD2AP protein expression remained unaltered (25). Still, it is remarkable that in proteinuric states, podocin and nephrin protein levels are severely decreased, whereas in the same cluster of molecules, CD2AP remains unimpaired. This might

suggest that upon damage to the podocyte, the binding of CD2AP with nephrin and podocin is disconnected, whereas CD2AP itself remains attached to the actin cytoskeleton (25). As observed in experimental models, the uncoupled proteins might be shed from the podocyte membrane and excreted in the urine (38,39), whereas CD2AP condenses in the scaffold of microfilaments in the effaced foot processes (25,40).

The glomerular podocalyxin protein expression in most proteinuric diseases was reduced compared with controls. This might suggest uncoupling of podocalyxin from the actin cytoskeleton as a result of rearrangements of the cytoskeleton. This phenomenon has been described in rat models of proteinuria, in which the complex linking podocalyxin to the actin cytoskeleton is disrupted (19).

In remarkable contrast to decreased protein levels in most patient groups, the mRNA levels in proteinuric states were generally increased compared with controls. Nephrin mRNA levels were significantly elevated in lupus nephritis, whereas only in diabetic nephropathy was there a slight decrease in nephrin mRNA levels. The latter finding is in line with other studies measuring nephrin mRNA expression in diabetic nephropathy by in situ hybridization (41). Our results are in sharp contrast with the study by Furness et al (42), which described a downregulation of nephrin mRNA in four patients with nephrotic syndrome. Patrakka et al (31) did not find significant changes in nephrin mRNA levels, as studied in proteinuric patients using in situ hybridization. In experimental studies, both up- and downregulation of nephrin mRNA in proteinuric states has been described (34,35,38,43). Following the same pattern as nephrin mRNA, the podocin mRNA levels showed an increase in most patient groups. Likewise, podoplanin mRNA levels were increased in most patient groups compared with controls. In PAN nephrosis of the rat, mRNA levels of molecules that bind the podocyte to the GBM are increased, suggesting an effort of the podocytes to remain attached to the GBM (37). Similarly, in human and experimental proteinuric states, the mRNA expression of α-actinin-4 is increased, probably reflecting a compensatory reaction of the podocyte (44,45). The rise in mRNA levels of nephrin, podocin, and podoplanin might likewise result from a compensatory reaction to the damage inflicted on the podocyte.

The pathogenic effects exerted on the glomerulus in the various renal disease entities studied differ in nature and severity. For example, putative circulating permeability factors might cause the loss of the glomerular permselectivity in MCD (46). In lupus nephritis and membranous nephropathy, subepithelial Ig deposits are formed, thereby inflicting damage on the podocyte, whereas in IgA nephropathy, more preferentially mesangial areas are affected (47). Diabetic nephropathy is thought to impair the charge selectivity of the glomerular filtration barrier and might thereby indirectly damage the podocyte (48). Damage to the podocyte leads, irrespective of its origin, to effacement of foot processes (40). Therefore, as a measure of podocyte damage, we examined the extent of foot process effacement by assessment of the mean FPW. The mean FPW was positively

correlated with proteinuria and podoplanin mRNA levels, whereas it was inversely correlated with nephrin protein levels. This suggests that the changes seen in protein expression are related to the severity of the damage inflicted on the podocyte. For instance, that IgA nephropathy is characterized primarily by damage to the mesangial areas and, to a lesser extent, to the podocytes might be reflected by the absence of significant changes in the podocyte-associated molecules at the protein level, as has been observed before (32). The observation that downregulation of nephrin protein is paralleled by foot process effacement is in line with results from a study by Huh et al (49) and Wernerson et al (50), which suggests that not the quantity of nephrin between adjacent foot processes is different but that merely the total glomerular amount of nephrin is decreased as a result of foot process effacement. The decreased protein staining of nephrin, podocin, and podocalyxin might also be explained by a reduction of podocytes, as has been observed in several diseases (51,52). Furthermore, the accessibility of the antigen might be diminished.

The development of a nephrotic syndrome and effacement of podocyte foot processes is nowadays known to occur upon mutations in a wide range of podocyte-associated molecules, including those investigated in this study. The molecular scaffold in the podocyte foot process therefore seems to be fragile, disassembling when one of its components is removed. Generally, in the current study, the protein expression of most podocyte-associated molecules was diminished in acquired proteinuric diseases, coinciding with an increase in mRNA levels. A divergence between mRNA and protein levels has been described for synaptopodin and α-actinin 4 in patients with nephrotic syndromes (24,44) and in podocyte-associated molecules in the PAN nephrosis rat model (37,45). There was no direct correlation between the level of proteinuria and the protein and mRNA expression of the molecules studied. However, nephrin protein levels and podoplanin mRNA levels were correlated with the effacement of podocyte foot processes, which might point in the direction of an association between the expression of podocyte-associated molecules and proteinuria. It is still unclear whether changes in expression of these podocyte-associated molecules are the underlying cause of the development of proteinuria. Taken together, there is a cluster of molecules, at both the protein and the mRNA levels, that seem to show a stereotypic reaction in proteinuric diseases. Besides taking into account the correlation between foot process effacement and protein and mRNA levels, the observed changes in expression levels merely seem to be the consequence of podocyte foot process effacement as a result of other causes and the subsequent reaction of the podocyte to this phenomenon.

Acknowledgments

We thank Dr. C. Antignac (Hôpital Necker, Paris, France) for kindly providing antibody p35 against the C-terminus of human podocin. Peter Neeskens is gratefully acknowledged for his indispensable help in the ultrastructural techniques and analyses. We thank Prof. Dr. L.A. van Es for critical comments on the manuscript.

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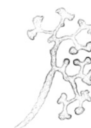

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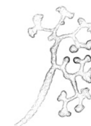

Selective Loss of Podoplanin Protein Expression Accompanies Proteinuria and Precedes Alterations in Podocyte Morphology in a Spontaneous Proteinuric Rat Model

3

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Am J Pathol. 2008 Aug;173(2):315-26

Abstract

To evaluate changes during the development of proteinuria, podocyte morphology and protein expression were evaluated in spontaneously proteinuric, Dahl salt-sensitive (Dahl SS) rats. Dahl SS rats on a low-salt diet were compared with spontaneously hypertensive rats (SHR) at age 2, 4, 6, 8, and 10 weeks. Blood pressure, urinary protein excretion, urinary albumin excretion, and podocyte morphology were evaluated. In addition, the expression of 11 podocyte-related proteins was determined by analyzing protein and mRNA levels. In Dahl SS rats, proteinuria became evident around week 5, increasing thereafter. SHR rats remained non-proteinuric. Dahl SS rats showed widespread foot process effacement at 10 weeks. At 8 weeks, expression and distribution of the podocyte proteins was similar between the two strains, except for the protein podoplanin. At 4 weeks, podoplanin began decreasing in the glomeruli of Dahl SS rats in a focal and segmental fashion. Podoplanin loss increased progressively and correlated with albuminuria ($r = 0.8$, $P < 0.001$). Double labeling experiments revealed increased expression of the podocyte stress marker desmin in glomerular areas where podoplanin was lost. Dahl SS rats did not show podoplanin gene mutations or decreased mRNA expression. Thus, podocyte morphology and the expression and distribution of most podocyte-specific proteins were normal in young Dahl SS rats, despite marked proteinuria. Our study suggests that decreased expression of podoplanin plays a role in the decrease of glomerular permselectivity.

Introduction

The permselectivity of the glomerular filtration barrier restricts passage of proteins into Bowman's space. Loss of permselectivity leads to proteinuria, which is common in renal diseases of diverse origin. Proteinuria is related to the progression of renal and cardiovascular disease (1-3) Although the details of glomerular filtration remain unknown, it is clear that the glomerular visceral epithelial cell or podocyte is an important component of the glomerular filtration barrier. Damage to podocytes is frequently involved in the pathogenesis of glomerular diseases (4).

Podocyte damage can be the result of changes in individual podocyte-associated proteins. Examples include mutations in the genes that encode nephrin, podocin, CD2AP, and α-actinin-4 (5-8). Such mutations can result in both congenital and hereditary forms of glomerular dysfunction. Podocyte damage also appears to be of pathogenic importance in acquired diseases. For example, in diabetic nephropathy and IgA nephropathy, podocyte loss is related to disease severity (9-11). Similarly, animal models have shown that loss of podocytes is related to the development of proteinuria and glomerulosclerosis (12-15).

A change in podocyte morphology often accompanies proteinuria. In the normal glomerulus, the podocyte has an arborized phenotype, and its terminal branches or foot processes cover the outer wall of the glomerular capillaries. In proteinuric states, this morphology is typically lost as the podocyte converts to a flatter epithelial cell, a process referred to as "foot process effacement". In foot process effacement, the cytoskeleton that normally supports the delicate architecture of the foot processes is condensed at the basal side of the flattened podocytes (16).

We previously studied the mRNA and protein expression of several podocyte-associated proteins in acquired proteinuric diseases (17). Based on the results of that study, we hypothesized that the changes in expression of podocyte proteins represented a compensatory reaction of the podocyte to the occurrence of proteinuria. Accordingly, we wanted to investigate how the expression of podocyte-associated proteins is regulated during the development of proteinuria, and how changes in expression are related to podocyte morphology. We studied proteins that have been shown to be involved in congenital and hereditary nephrotic syndromes, as well as podocyte proteins that have been studied in animal models. Among these is the glycoprotein podoplanin, of which the expression was previously found to change in the puromycin aminonucleoside nephrosis model of the rat (18). Studying human kidney biopsy samples does not allow the rigorous evaluation of changes over time. In this study, we therefore used the Dahl salt-sensitive rat (Dahl SS) proteinuric model (19) to evaluate changes in podocyte morphology and expression of podocyte-associated proteins during the development of proteinuria.

Materials and Methods

Animals and study design

We compared the spontaneously proteinuric Dahl SS rat strain with non-proteinuric spontaneous hypertensive (SHR) rats. Male Dahl SS and SHR rats were obtained from colonies at the Freie Universität Berlin as previously reported (19). All animals were fed a low-salt diet containing 0.2 percent NaCl by weight; on this diet, Dahl SS rats develop mild spontaneous hypertension comparable to SHR rats. To study changes in expression of podocyte-associated proteins during the development of proteinuria, groups of rats (n = 5 to 8 rats per group) were studied at 2, 4, 6, 8, and 10 weeks of age. Experiments were performed in accordance with institutional guidelines.

Urinalysis and blood pressure measurements

Urinalysis was performed in each rat. Rats that were 4 weeks of age or older were placed in metabolic cages for a 24-hour period. In the 2-week-old rats, a urine sample was obtained by bladder punction before the perfusion procedure described below. Urinary albumin excretion was subsequently determined by enzyme-linked immunosorbent assay (20). Urinary protein excretion rates were determined using the Bradford method. In rats of 6 weeks of age or older, systolic blood pressure was determined using the tail-cuff method, as described previously (20). Three blood pressure measurements were performed each day on two consecutive days.

Perfusion, tissue preservation and isolation of glomeruli

Rats were anesthetized using intraperitoneal injection of ketamine/xylazine. After the abdomen was opened, the aorta was ligated below the diaphragm and both kidneys were perfused with PBS. After clamping the vascular pole, the right kidney was removed. For electron microscopy, small pieces of the cortex of the right kidney were immersion-fixed for 24 hours in 0.1M glutaraldehyde containing cacodylate and were processed according to standard procedures. For histology and immunohistochemistry, part of the remaining right kidney was embedded in paraffin, and the other part was snap frozen and stored at –80°C.

The left kidney was used for isolation of glomeruli. Because the standard differential sieving method is not suitable for isolation of glomeruli in very young animals, we used the magnetic retraction method to isolate glomeruli from all animals (21). Briefly, the left kidney was perfused with sonicated 1.25% suspension of Fe_3O_4 (Sigma-Aldrich Chemicals, Zwijndrecht, The Netherlands) in PBS, removed, and placed in ice-cold PBS. The kidney was then cut into pieces and pressed through a 106 μm (weeks 2 and 4) or 150 μm (weeks 6 to 10) mesh filter. The glomeruli were isolated by holding the suspension to a magnet. The fluid was discarded, and the glomeruli

were resuspended in fresh PBS. After repeating this procedure two times, glomeruli were pelleted and stored at –80°C until RNA extraction.

Table 1. Primer sequences

Name	Symbol	mRNA sequence	Forward	Reverse	Amplicon size
α-actinin-4	Actn4	NM_031675	GGCTATGAAGAATGGCTGCTGAATG	AGGGTGGCTGTCTCGTAGTCC	151
CD2-associated protein	Cd2ap	NM_181475	TCCAGCGAATCAGCACCTACG	CACTCCACCAGCCTTCTTCTACC	197
dystroglycan	Dag1	XM_343483	GATGGCACGGCTGTTGTTGG	GCACTCACTGAGATGTAATGGACAC	199
ezrin	Vil2	NM_019357	CTGGACGACCGTAACGAGGAG	CTTGCCGCATGTTCTCATTGTG	156
GLEPP1	Ptpro	NM_017336	TGGATGGTCGTGGCAGAAGG	TGGAGGCAGGCTAAGGATGG	107
hsp-27	Hspb1	NM_031970	AAGGAAGGCGTGGTGGAGATC	ACCTGGAGGGAGCGTGTATTTC	101
megalin	Lrp2	NM_030827	CACGACCGCTGCTTACAACTG	GATGGCATGGCACCGATTCAC	128
NEPH1	Kirrel1	NM_207606	GGATGGCGGTAAGGTGGAGTG	CGTTATTGATGGTGAGAGTGGACAG	163
nephrin	Nphs1	NM_022628	CGTCAGCATCAGCAGCAACC	AGCCACATCTTCCAGCCTCTC	72
podocalyxin	Podxl	NM_138848	GGCTGTGTTTGAACTGCTGAAGG	ACGATGGTGATGATGAGAGGAAGG	135
podocin	Nphs2	NM_130828	TGGACTCAGTGACCTGTGTTTGG	CAGCAATCACCCGCACTTTGG	138
podoplanin	Pdpn	NM_019358	CCAGCCACTCCACGGACAAG	GGGTCACTACAGCCAAGCCATC	101
ras homolog gene family, member A	Rhoa	NM_057132	GCACACAAGGCGGGAGTTAG	CGTCTTTGGTCTTTGCTGAACAC	121
synaptopodin	Synpo	NM_021695	AGCCTAAGGTGACGCCGAATC	TCTCTGCCTCCGCTTCTCATC	70
wilms tumor 1	Wt1	NM_031534	TGTGACTTCAAGGACTGCGAGAG	GGTGTGGGTCTTCAGGTGGTC	143
hypoxanthine guanine phosphoribosyl transferase	Hprt	NM_012583	GGCTATAAGTTCTTTGCTGACCTG	AACTTTTATGTCCCCCGTTGA	138
TATA box binding protein	Tbp	NM_001004198	ACCGTGAATCTTGGCTGTAAACTTG	GCAGTTGTTCGTGGCTCTCTTATTC	122
hydroxymethylbilane synthase	Hmbs	NM_013168	TGAAGGATGTGCCTACCATACTACC	GCAAGGTTTCCAGGGTCTTTCC	123

RNA isolation, cDNA synthesis and real-Time PCR

RNA was isolated from the glomeruli using the TRIzol (Invitrogen) method. Total RNA (0.5 µg) was reverse transcribed into cDNA using Avian Myeloblastosis Virus reverse transcriptase (Roche Diagnostics).

Primers (Isogen Bioscience) for 14 podocyte-expressed genes were designed using BeaconDesigner 4.0 software (PREMIER Biosoft International). To prevent genomic contamination, all primers, except those for the synaptopodin gene that contains only one exon, were chosen to span at least one splice-site. Primers sequences are listed in Table 1 . Real-time PCR was performed using an iCycler real-time PCR machine with iCyclerIQ 3.1 software (Bio-Rad laboratories). Sybrgreen was used as the fluorescent dye. The mean expression levels of three housekeeping genes (Hmbs, Tbp, and Hprt) were used to correct for variations in the quantity of input cDNA.

Immunohistochemistry, immunofluorescence and morphometrics

Three-micron paraffin sections were deparaffinized, rehydrated, and used for immunostaining after appropriate antigen retrieval procedures. Three-micron cryosections were used untreated or after fixation with acetone/ethanol for 5 minutes and 100% ethanol for 10 minutes.

Endogenous peroxidase activity was blocked for 15 minutes in 0.1% H_2O_2 in water. After washing with PBS, sections were incubated with primary antibodies diluted in 1% bovine serum albumen in PBS for 2 hours. Details regarding antibodies, fixation, and antigen retrieval are listed in Table 2. After 1 hour incubation with the secondary antibody, the slides were developed with diaminobenzidine. Slides were counterstained with hematoxylin, dehydrated, and mounted. To minimize variations in intensity, staining for each antibody was performed on all sections during one session.

Podoplanin staining was evaluated on a semiquantitative scale: 0, normal intensity and pseudo-linear staining pattern; 1, loss of staining <25 percent of the glomerular surface or a granular staining pattern; 2, loss of staining involving 25 to 75 percent of the glomerular surface; and 3, loss of staining in >75 percent of the glomerular surface.

Podoplanin and desmin were costained on paraffin sections and cryosections using the appropriate Alexa 466- and 536-conjugated secondary antibodies. Staining was evaluated with a Zeiss LSM 510 confocal microscope.

For morphometric analysis of the size of the glomeruli and the number of podocytes, we used slides stained for WT-1, a podocyte-specific transcription factor that is used to identify and count podocytes in tissue sections (22). Slides stained for WT-1 were evaluated with a Zeiss Axioplan microscope equipped with a Sony DXC-950P 3CCD color camera (Sony Corporation, Tokyo, Japan). Ten randomly chosen regions of the outer glomerular cortex were photographed at x200 magnification. The surface area of all glomeruli in the photographs was determined using ImageJ 1.34 software (National Institutes of Health, http://rsb.info.nih.gov/ij). The number of WT-1 positive nuclei in each glomerular cross section was counted using the same software. The number of podocytes per glomerulus and the glomerular volume per podocyte were calculated from

Table 2: Antibodies

Epitope	Cryo/paraffin	Antigen retrieval	Primary Antibody, dilution and source	Secondary Antibody	Reference
α3 integrin	Cryo	-	Rabbit anti-α3 integrin, 1:800, Chemicon cat# AB1920	Anti-rabbit Envision	
α-actinin-4	Cryo	-	Rabbit polyclonal antibody 6A3, 1:4000	Anti-rabbit Envision	(8)
α-dystroglycan	Paraffin	Citrate	Mouse mAb VIA4-1, 1:150, Upstate cat# 05-298	Anti-mouse Envision	
Desmin	Paraffin/Cryo	Tris/EDTA	Mouse mAb clone 33, 1:750	Anti-mouse Envision, Alexa	
Ezrin	Paraffin	Tris/EDTA	Mouse mAb, 1:100	Anti-mouse Envision	
Nephrin (EC domain)	Cryo	-	Mouse mAb 5-1-6, 1:200	Anti-mouse Envision	(51)
Podocalyxin	Paraffin	Proteinase K	Mouse mAb 5A, 1:300	Anti-mouse Envision	(52)
Podocin (C-terminal part)	Paraffin	Proteinase K	Rabbit polyclonal antibody P35, 1:3000	Anti-rabbit Envision	(53)
Podoplanin	Paraffin/Cryo	Proteinase K	Rabbit polyclonal anti-rat podoplanin, 1:3000	Anti-rabbit Envision, Alexa	(54)
Synaptopodin	Paraffin	Citrate	Mouse mAb G1D4, 1:4, Progen cat# 65194	Anti-mouse Envision	
WT-1	Paraffin	Tris/EDTA	Mouse mAb WLM04, 1:50, Abcam cat# ab3236	Anti-mouse Envision	

Envision: DakoCytomation, Glostrup, Denmark. Cat# K4001 (anti-mouse Envision HRP) and K4003 (anti-rabbit Envision HRP); Proteinase K: DakoCytomation, Glostrup Denmark. Cat# S3020; Citrate: 0.1M Citrate buffer, pH 6.0; Tris/EDTA: 0.1M Tris/EDTA, pH9.0

these measurements (22). Periodic acid-Schiff staining was performed to determine changes in morphology.

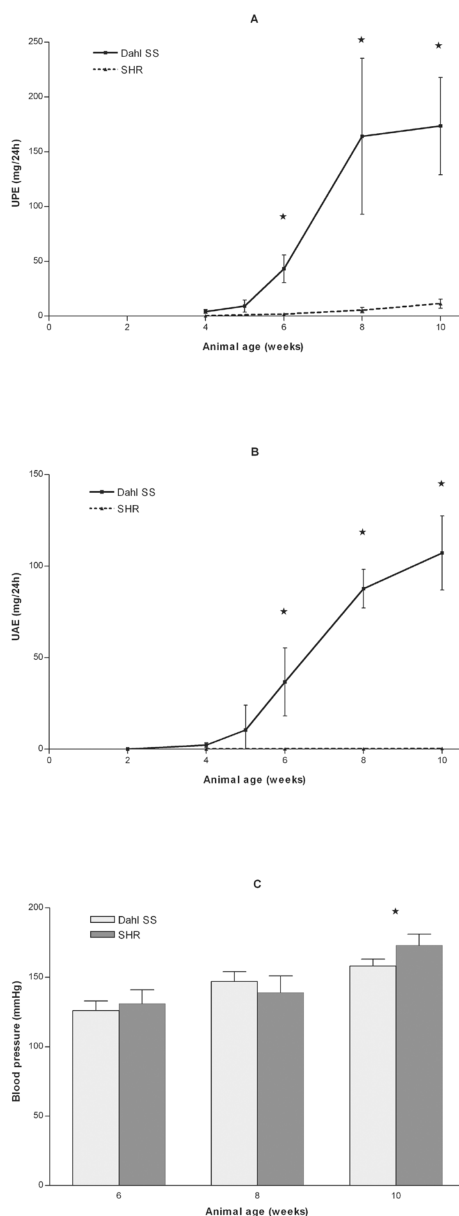

Figure 1. Urinary protein excretion (A), urinary albumin excretion (B) and blood pressure (C) in Dahl SS and SHR rats. As expected, SHR rats remained non-proteinuric throughout the time course of the study. Dahl SS rats developed proteinuria and albuminuria between 4 and 6 weeks of age (A and B). In Dahl SS rats, measurements were taken at an additional time point, 5 weeks of age, to obtain a more detailed view of the early phase of proteinuria development in these rats. Both rat strains developed hypertension starting at week 8. At 10 weeks of age, the blood pressure of SHR rats was significantly higher than that of Dahl SS rats (C). An asterisk indicates $P < 0.05$.

Sequencing

To check for genetic differences between the Dahl SS and SHR rats, we sequenced the full-length podoplanin cDNA in a representative rat from each strain. The sequences of the primers used are available on request.

Laser capture microscopy

We used laser capture microscopy to determine whether there were differences in podoplanin mRNA expression in glomeruli that showed a difference in podoplanin protein expression. Four-micron cryosections were obtained from four 8-week-old Dahl SS rats. One section was used for immunohistochemical staining for podoplanin to identify glomeruli that had either completely lost or retained podoplanin expression. The cross-section of the same glomerulus was identified in the sequential unstained section and isolated by laser capture microdissection using a PALM Laser-MicroBeam system (Wolfratshausen, Germany). RNA was isolated from the isolated glomeruli, and quantitative measurements of the expression of podoplanin and RhoA mRNA were performed as described above.

Electron microscopy and immunoelectron microscopy

Small pieces of the cortex were fixed in 1.5% glutaraldehyde and 1% paraformaldehyde for

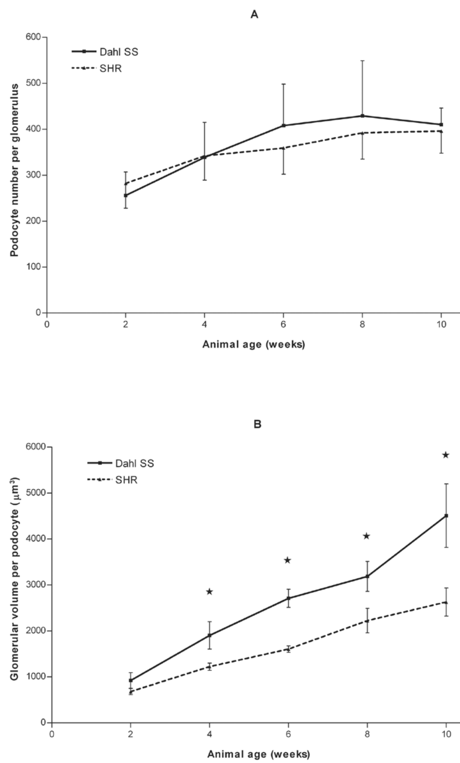

Figure 2. Number of podocytes per glomerulus (A) and glomerular volume per podocyte (B) in Dahl SS and SHR rats. The total number of podocytes per glomerulus increased over time in both Dahl SS and SHR rats, and remained stable from 6 to 8 weeks onwards at 350 to 400 podocytes per glomerulus. Although there was no difference in absolute number of podocytes per glomerulus, the glomerular volume per podocyte was higher in Dahl SS compared with SHR rats. The glomerular volume per podocyte is an index used to indicate the glomerular volume that one podocyte 'serves' and is calculated as the mean glomerular volume divided by the mean number of podocytes per glomerulus. This difference was statistically significant from week 4 onward. An asterisk indicates $P < 0.05$.

24 hours and then stored in 0.1 M cacodylate buffer with 6% sucrose. After postfixation in 1% reduced osmium in 0.1 M cacodylate buffer, the samples were dehydrated and embedded in EPON. Ultrathin sections were cut using a Leica Ultracut microtome and mounted on uncoated copper grids. Sections were contrasted with uranyl acetate and lead citrate before evaluation with a JEOL JEM-1011 electron microscope equipped with a digital camera.

Random pictures of 2 to 3 glomeruli were taken in two rats per group using x15,000 magnification. Using image analysis, we determined the extent of foot process effacement by measuring the average foot process width, as described previously (17).

For immunoelectron microscopy, small pieces of the cortex from 8-week-old Dahl SS rats were fixed in 4% paraformaldehyde in 0.1 M PBS and stored in 0.1% paraformaldehyde in 0.1 M PBS. Immunogold labeling was performed on ultrathin frozen sections as described previously (18).

Statistical analysis

Data are reported as the mean ± SD. Analysis of variance with a Least Significant Difference posthoc correction was used to test for differences between groups. Correlations were tested using Pearson's or Spearman's tests, where appropriate. $P < 0.05$ was considered significant.

Proteinuria and blood pressure

Starting at 4 weeks, proteinuria was increasingly evident in Dahl SS rats. As expected, SHR rats remained non-proteinuric throughout the time course of the study (Figure 1, A and B). Both rats developed hypertension starting at the age of 8 weeks (Figure 1C). The blood pressure of the 10-week old SHR rats was significantly higher than that of Dahl SS rats of the same age (P < 0.05).

Glomerular volume, podocyte number, and light microscopy

The number of podocytes per glomerulus, as determined using WT-1 staining, was not different between the two rat strains (Figure 2A). The glomerular volume was higher in Dahl SS rats than in SHR rats. The glomerular volume per podocyte (22) was also higher in Dahl SS rats (Figure 2B); this difference was statistically significant from 4 weeks of age onwards. Fibrotic changes in glomeruli or in the interstitium were not observed at any time point.

Electron microscopy

Throughout the first 8 weeks of life, both the Dahl SS and the SHR rats generally showed normal ultrastructural glomerular morphology. In 6- and 8-week-old Dahl SS rats, subtle coarsening of the foot processes was occasionally seen, together with protein droplets in the podocyte cell bodies and major processes (Figure 3A).

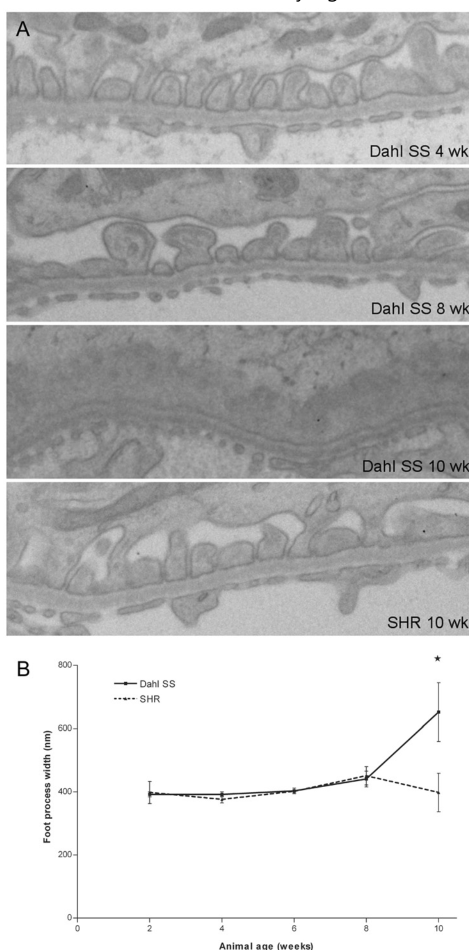

Figure 3. Electron microscopy of podocytes in Dahl SS and SHR rats (A) and evaluation of the mean foot process width (B). In 4-week-old Dahl SS rats (A, top panel), the podocyte ultrastructure was normal, showing regularly spaced foot processes. Occasionally, protein droplets were observed in podocyte major processes and cell bodies (arrowheads). At 8 weeks, subtle coarsening of the foot processes was observed sporadically in Dahl SS rats (A, second panel). At 10 weeks of age, widespread foot process effacement was observed in segmental areas of the glomerulus, with condensation of the actin cytoskeleton at the basal site of the effaced processes (arrow). Microvillous transformation of podocytes was observed frequently at this time point. In contrast, the podocytes of SHR rats had normal ultrastructure throughout the time course studied (A, bottom panel). Original magnification: x 15,000. Quantification of the mean foot process width showed significant effacement only in the 10-week-old Dahl SS rats (B). An asterisk indicates P < 0.05.

Ten-week-old Dahl SS rats showed more widespread foot process effacement. Quantification of the mean foot process width underscored the normal ultrastructure of the podocyte in the first 8 weeks of life (Figure 3B). Detachment of podocytes from the glomerular basement membrane was not seen at any time point, and alterations in the glomerular basement membrane morphology were not observed.

Protein expression

Immunohistochemistry showed that expression of α3-integrin, α-actinin-4, α-dystroglycan, ezrin, podocalyxin, podocin, and synaptopodin, was similar at all time points in both rat strains (Figure 4). Expression of the nephrin protein showed a normal pseudo-linear staining pattern throughout the first 8 weeks of life; however, by 10 weeks of age, expression became more granular in a

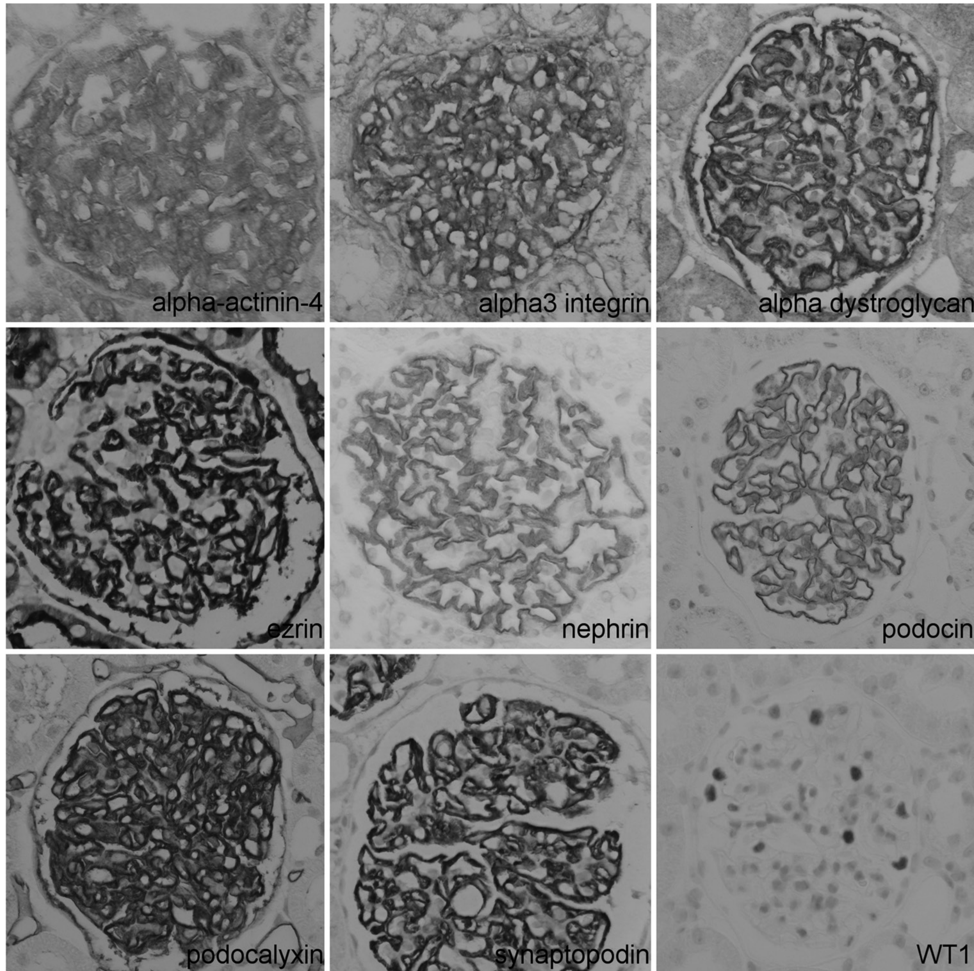

Figure 4. Immunohistochemistry of podocyte proteins. Despite marked proteinuria, 10-week-old Dahl SS rats showed normal expression of α-actinin-4, α3 integrin, α-dystroglycan, ezrin, nephrin, podocin, podocalyxin, synaptopodin, and WT1. Original magnification: x 400.

Figure 5. Podoplanin protein expression in Dahl SS glomeruli was progressively lost in a focal and segmental fashion. Loss of podoplanin protein expression was first seen in 4-week-old Dahl SS rats and increased thereafter. In contrast, podoplanin protein expression remained normal in SHR rats throughout the time course of the study. The upper row of images in A shows podoplanin staining in Dahl SS rats at the indicated time points and in a 10-week-old SHR rat. The lower row of images in A shows desmin staining in Dahl SS rats at the indicated time points and in a 10-week-old SHR rat. Starting at 6 weeks, desmin expression was visible in the extramesangial areas of Dahl SS glomeruli. Expression level increased with age. No change in desmin expression was observed in aging SHR rats. Sequential sections of the kidney of a 10-week-old Dahl SS rat stained with anti-rat podoplanin antibodies, anti-podocin antibodies, and with PAS show that loss of podoplanin is not related to morphological alterations observed by light microscopy or to alterations in podocin expression (B). Nephrin expression was diminished sporadically in segmental parts of glomeruli, but only in 10-week-old Dahl SS rats (C). Original magnification: x 400.

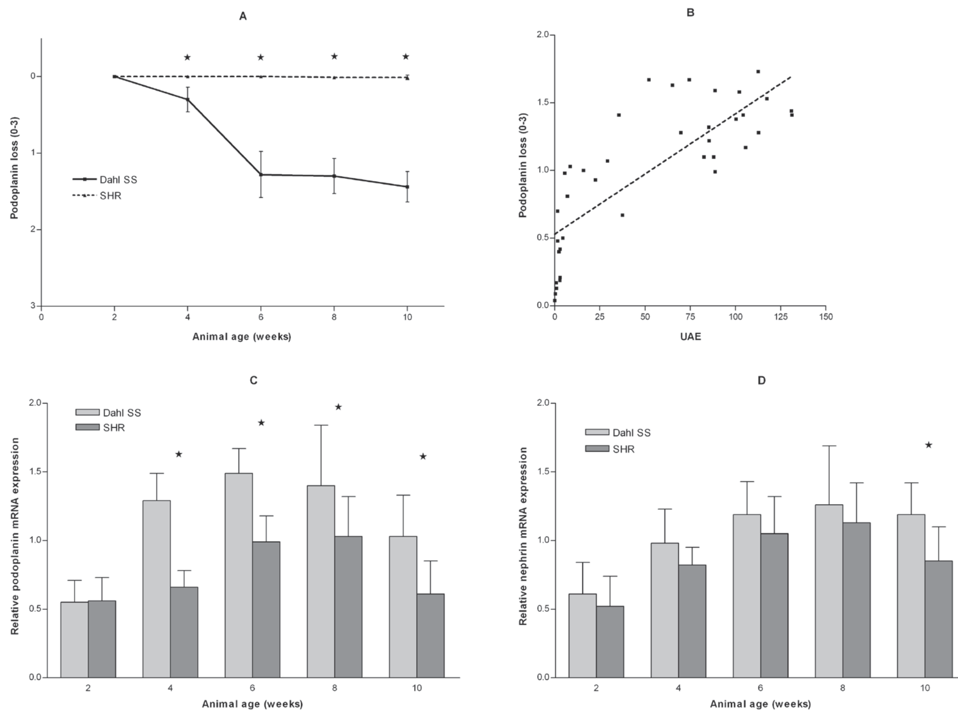

Figure 6. Loss of podoplanin protein expression was determined using a semiquantitative method. The loss of podoplanin protein staining in Dahl SS samples was statistically significant from week 4 onward (A). The extent of loss of podoplanin expression was positively correlated with urinary albumin excretion rates (r = 0.8, P < 0.001) B: In contrast, there was a slight but significant increase in podoplanin mRNA expression levels in Dahl SS rats compared with SHR rats at weeks 4 to 10 (C). Nephrin mRNA expression was increased only in 10-week-old Dahl SS rats (D). An asterisk indicates P < 0.05.

focal and segmental fashion in Dahl SS rats (Figure 5C). In contrast to all other proteins studied, and only in Dahl SS rats, expression of the podoplanin protein decreased in a focal and segmental fashion. The decrease in podoplanin expression started as early as week 4 and increased with age (Figure 5A and Figure 6A). There was a strong correlation between development of albuminuria and loss of podoplanin protein expression (r = 0.8, P < 0.001, Figure 6B). The loss of podoplanin protein expression was not related to changes in glomerular morphology, and did not coincide with changes in expression of other podocyte-associated proteins (Figure 5B). Podoplanin expression in endothelial cells in intrarenal lymphatic vessels was not diminished.

Expression of the intermediate filament desmin can be used as a marker of podocyte stress or damage in rat podocytes. Indeed, we found an increase in desmin protein expression during the time course (Figure 5A). When desmin and podoplanin were costained, we found that desmin expression was absent in glomeruli that retained podoplanin expression. In contrast, segmental loss of podoplanin was mirrored by an increase in desmin expression in that particular segment (Figure 7). Desmin expression seemed to lag behind podoplanin loss.

mRNA expression

We also investigated the expression of 14 podocyte-expressed genes at the mRNA level (Table 1), revealing varied expression patterns. In both strains, the expression of podocyte-specific genes was lowest at the early time points. At 4 weeks of age, the podoplanin and ezrin genes were more highly expressed in Dahl SS than in SHR rats. From 4 to 10 weeks, the podoplanin mRNA expression levels were significantly increased in Dahl SS rats compared with SHR rats (Figure 6C), mirroring the decreased podoplanin protein expression in Dahl SS at these time points (Figure 5A). Other genes (NEPH-1, podocin, synaptopodin, α-actinin-4) showed late up-regulation (at 8 to 10 weeks) of mRNA expression in Dahl SS rats as compared with SHR rats.

Only at 10 weeks were nephrin mRNA expression levels significantly elevated in Dahl SS rats compared with SHR rats ($P < 0.05$, Figure 6D), again mirroring the nephrin protein staining results in Dahl SS rats at this time point (Figure 5C). Despite marked proteinuria, the pivotal podocyte protein transcription factor WT-1 was not differentially regulated (data not shown).

Sequencing and laser capture microdissection

Sequencing the full-length podoplanin cDNA in a representative Dahl SS and SHR rat did not reveal mutations in the Dahl SS podoplanin gene. There was no difference in podoplanin mRNA expression in glomeruli that had completely lost podoplanin protein expression versus those that had retained normal podoplanin expression (Figure 8). Podoplanin has been linked to the activity of the small GTPase RhoA (23) so we evaluated whether such a link existed in glomeruli. We found that RhoA mRNA levels were increased in sections of glomeruli that lacked podoplanin expression (Figure 8), although this did not reach statistical significance.

Immunoelectron microscopy

Immunogold labeling of podoplanin in 8-week-old Dahl SS rats revealed that podoplanin was extensively expressed in areas that showed no foot process effacement. Conversely, foot process effacement was accompanied by a loss of podoplanin expression (Figure 9).

Discussion

In this study, spontaneous development of proteinuria in Dahl SS rats preceded changes in podocyte morphology and changes in expression of most of the observed podocyte-specific proteins. In contrast, focal and segmental loss of the podoplanin protein accompanied proteinuria. The early loss of podoplanin expression did not coincide with changes in the expression of other

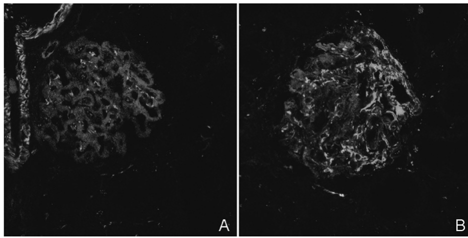

Figure 7. Podoplanin (red) and desmin (green) were costained in kidney sections from 8-week-old Dahl SS rats. Normal glomeruli show desmin expression in renal blood vessels and glomerular mesangium and podoplanin expression in podocytes. Desmin and podoplanin did not colocalize (A). In glomeruli that showed segmental loss of podoplanin expression, increased desmin expression was observed in the podoplanin-negative areas (B). Original magnification: x 630.

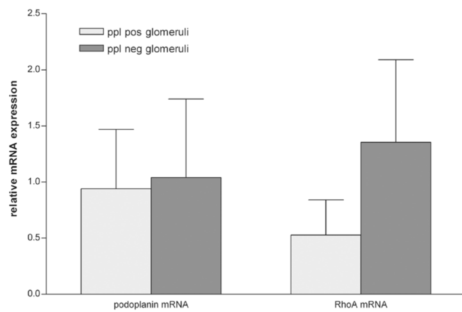

Figure 8. Podoplanin mRNA expression in podoplanin positive and negative glomeruli. In 8-week-old Dahl SS rats, podoplanin mRNA expression was not different in glomeruli that showed extensive loss of podoplanin protein (ppl) expression compared with glomeruli that had retained podoplanin expression. RhoA mRNA expression was higher in glomeruli that no longer expressed the podoplanin protein, although this difference did not reach statistical significance.

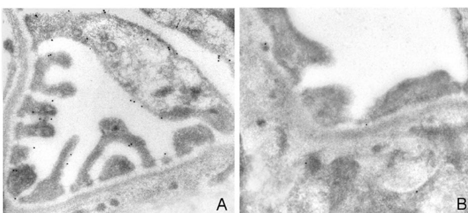

Figure 9. Immunogold electron microscopic analysis of podoplanin labeling in 8-week-old Dahl SS rats. Podoplanin labeling was most prominent in glomerular areas that showed no foot process effacement (A). Podoplanin labeling was weak or absent in areas in which foot process effacement was observed (B).

podocyte proteins and could not be explained by mutations in the podoplanin gene or by decreased transcription.

Proteinuria is an important risk factor for the progression of renal disease (1,2), and understanding its pathogenesis may help reduce the burden of chronic renal disease. Elucidation of the molecular basis of congenital and hereditary proteinuric diseases has contributed significantly to our understanding of glomerular function in health and disease. We previously studied the expression of podocyte-associated proteins in human acquired proteinuric diseases, and found that the mRNA expression of these was upregulated, whereas the protein expression was down-regulated. These changes correlated with the extent of foot process effacement. Based on these results, we hypothesized that the changes in expression represented a compensatory reaction of the podocyte to damage (17). In the current study, we used a rat model to determine how the observed changes may have developed over time.

Proteinuria is often, but not always, accompanied by podocyte foot process effacement. A remarkable finding in the current study was that foot process effacement early after the development of proteinuria was subtle, and the difference in mean foot process width between SHR and Dahl SS rats only became statistically significant at 10 weeks of age. There are several reports in the literature that support this temporal uncoupling of proteinuria and foot process effacement: similar observations were made in young proteinuric Dahl SS rats (19), and in other spontaneous proteinuric rat strains (24) as well as in experimental models (25,26). Some reports

have indicated that in humans, the extent of foot process effacement is not related to the severity of proteinuria (27,28). Others have reported patients and families with nephrotic syndromes that did not show foot process effacement (29-31).

Our observations in the spontaneously proteinuric rat model studied here are in agreement with the pattern we found in humans in which the mRNA expression levels of several podocyte proteins were up-regulated during the development of proteinuria. For example, only at time points at which proteinuria was present in combination with more widespread foot process effacement (ie, at week 10) changes in nephrin protein expression were seen, paralleled by increased nephrin mRNA expression levels (Figures 5C and 6D). Although the etiology of proteinuria in the Dahl SS rat is probably different from that in human disease, these findings support the hypothesis that changes in nephrin expression are secondary to proteinuria development and foot process effacement.

In remarkable contrast to the general pattern of most of the podocyte-associated proteins studied, the glycoprotein podoplanin showed changes in expression with a clear temporal relationship to the development of proteinuria. Increasing loss of glomerular podoplanin expression was highly correlated with urinary albumin excretion ($r = 0.8$, $P < 0.001$, Figure 6B). Observation of rats up to 6 weeks of age showed that the onset of decreased glomerular podoplanin expression preceded detectable morphological alterations in the podocytes. At later time points (from 8 weeks onwards), when proteinuria had already progressed appreciably, immunoelectron microscopy showed that foot process effacement coincided with the loss of podoplanin; in contrast, areas in which podoplanin expression was retained showed normal ultrastructural morphology (Figure 9). The semiquantitative nature of the immunoelectron microscopy experiments did not allow us to draw conclusions with regard to more subtle changes in podoplanin expression in areas without foot process effacement. The expression of the slit diaphragm-associated proteins nephrin and podocin appeared unchanged up until weeks 8 (nephrin) and 10 (podocin) of age and was not related to the expression of podoplanin (Figure 5), suggesting that loss of podoplanin expression was not related to changes in the slit diaphragm.

Expression of podoplanin in the glomerulus was first reported by Breiteneder-Geleff et al, who described a 43-kDa glycoprotein that was specifically down-regulated in puromycin aminonucleoside nephrosis in the rat (18). Since down-regulation of this protein was related to foot process effacement, it was named podoplanin. The relationship between foot process effacement and podoplanin expression was further substantiated by the observation that injection of antibodies directed against a specific podoplanin epitope caused reversible proteinuria and foot process effacement, but only if divalent IgG antibodies were used (32). Homologs of podoplanin (T1-, P2.26, and E-11) are expressed in other cell types, including type I alveolar cells in the lung, ke-

ratinocytes, and osteoblasts (33-35). In recent years, podoplanin has received more interest as a marker of lymphatic endothelial cells (36) and various tumors (37).

The mechanism underlying the involvement of podoplanin in glomerular pathology has remained unclear. In the current study, we found that focal and segmental loss of podoplanin protein coincided with the occurrence of proteinuria. The down-regulation of podoplanin at the protein level in Dahl SS rats could not be explained by decreased mRNA expression, which was actually increased during the development of proteinuria compared with non-proteinuric SHR rats. In addition, sequencing of the full-length Dahl SS podoplanin cDNA did not reveal any differences between Dahl SS and SHR rats that could explain the down-regulation of podoplanin protein in Dahl SS rats.

Although the exact extra- and intracellular interactions of podoplanin are currently unknown, several studies have indicated that podoplanin is involved in cell motility and actin cytoskeleton modeling. Ectopic expression of podoplanin in cultured cells increases the formation of cell extensions as well as increasing cell adhesiveness and migration (38,39). Furthermore, podoplanin expression in MCF7 cells induces filopodia formation, loss of stress fibers, and relocalization of actin to the newly formed filopodia; relocalization is mediated by inhibition of RhoA (23). Studies indicate that podoplanin and ezrin colocalize in subcellular compartments (23,39), and one study found that podoplanin and ezrin could be coimmunoprecipitated (39). Although the details of the interaction remain to be elucidated, these studies suggest a role for podoplanin in actin cytoskeleton modeling. In our study, ezrin did not show an altered expression pattern. However, immunohistochemical staining may not detect protein modifications such as phosphorylation.

How could down-regulation of this protein with actin-modeling properties relate to the development of proteinuria? The temporal relationship between podoplanin down-regulation and development of proteinuria does not necessarily imply a causal involvement of podoplanin dysregulation in the pathogenesis of proteinuria. However, in the current model, because no marked regulation of the expression of other podocyte proteins has been observed, it is most likely that podoplanin expression is specifically regulated. In puromycin aminonucleoside nephrosis in the rat, podoplanin is also down-regulated (18). This suggests that either down-regulation of podoplanin causes proteinuria, or that the expression of podoplanin is actively down-regulated by the podocyte in response to proteinuria. Until now, it has not been possible to study the effect of the absence of podoplanin in glomeruli, since podoplanin knockout mice have an embryonic lethal phenotype (38). However, injection of divalent anti-podoplanin IgG antibodies in rats induces rapid foot process effacement and proteinuria (32), indicating that changes in podoplanin can indeed be the initial step in the development of proteinuria. We recently found that podoplanin protein expression is also selectively lost in proteinuric Munich Wistar Frömter rats (40). This may indicate that loss of podoplanin is a more general phenomenon in proteinuria in rats.

It has been suggested that foot process effacement and formation of stress fibers are reactions of the podocyte to prevent dilation of capillaries and further leakage of proteins (41). Recently, Morigi et al showed that following protein load, podocytes reorganize their cytoskeleton in a RhoA-dependent manner (42). Similarly, Zhang et al reported that complement-mediated injury of cultured podocytes increases RhoA activity, resulting in a change in cellular morphology that mimics foot process effacement (43). Thus, the events that take place during foot process effacement (ie, stress fiber formation and foot process effacement in a RhoA-dependent fashion) seem to be the reverse of those brought about by the actin remodeling properties of podoplanin (prevention of stress fiber formation, filopodia formation, and RhoA inhibition). The loss of podoplanin we observed thus suggests a scenario in which the podocyte actively remodels its shape and down-regulates proteins, including podoplanin, which normally would promote foot process formation. Indeed, we found that RhoA mRNA levels were up-regulated in microdissected glomeruli that showed a loss of podoplanin protein expression compared with those that had retained podoplanin (Figure 8).

The extracellular domain of podoplanin is presumably negatively charged (32). Given the predominant localization on the apical membrane of the foot processes, it has been suggested that podoplanin may have a function comparable to that of podocalyxin, another negatively charged protein that is thought to serve as a spacer molecule between adjacent foot processes (32,44). Loss of podocalyxin is associated with foot process effacement (45), and it could be hypothesized that loss of podoplanin has similar consequences.

As the name implies, the Dahl SS rat is known for developing severe hypertension when raised on a high-salt diet. The development of hypertension is delayed and diminished when the rats are fed a low-salt diet, as in the current study. Although development of hypertension aggravates the rate of disease progression, several lines of evidence suggest that the development of proteinuria is uncoupled from the development of hypertension. First, proteinuria in Dahl SS rats develops before the onset of hypertension (46), starting from about 4 weeks of age. Furthermore, micropuncture experiments indicate that proteinuria is not explained by increased intraglomerular pressure (46). From a genetic point of view, development of proteinuria is independent of the development of hypertension (19,47). Recently, Nagase et al reported that in Dahl SS rats, podocytes are injured only on salt loading, as judged by de novo expression of desmin and B7–1 and down-regulation of nephrin protein expression (48). The authors suggested that podocyte injury might underlie the development of proteinuria in these rats. We, too, observed desmin expression in the podocytes of Dahl SS rats in the absence of salt loading, most notably in glomerular segments that showed a loss of podoplanin protein expression. However, this does not necessarily implicate podocyte damage as the cause of proteinuria in the Dahl SS rat. Desmin protein expression may also reflect other injury, such as increased mechanical strain as a result of the increased glomerular volume covered per podocyte (14). Furthermore, increased protein trafficking through

the glomerular filtration barrier may itself be harmful for podocytes and may promote expression of podocyte stress markers (42,49,50).

In conclusion, we found that in the spontaneously proteinuric Dahl SS rat, significant proteinuria was detected several weeks before widespread changes in podocyte morphology were observed. Moreover, segmental loss of podoplanin protein expression accompanied proteinuria and preceded widespread podocyte alterations.

Acknowledgements

We thank Dr. Schweikert and Dr. Beggs for the anti-α-actinin-4 antibody, Dr. Antignac for the anti-podocin antibody, and Dr. Mittienen for the anti-podocalyxin antibody. We thank Peter Neeskens and Frans Prins for their help with the electron microscopy studies.

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Glomerular expression profiling in spontaneously proteinuric rats reveals differential expression of genes associated with cytoskeleton and protein overload

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submitted for publication

Abstract

To get more insight in cause and consequences of proteinuria, we studied glomerular gene expression patterns before and after the onset of increased urinary albumin excretion (UAE) in a proteinuric rat strain.

Spontaneously proteinuric Dahl salt-sensitive rats (Dahl SS) were compared to non-proteinuric, spontaneously hypertensive rats (SHR). In Dahl SS, UAE significantly increased starting from week 5 of age. Glomerular RNA profiles of 4- and 6-week-old rats were studied using Affymetrix microarray chips. Gene expression was further studied by quantitative PCR and immunohistochemistry.

398 genes were upregulated and 210 genes were downregulated in Dahl SS compared to SHR. The data were analyzed using three main approaches, and the following results were found: 1) 115 genes, located on quantitative trait loci previously associated with proteinuria, were differentially expressed between the rat strains. These included hedgehog-interacting protein and outer dense fiber 3; 2) Several pathways related to changes in the cytoskeleton were represented in the list of differentially expressed genes. Expression of the actin-regulating protein dynamin was increased in proteinuric rats and in patients with proteinuric disease; 3) The expression pattern of a group of 13 genes, of which the expression was previously found to be regulated in tubular epithelial cells upon protein loading, was differentially regulated in glomerular samples of Dahl SS versus SHR rats ($P < 0.03$). Expression of the podocyte stress marker desmin co-localized with albumin resorption droplets in podocytes.

The list of genes differentially expressed between proteinuric and non-proteinuric rat strains is predominated by those encoding for cytoskeletal proteins. Secondly, expression differences between strains may result partly from increased protein trafficking through the glomerular filtration barrier.

Introduction

The initial step in the production of urine is glomerular filtration, in which water and small solutes are filtered but macromolecules and cells are retained within the glomerular capillaries. Loss of the glomerular filtration barrier permselectivity results in the loss of proteins into the urine, proteinuria. Proteinuria is an important symptom of various kidney diseases, and its presence is related to the progression of renal and cardiovascular diseases (1,2). Knowledge about the molecular determinants of glomerular filtration has advanced considerably through the discovery of mutations in genes that underlie hereditary proteinuric kidney diseases (3). Nevertheless, the understanding of the development of proteinuria in the majority of patients that do not have such genetic mutations remains limited.

The Dahl salt-sensitive (Dahl SS) rat strain has been used as a model for the development of salt-sensitive hypertension. Sterzel et al (4) showed that prior to development of hypertension and independent of salt loading, Dahl SS rats also show an increased urinary albumin and protein excretion. Several groups have attempted to dissect the genetic basis of the increased urinary albumin excretion (UAE) in Dahl SS rats. Using the spontaneously hypertensive rat (SHR) as a reference strain, genetic linkage analysis yielded several quantitative trait loci (QTLs) of interest for the increased UAE (5-8). In several analyses by Kreutz' group a QTL for UAE was located on rat chromosome 19 (5,6), in both high and low salt conditions. Consomic strains in which the SHR chromosome 19 was introgressed into the Dahl SS background, showed a considerable amelioration of the albuminuric phenotype (9). The identification of genomic regions is helpful in elucidating the polygenetic nature of the UAE, and can demonstrate the linkage of other genetically determined phenotypes such as hypertension to the development of proteinuria. However, the relatively low resolution of the linkage analysis impairs the identification of the genes that play a role in the development of proteinuria.

It is hypothesized that proteinuria, once it has developed, exerts a toxic effect on proximal tubular epithelial cells. Although the exact nature of the compounds that cause this effect (albumin, albumin-bound factors, or other macromolecules) is subject of discussion, several studies have indicated that upon proteinuria proximal tubular epithelial cells acquire a pro-inflammatory and pro-fibrogenic state (10,11). Some studies have indicated that an increased passage of proteins through the glomerular filtration barrier may have a similar toxic effect on podocytes (12). Indeed, proteinuria is almost always accompanied by changes in podocyte cell structure, and reorganization of the podocyte cytoskeleton (13,14).

We used a microarray approach to get insight in the gene expression differences that occur in the glomerulus during the development of proteinuria. The results of the microarray experiments were used in several ways. Combination of the microarray data and previously identified genomic

regions was used to identify genes that could be of interest for the development of the increased UAE. Next to studying single genes, we used pathway analysis to identify groups of genes that differentiate between the proteinuric and non-proteinuric rat strains. These approaches yielded further genes of interest and pathophysiological concepts that were studied in more detail.

Materials and Methods

Animal studies

We compared the spontaneously proteinuric Dahl SS rat strain with non-proteinuric spontaneously hypertensive rats (SHR). Male Dahl SS and SHR rats were obtained from colonies at the Freie Universität Berlin as reported (5). The animals were fed a low-salt diet of 0.2% NaCl by weight content to prevent early development of hypertension. For the microarray studies, we used 4- and 6-week-old rats. In follow-up experiments, groups of rats that were 2, 4, 6, 8, and 10 weeks of age were studied (n = 5 to 8 per group). Systolic blood pressure, proteinuria, and albuminuria were recorded. Following described methods (15), tissues were collected under ketamin/xylazine anesthesia, and glomeruli were isolated using perfusion with iron oxide followed by magnetic retraction. Experiments were performed in accordance with institutional guidelines.

Microarrays

For the microarray experiments Affymetrix GeneChip Rat Genome 230 2.0 arrays were used (Affymetrix, www.affymetrix.com). These high-density gene chips contain 31,042 probesets, representing approximately 28,000 different genes and ESTs, on a single chip.

We compared 4- and 6-week-old Dahl SS and SHR rats, studying two rats from each strain at each time point. Total RNA from isolated glomeruli was purified with Qiagen RNeasy mini columns (Qiagen, www.qiagen.com). Quality and purity of the RNA was controlled by gel electrophoresis and measurement of the 260/280 ratio, which was between 1.8 and 2.1 in all samples. Biotin-labeled cRNA was produced using the Ambion MessageAmp II-Biotin Enhanced kit (Ambion, www.ambion.com), according to the manufacturer's instructions. Briefly, 1µg of total RNA was reverse transcribed in the presence of T7 oligo(dT) primer, followed by second-strand cDNA synthesis. After purification, in vitro transcription of the double stranded cDNA was performed in the presence of T7 RNA polymerase and biotin-11-UTP. Following another round of purification, 20 µg of cRNA was fragmented and used for hybridization to the chips. Hybridization and scanning of the chips was performed at the Leiden Genome Technology Center (www.lgtc.nl).

The data discussed in this publication have been deposited in NCBI's Gene Expression Omnibus (16) and are accessible through GEO Series accession number GSE13810 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc= GSE13810)

Preprocessing and differential expression analysis

Preprocessing, differential expression analysis, and parts of the pathway analysis were performed in the R-software environment (www.r-project.org). All R-packages used are available at the bioconductor website (www.bioconductor.org).

Preprocessing of the data was performed with the GC Robust Multiarray Average (GCRMA) package. GCRMA – a modification of RMA (17) – calculates expression values using background adjustment, quantile normalization, and summarization of the probe intensities, and uses the probe sequence information (e.g., GC-content) to estimate the non-specific binding related to the background noise (18). Quality control was performed using functions in the affyPLM package in R, and included evaluation of RNA degradation plots, relative log expression, and normalized unscaled standard errors. All chips were of good quality with regard to these parameters.

To identify statistically significant differences in gene expression levels we used the Linear Models for Microarray Data (Limma) package (19,20). Limma first fits a model to each gene in the different arrays, as in a standard ANOVA approach. With a contrast matrix, the differently expressed genes can subsequently be extracted. We used Benjamin and Hochberg's method to correct for multiple testing. Comparisons of strain differences and time differences within strains were made. A differential gene expression of more than 1.5 fold, with an adjusted P-value < 0.01 in strain comparisons, and an adjusted P-value < 0.05 in time comparisons, was considered significant. In the text and tables, fold changes are given as base 2 logarithmic values.

Comparison to genomic studies

Previous genetic analysis of the strains that we studied has yielded several QTLs of importance for the development of urinary albumin excretion. To integrate these genetic analyses with the microarray data, we obtained the QTLs found by Poyan Mehr et al (5), Siegel et al (6), and Garrett et al (7,8) from the rat genome database (RGD, http://rgd.mcw.edu). Of the genes that showed a differential expression in the array experiments, we evaluated those that were located within a chromosomal location identified in the previous genetic linkage analyses, using information in the RGD and Ensemble (www.ensembl.org) databases.

Pathway analysis

For pathway analysis, we used several complimentary approaches. For the evaluation of known pathways, we used the online pathway analysis program Gene Ontology Tree Machine (GOTM, bioinfo.vanderbilt.edu/gotm)(21) and the global test. GOTM uses a hypergeometric test to evaluate whether the genes of a certain Gene Ontology (GO) category are overrepresented in the

group of differentially expressed genes as defined using Limma (21). In a complementary approach, the global test was used to evaluate whether genes within a certain gene set (ie, a GO category or KEGG pathway, www.genome.jp/kegg) were differentially expressed (22). The global test assigns a p-value to this association. In addition, a so-called comparative p-value can be calculated. In this calculation, the association of the group of genes of interest is compared to that of a large number of randomly selected groups of genes of the same size. The benefit of the global test approach is that it also allows identification of pathways in which many genes are only moderately regulated.

The effect of proteinuria on gene regulation in proximal tubular epithelial cells has been extensively studied. From such studies, we extracted a group of well-substantiated genes that were shown to be regulated in response to proteinuria in mouse, rat, and human proximal tubular epithelial cells. We identified the rat homologue of these genes, and used the global test to

Table 1. Primer sequences

Name	Symbol	mRNA sequence	Forward	Reverse	Amplicon size
hedgehog-interacting protein	Hhip	XM_238042	CCTTTGGTTGGTGGATTCGTTTAC	TTGCTTAGTCACTGGGCTTTGC	119
osteoglycin	Ogn	NM_001106103	TGTGCCTCCTAATTTACCAGAAAG	TACCGAGTGTCATTAGCCTTGC	105
dipeptidylpeptidase 6	Dpp6	NM_022850	CCTATGTCAGTGCTTCCTTCAGTC	CTTCTCTTATCCGTGGTATTGTGC	105
minichromosome maintenance deficient 6	Mcm6	NM_017287	GGCTGGGCTTTGCTGAATACTG	GCTGACTCATCC-TCCTCTTCTTCC	85
rac GTPase-activating protein 1	Racgap1	NM_001108112	TCCAGATCCAGTGACGATGTTCC	GGCAGTCCATGTTCTCTTGTTCC	124
patched	Ptch1	NM_053566	GTGGTCATCCTGATTGCGTCTG	CATAGCCCTGTGGTTCTTGTCC	102
dynamin 1	Dnm1	NM_080689	TTGATGAGAAGGAACTGCGAAGG	AAGCGAGGTCAGGAGTGAAGAG	89
cell division cycle associated 1	Cdca1	NM_001012028	AGCAAGTAACCGCCATTAATCAAG	GATGCCCTCATGGTACTTCTCC	145
chondroitin sulfate proteoglycan 2	Cspg2	XM_001058160	ACCTGCTATCCTACTGAGACTTCC	CCGACAAGGGTTAGAGTGACATTC	104
ras homolog gene family, member A	Rhoa	NM_057132	GCACACAAGGCGGGAGTTAG	CGTCTTTGGTCTTTGCTGAACAC	121
desmin	Des	NM_022531	CCTACACCTGCGAGATTGATG	GCGATGTTGTCCTGATAGCC	114
vimentin	Vim	NM_031140	GCTGCCCTGCGTGATGTC	ATTCGTTTGACTCCTGCTTTGC	156
filamin beta	Flnb	XM_224561	GCTGCTGACTTCCTTCTGG	GTTGCCGTTCTTCTTGATGC	183
kinesin family member 23	Kif23	XM_236313	GTGACTGAACCCA-AACCTGAGAAG	CAAGAGTTAGAGCGTCTGTGAAGC	179
rab8b	Rab8b	NM_153317	CCTGCCTCCTGTTCCGCTTCTC	GCCCGCCGTGTCCCATATCTG	133
polyamine modulate factor 1 binding protein 1	Pmfbp1	NM_134393	GGCTGGAGAGGAGTATGC	TTGACGGACGACTGTAGG	196
hypoxanthine guanine phosphoribosyl transferase	Hprt	NM_012583	GGCTATAAGTTCTTTGCTGACCTG	AACTTTTATGTCCCCCGTTGA	138
TATA box binding protein	Tbp	NM_001004198	ACCGTGAATCTTGGCTGTAAACTTG	GCAGTTGTTCGTGGCTCTCTTATTC	122
hydroxymethylbilane synthase	Hmbs	NM_013168	TGAAGGATGTGCCTACCATACTACC	GCAAGGTTTCCAGGGTCTTTCC	123

evaluate whether this group of genes was also differentially expressed in the glomeruli of Dahl SS and SHR rats.

RNA isolation and QPCR

RNA was isolated from the glomeruli using the TRIzol (Invitrogen) method. Total RNA (0.5μg) was reverse transcribed into cDNA using AMV reverse transcriptase (Roche Diagnostics).

Primers (Isogen Bioscience) were designed using BeaconDesigner 6.0 software (PREMIER Biosoft International). To prevent genomic contamination, all primers were chosen to span at least one splice-site. Sequences of the primers are listed in Table 1. Real-time PCR using Sybrgreen as the fluorescent dye was performed using a iCycler real-time PCR machine with iCyclerIQ 3.1 software (Bio-Rad laboratories).

Immunohistochemistry

A rabbit polyclonal anti-rat albumin antibody (Nordic Immunological Laboratories, www.nordiclabs.nl) was used for immunofluorescent localization of albumin in formalin-fixed paraffin-embedded tissue after antigen retrieval by boiling the slides in Tris/EDTA buffer for 10 minutes. A mouse monoclonal anti-desmin antibody (clone 33, diluted 1:750) was used in albumin and desmin double-labeling studies. As secondary antibodies, Alexa-conjugated anti mouse and anti-rabbit antibodies were used as appropriate. The immunofluorescent staining was evaluated using a Zeiss LSM 510 confocal microscope (Carl Zeiss, Germany).

For immunohistochemical localization of dynamin in rat and human tissues, we used the mouse anti-dynamin antibody Hudy 1 (Upstate biotechnology, catalog # 05-319, www.upstate.com, diluted 1:80). As secondary antibody, we used anti-mouse Envision (Dako cytomotion, Glostrup, Denmark). Staining was performed using standard procedures as described previously (23). Specificity of the staining was tested through replacing the antibody by serum of the same species, in the same dilution as the secondary antibodies. For quantification of the staining of renal biopsies, we used both a digital image analysis and a semi-quantitative approach (scoring on a scale of 1 to 4). The results of both approaches were comparable and correlated highly with each other.

Biopsies from patients with acquired proteinuric kidney diseases

Biopsies from patients with various proteinuric kidney diseases were studied in comparison to controls. Disease categories included minimal change disease (n = 8), focal segmental glomerulosclerosis (n = 3), IgA nephropathy (n = 3), lupus nephritis (n = 6), diabetic nephropathy (n = 6), other diseases (including post-streptococcal glomerulonephritis, membranous nephropathy, light chain excretion nephropathy, n = 6). For controls, biopsies from patients with interstitial nephritis or with no glomerular pathology, tissue from kidneys unsuitable for transplantation for technical reasons, and tissue from kidneys obtained at autopsy was used (n = 16).

Results

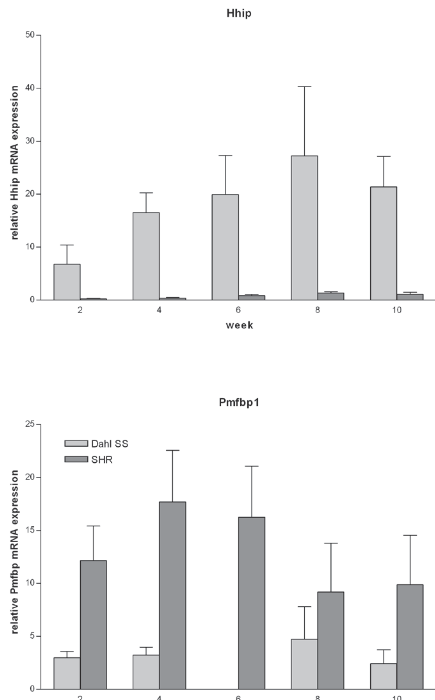


Figure 1. mRNA expression of hedgehog-interacting protein (Hhip, A) and polyamine modulated factor binding protein 1 (Pmfbp1, B) in glomeruli of Dahl SS and SHR rats of various age. Hhip was upregulated in Dahl SS rats at all time points, and also before the development of overt proteinuria (week 2). Pmfbp1 was downregulated in Dahl SS rats compared to SHR.

Animal phenotype

The animals used for the microarray experiments had normal systolic blood pressure (125 mmHg for 6-week-old SHR and DS rats). The UAE was 2.9 ± 0.0 and 32 ± 4.5 mg/24h for the 4- and 6-week-old Dahl SS rats, respectively, and < 0.1 mg/24h for the SHR rats at both time points. These values reflect those of the total group (n = 5-8) (15). Over the studied 10-week time course, the Dahl SS rats showed increasing UAE rates, while the SHR rats remained non-albuminuric.

Differential regulation of genes

We first compared the differential regulation of genes between the two rat strains, irrespective of the time points. Using a false discovery rate of < 0.01 and a 1.5 fold change as cut-off, 366 genes were found to be significantly upregulated, and 151 downregulated. The top-20 of up- and downregulated of these are listed in Table 2.

Comparing the differential regulation in time, first looking at the strains individually, we found that the gene expression levels of the Dahl rats were relatively similar at 4 and 6 weeks: only 16 and 7 genes were up- and downregulated, respectively, between 4 and 6 weeks. In contrast, the non-proteinuric SHR rat showed substantial regulation of genes between 4 and 6 weeks, with an upregulation of 397 genes and a downregulation of 210 genes at 6 weeks.

We subsequently evaluated which genes showed a different pattern of regulation in time between the two rat strains. For example, this would identify genes that over time are downregulated in the Dahl rat, while they are upregulated in the SHR. In total, 63 genes were significantly upregulated in the Dahl rat in comparison to the SHR, while 85 were downregulated.

We validated the results of the microarray using QPCR in individual rats. Measurements of 16 different genes showed that the direction of changes (up- or downregulation) was always consistent. The extent of changes showed some variability between the microarray and QPCR, but there was no consistent under- or overestimation of the regulation in the array results.

Table 2. Top 20 up- and downregulated genes

DahlSS vs SHR: Upregulated			
Gene name	Symbol	RNO	Fold change
similar to hedgehog-interacting protein	RGD1564108	19q11	5.2
osteoglycin	Ogn	17p14	4.8
EST		11q11	2.3
SUMO/sentrin specific protease 5	Senp5	11q22	4.2
EST			4.0
osteoglycin	Ogn	17p14	4.4
similar to vacuolar protein sorting 13C protein	RGD1560364	8q24	3.4
Similar to teratocyte-specific carboxylesterase	RGD1564156	8q24	4.7
EST			2.1
RT1 class II, locus Bb	RT1-Bb	20p12	7.6
zinc finger homeobox 1b	Zfhx1b	3q12	3.9
RT1 class Ib, locus Aw2	RT1-Aw2	20p12	6.4
histone 2a	H2a	17q11	1.4
tetraspanin 18	Tspan18	3q24	2.7
EST		9q13	1.9
Cut-like 1 (Drosophila)	Cutl1	12q12	1.3
EST	RGD1310127	1q36	1.2
Similar to Leucine rich repeat and sterile alpha motif containing 1	RGD1564403	3p11	1.5
GRAM domain containing 3	Gramd3	18q12.1	0.6
Mindbomb homolog 1 (Drosophila)	Mib1	18p13	1.4

DahlSS vs SHR: Downregulated			
Gene name	Symbol	RNO	Fold change
aldo-keto reductase family 1, member B8	Akr1b8	4q22	-4.5
EST			-4.3
similar to interferon regulatory factor 10	RGD1562711	3q41	-3.9
EST			-3.7
acyl-Coenzyme A oxidase 2, branched chain	Acox2	15p14	-3.7
similar to RIKEN cDNA 4921520P21; DMRTC1	LOC363483	Xq31	-3.4
transmembrane protein 14A	Tmem14a	9q13	-3.0
torsin family 1, member B	Tor1b	3p12	-3.0
EST			-2.9
polyamine modulated factor 1 binding protein 1	Pmfbp1	19q12	-2.8
RT1 class Ib, locus Aw2	RT1-Aw2	20p12	-2.7
similar to hypothetical protein FLJ20647	RGD1305326	2q43	-2.6
EST			-2.5
solute carrier family 2, member 5	Slc2a5	5q36	-2.5
nuclear protein 1	Nupr1	1q36	-2.4
GNAS complex locus	Gnas	3q42	-2.4
apolipoprotein B	Apob	6q14	-2.4
spectrin alpha 1	Spna1	13q24	-2.4
protein kinase C, beta 1	Prkcb1	1q36	-2.4
EST			-2.3

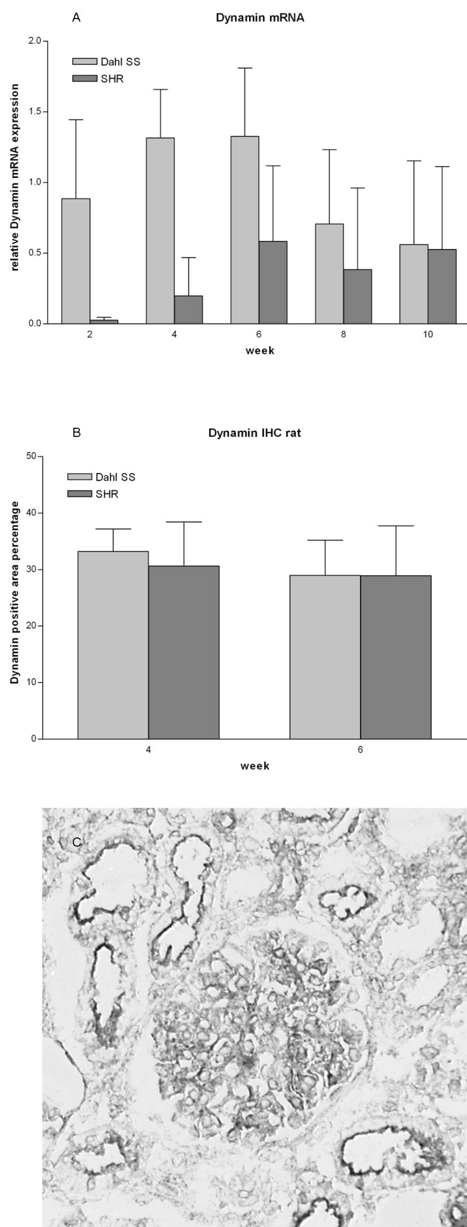


Figure 2. Dynamin expression in rat kidneys. The microarray showed a higher dynamin mRNA expression in 4- and 6-week-old Dahl SS rats compared to SHR. This was confirmed by QPCR (A). At later time points (8 and 10 weeks), dynamin upregulation was not significant. There was no clear increase in dynamin protein expression in Dahl SS rats (B). Dynamin protein was seen in the glomerulus and at the tubular brush border (C).

Relation to previously defined QTLs

Of all genes and ESTs with a known chromosomal location, which were differentially regulated between the two strains, we identified those genes that were located on QTLs know to be involved in the development of proteinuria in the studied rat strains (5-8). Of all up- and downregulated genes, 82 and 33 were located on such genomic regions. Within the UAE QTL on chromosome 19 (5,6), we identified five upregulated, and three downregulated probes. Of the five upregulated probes, three recognized hedgehog-interacting protein (Hhip) (5.2 times). The other two probes were ATP-binding cassette sub-family B (MDR/TAP) member 10 (Abcb10) (1.5 times), and integrin beta 1 (Itgb1) (0.9 times). The downregulated genes included polyamine modulated factor binding protein 1 (Pmfbp1, also called ODF3) (-2.8 times), WW domain-containing oxidoreductase isoform 2 (RGD1565791) (1.1 times), and hypothetical protein MGC3207 (RGD1307789) (-0.71 times). Of these, we validated the expression of Hhip and Pmfbp1 by QPCR. Dahl SS rats showed an increase in Hhip at all time points, while a downregulation of Pmfbp1 was seen. At week 6, levels of Pmfbp1 were undetectable in Dahl SS rats, whereas SHR rats showed a stable expression throughout the studied time course (Figure 1).

Pathway analysis

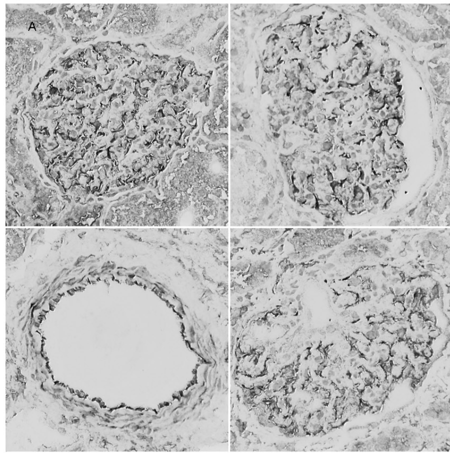
Pathway analysis was performed to get insight in the biological processes that were represented by the differentially regulated genes. GOTM analysis of the differentially regulated genes in time showed that cell-cycle associated genes were significantly enriched in the SHR rat, while

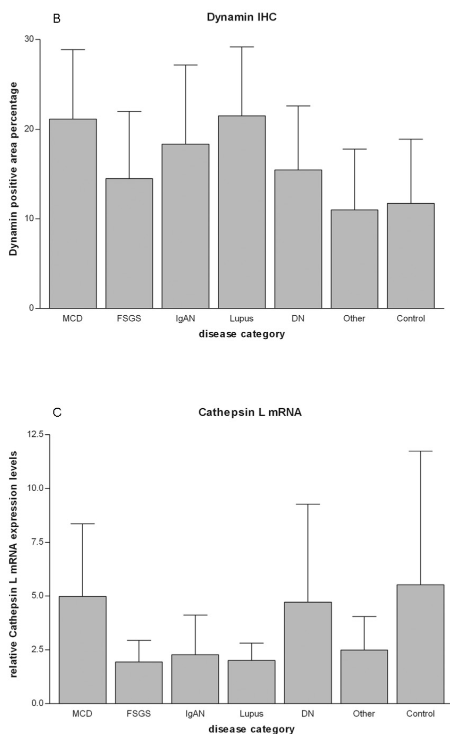
Table 3. Differential expression of cytoskeleton-related genes

Gene name	Symbol	RNO	Fold change	Function
periplakin	Ppl	10q12	2.06	intermediate filament binding
filamin, beta	Flnb	15p14	0.90	actin binding
dynamin 1	Dnm1	3p11	1.75	actin dynamics regulation
supervillin	Svil	17q12	1.39	actin binding
moesin	Msn	Xq31	1.95	actin filament -- membrane cross-linking
ARP1 actin-related protein 1 homolog A (yeast)	Actr1a	1q54	0.63	cytoskeleton organization
Rho guanine nucleotide exchange factor (GEF) 17	Arhgef17	1q32	1.07	actin cytoskeleton organization and biogenesis
CAP, adenylate cyclase-associated protein 1 (yeast)	Cap1	5q36	0.79	actin cytoskeleton organization and biogenesis
myosin Ib	Myo1b	9q22	0.99	actin binding
myosin IC	Myo1c	10q24	0.83	actin binding
tropomyosin 1, alpha	Tpm1	8q24	1.16	actin filament capping
parvin, alpha	Parva	1q33	1.39	actin cytoskeleton organization and biogenesis
microtubule-associated protein, RP/EB family, member 1	Mapre1	3q41	1.14	regulation of microtubule polymerization
caldesmon 1	Cald1	4q22	1.07	actin binding
Src homology 2 domain-containing transforming protein C1	Shc1	2q34	0.65	actin cytoskeleton organization and biogenesis
thymoma viral proto-oncogene 1	Akt1	6q32	1.44	cell projection organization and biogenesis
actin, beta	Actb	12p11	0.62	cytoskeleton organization
echinoderm microtubule associated protein like 4	Eml4	6q12	0.98	microtubule stabilization
kinesin light chain 1	Klc1	6q32	0.67	microtubule motor activity
signal-regulatory protein alpha	Sirpa	3q36	1.03	actin filament organization
mitogen activated protein kinase kinase kinase 1	Map3k1	2q14	0.83	actin filament polymerization
filamin, beta	Flnb	15p14	1.07	actin binding
actin related protein 2/3 complex, subunit 1B	Arpc1b	12p11	0.92	cytoskeleton organization
tropomyosin 4	Tpm4	16p14	1.45	actin binding
A kinase (PRKA) anchor protein 2	Akap2	5q24	0.59	actin filament organization
WD repeat domain 44	Wdr44	Xq12	1.00	
plastin 3 (T-isoform)	Pls3	Xq14	1.33	actin filament organization
polyamine modulated factor 1 binding protein 1	Pmfbp1	19q12	-2.81	cytoskeleton organization and biogenesis
spectrin alpha 1	Spna1	13q24	-2.39	cytoskeleton organization

Genes are listed with their chromosomal location and function. Fold changes are indicated as base 2 logaritmic value. A positive value indicates upregulation in Dahl SS vs SHR.

this was not the case in Dahl rats. Cell cycle related genes were also significantly enriched in the list of genes that showed a differential expression in time between SHR and Dahl SS strains.





Pathway analysis showed a relative enrichment of genes that were differentially expressed between the two strains in 49 GO categories. Nineteen of these GO categories were related to cytoskeletal changes. In a complementary approach we used the global test to evaluate KEGG pathways, as well as GO categories. This analysis also showed that the differences in gene regulation between the Dahl SS and SHR could in part be explained by regulation of cytoskeletal genes (KEGG pathway 04810, $P < 0.03$, comparative p value 0.17); GO:0005856, cytoskeleton, $P < 0.05$, comparative P value 0.21). The differentially expressed cytoskeletal genes are listed in Table 3.

Expression of dynamin in rat and human glomeruli

Dynamin is a cytoskeleton-associated gene that we identified as one of the genes that was upregulated in the Dahl SS rats compared to the SHR rat. Because recent studies showed that the actin-remodeling properties of dynamin may be important in podocytes (24), we decided to further evaluate the expression and distribution of dynamin in our model. Evaluation in individual rats confirmed the upregulation of dynamin mRNA expression in 4- and 6-week-old Dahl SS rats. At later time points upregulation of dynamin mRNA was not statistically significant (figure 2A). At the protein level, dynamin expression

Figure 3. Dynamin expression in human kidneys. The protein distribution of dynamin in human tissue (A) was similar to that in rats (figure 2C): dynamin was expressed in the glomerulus in a podocyte-like pattern (A, panel 1 and 2); the endothelial cells of large vessels were positive, while PTC and intraglomerular vessels did not show immunoreactivity (A, panel 3 and 4). Using digital image analysis, the protein expression of dynamin in various acquired proteinuric disease was quantified (B). Dynamin protein expression was increased in minimal change disease and in lupus nephritis compared to controls. The expression of cathepsin L mRNA was decreased in most acquired proteinuric diseases (C).

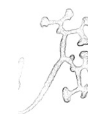


Table 4. Genes with differential expression in proximal tubular epithelial cells in proteinuric conditions

Model (in vivo, in vitro)	Species	Protein that evoked response	Method (RT PCR, ISH, microarray)	Genes regulated	Reference
Protein overload after uninephrectomy	Rat	proteinuria	RT PCR, in situ PCR	Ang II, ACE, renin (downregulated)	(40)
Proteinuric diseases	Human	proteinuria	ISH in biopsies	HO-1	(41)
PTEC	Human	HSA	RT PCR	ET-1, TGF-beta	(42)
PTEC, mouse protein overload	Human, mouse	HSA, mouse proteinuria	RT PCR	Fractalkine (CX3CL1)	(43)
Passive heymann nephritis, 5/6 NX	Rat	proteinuria	RT PCR	MCP-1	(44)
PTEC	Rat	BSA (delipidated)	RT PCR	MCP-1	(45)
PTEC	Rat	BSA, transferrin	RT PCR	MCP-1	(46)
Protein overload proteinuria	Rat	proteinuria	RT PCR	MCP-1	(47)
Protein overload proteinuria	Rat	proteinuria	RT PCR	MCP-1, osteopontin (ssp1)	(48)
Adriamycin nephropathy	Rat	proteinuria	RT PCR whole cortex	MCP-1, RANTES	(49)
Membranous nephropathy	Human	proteinuria	ISH	MCP-1, RANTES, TGF-beta, PDGF	(50)
Membranous nephropathy, MCD	Human	proteinuria (not albuminuria)	ISH	MCP-1, RANTES, TGF-beta	(51)
Protein overload proteinuria	Rat	proteinuria	ISH	TIMP-1	(52)
PTEC	Human	serum proteins		Fibronectin	(53)
PTEC	Human	albumin	Microarray	EGF receptor	(54)

PTEC – proximal tubular epithelial cell; MCD – minimal change disease; HSA – human serum albumin; BSA – bovine serum albumin.

was observed at the tubular brush border and in glomerular podocytes (Figure 2C). The protein expression of dynamin in rat glomeruli was slightly but not significantly elevated in 4-week-old Dahl SS rats compared to SHR. At 6 weeks, we could not find a difference in glomerular dynamin expression between the two strains (figure 2B).

As in rat kidneys, tubular epithelial brush borders were dynamin-positive in human renal tissue. Endothelial cells of larger vessels showed expression of dynamin as well. In human glomeruli, dynamin was present in podocytes and occasionally in parietal epithelial cells. Glomerular endothelial cells were not labeled (figure 3A). We observed an increase in dynamin protein expression in proteinuric kidney diseases, with minimal change disease and lupus nephritis showing a statistically significant increase ($P < 0.01$). Results of the digital image analysis measurements of glomerular dynamin expression are depicted in figure 3B.

Recent studies suggest that proteolytic cleavage of dynamin by cathepsin L may underlie the development of proteinuria (24). We therefore evaluated the mRNA expression of cathepsin L in proteinuric kidney diseases. In most renal diseases studied, we found a downregulation of cathepsin L at the mRNA level that was inversely correlated to the extent of proteinuria ($r = -0.39$, $P = 0.018$; figure 3C).

Proteinuria-induced glomerular gene expression patterns

Increased albumin trafficking through the glomerular filtration barrier within the context of proteinuria may have toxic effects on podocytes. We performed an immunostaining to visualize albumin within the glomerulus. As expected, albumin droplets were more often seen in Dahl SS rat glomeruli than in those of SHR rats. Co-staining of albumin and the podocyte stress marker desmin in Dahl SS tissues revealed that desmin accumulated in a podocyte-pattern in glomeruli or segments of glomeruli that showed the most extensive albumin accumulation (Figure 4).

These observations may support the hypothesis that increased albumin passage through the glomerular filtration barrier in Dahl SS rats is associated with podocyte stress. The intraglomerular albumin may consequently exert alterations in glomerular gene expression. From the literature we compiled a list of 13 genes of which the expression was found to be changed in proximal tubular epithelial cells upon proteinuria (Table 4). Using the global test, we found that the expression pattern of this group of genes was also significantly different in glomeruli of Dahl SS compared to SHR rats ($P < 0.03$ and $P < 0.008$ if time was taken as a covariate, Figure 5). If many genes are differentially expressed between groups of interest, a random group of genes may also be expected to be significantly associated with the difference between the two groups. We therefore compared this group of 13 'proteinuria-related' genes to 1,000 randomly selected groups of genes of the same size. We found that the group of proteinuria-related genes performed better than ~80 percent of the randomly selected groups. This indicates that the association between the expression pattern of the group of proteinuria-related genes with the difference between Dahl SS and SHR rats is specific.

Discussion

We used microarray technique to obtain more insight in gene expression differences during the spontaneous development of proteinuria in the Dahl SS rat. We report the following findings: i) we found and validated several genes that are differentially expressed and located within genomic regions previously identified to be associated with proteinuria; ii) pathway analysis showed that differences between the proteinuric and non-proteinuric rat strains are for a large part dependent on cytoskeletal genes; iii) from all genes involved in the regulation of the cytoskeleton, we further investigated the upregulation of dynamin expression in glomeruli of the Dahl SS rat and in human kidney diseases. iv) genes known to be differentially expressed upon proteinuria in tubular epithelial cells are differentially regulated in Dahl SS compared to SHR rats.

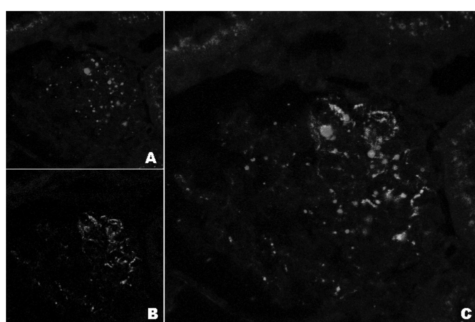


Figure 4. Co-immunostaining of desmin (green) and albumin (red) in a section of an 8-week-old proteinuric Dahl SS rat. Increased desmin expression is seen mostly in areas with extensive albumin accumulation. Desmin (A), albumin (B), merge (C).

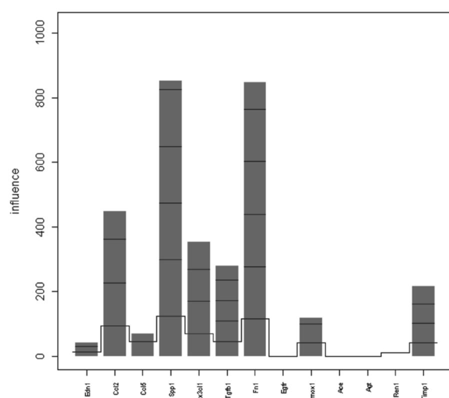


Figure 5. Proteinuria-induced gene expression patterns. We selected a group of genes of which expression was previously found to be induced in the tubulointerstitial compartment upon proteinuria. We used the global test to evaluate whether the glomerular expression of these genes was altered in proteinuric Dahl SS rats. The glomerular expression of the group of genes significantly differed between proteinuric Dahl SS rats and SHR ($P < 0.03$). In the graph, the contribution of the individual genes on the test result can be seen. On the x-axis, the individual genes are listed. The reference line marks the expected expression of the gene if it is not differently regulated (null-hypothesis); the height of the bar indicates the relative contribution of the individual gene to the test result; the marks indicate the number of standard deviations that the bar exceeds the expected (null-hypothesis) height. The graph shows a relatively large contribution of ssp1 (osteopontin) and fibronectin to the overall test result.

To find genes of interest for the development of proteinuria in the Dahl SS rat, we selected differentially expressed genes that are located on genomic regions previously identified in studies by Kreutz and Garrett et al (5-8). In these studies, a QTL on rat chromosome 19 was shown to be of importance for the development of proteinuria in the Dahl SS rat (5,6,9). We found that the mRNA expression of hedgehog-interacting protein (Hhip) and polyamine modulated factor binding protein 1 (Pmfbp1, also named outer dense fiber 3, ODF3), located within this genomic region, were significantly up- and downregulated, respectively. Although we confirmed the differential regulation of the mRNA transcripts in a time course analysis of individual rats, we have not been able to study the expression and distribution of the respective proteins.

Hedgehogs (Hh) are signaling molecules that play a role in tissue morphogenesis through influence on differentiation and proliferation of cells. Hh signaling is initiated by binding of Hh to the membrane receptor Patched, which via an elaborate pathway results in the transcriptional activation of target genes by Gli proteins (25). Hhip is a transcriptional target of Hh signaling and is expressed at the plasma membrane of Hh responsive cells where it is able to bind hedgehogs, thus preventing binding of hedgehogs to Patched (26). In the embryonic kidney, sonic Hh is thought to control the expression of various genes that play a role in the branching morphogenesis (27). Little is known about the activity of the Hedgehog signaling pathway in the adult kidney. Humphreys et al showed that in mice sonic Hh expression is limited to the renal medulla; Indian Hh is expressed in proximal

tubular epithelial cells, but its presence is dispensable for normal renal development and function (Humphreys et al, J Am Soc Nephrol 2006, 665A). Thus, it remains unknown whether increased glomerular Hhip expression has an effect on glomerular hedgehog signaling.

Pmfbp1, also termed outer dense fiber 3 (ODF3), is a coiled-coil protein that was originally identified in rat spermatozoa. Petersen et al (28) found that pmfbp1 is also expressed in the brain, and suggested that the protein is a component of the cytoskeleton. With regard to the organization of their cytoskeleton, podocytes show important similarities to neurons (29). It would be of interest to see whether the glomerular mRNA expression of pmfbp1 is indeed of podocyte origin.

Pathway analysis revealed significant overrepresentation of cytoskeletal genes in the group of genes with a differential expression between the Dahl SS and SHR. This is not surprising regarding the fact that proteinuria is mostly accompanied by profound alterations of the podocyte cytoskeleton. Widening and shortening of foot processes leads to loss of the complex cellular architecture (30,31). The organization of the actin cytoskeleton changes, and an electron dense band of filaments is formed at the base of the flattened cells (31). In a previous study, we found that the onset of proteinuria at five weeks of age precedes the first signs of foot process effacement in Dahl SS rats (15). However, cytoskeleton gene expression differences were already present at week 4, and persisted through week 6. We studied several cytoskeletal genes at the mRNA level, and found a consistent upregulation of genes involved in actin filament, intermediate filament, and microtubule based cytoskeletal networks in Dahl SS compared to SHR rats.

One of the proteins that has recently been implicated in the podocyte actin cytoskeleton rearrangement in proteinuria is the GTPase dynamin (24). We found an upregulation of dynamin mRNA in glomeruli of 4- and 6-week-old Dahl SS rats compared to SHR rats of the same age, although this difference was not clearly present at the protein level. In patients with acquired proteinuric kidney diseases, we did observe an upregulation of dynamin at the protein level. Reiser et al suggested that the cysteine protease cathepsin L is able to cleave and thereby inactivate dynamin (24). We found a downregulation of cathepsin L mRNA levels in patients with proteinuric diseases that was correlated to proteinuria. Taken together, these results may suggest that the absence of cathepsin L leads to the increased levels of dynamin protein. However, this pattern – downregulation of cathepsin L in combination with upregulation of dynamin – contrasts the previous findings by Reiser et al that cathepsin L activity is increased in LPS and polyamine nucleoside-induced nephrotic syndromes in rodents (32). The time frame in which proteinuria and foot process effacement develops is much shorter in these experimental models (days), in comparison to the spontaneous development of proteinuria in Dahl SS rats (weeks). The differences in gene and protein regulation in the current study and that of Reiser et al may be a reflection of this temporal regulation. For example, upregulation of dynamin may be a compensatory reaction to proteinuria, similar to our previous observation that certain critical podocyte proteins

are upregulated in acquired proteinuric diseases (23). We cannot exclude the possibility that the changes in cathepsin L and dynamin mRNA exression are separately influenced by another factor.

We observed that there was little differential regulation over time in the Dahl SS rats in comparison to the SHR rats. Pathway analysis of SHR rats indicated that cell-cycle and cytokinesis promoting genes were upregulated in the SHR rats compared to Dahl SS rats. In a previous study, we found that the glomerular volume per podocyte is increased in Dahl SS rats compared to SHR rats of the same age, i.e. there is a relative paucity of podocytes in Dahl SS glomeruli (15). Several studies have suggested that a shortage of podocytes may increase the susceptibility of the glomerulus to damage. Observational studies in human renal disease have already suggested a link between proteinuria and glomerular podocyte number (33). Macconi et al (34) found a similar relationship between relative "podocytopenia" (35) and proteinuria in Munich Wistar Fromter rats. In an experimental setting, Wharram et al (36) have studied the effects of podocyte depletion in rats: they developed a transgenic rat in which podocytes were depleted in a dose dependent fashion. A small percentage of podocyte depletion led to transient proteinuria, whereas removal of over twenty percent of podocytes resulted in persistent proteinuria, with increasing extent of glomerular damage and podocyte loss. We suggest that the lack of cell-proliferation response in Dahl SS rats that we observed using microarray analysis may relate to the development of proteinuria.

Furthermore, the observation that the Dahl SS rat showed little differential expression between weeks 4 and 6 may indicate that an impaired gene expression response underlies the development of proteinuria in the Dahl SS rats. Alternatively, these findings may indicate that the detection of protein in the urine lags behind the development of proteinuria-related gene expression changes in the gomerulus. We wanted to test whether the differential glomerular expression of genes between Dahl SS and SHR rats could be related to the increased protein trafficking through the glomerular filtration barrier. Gene expression changes upon protein loading are known to occur in tubular epithelial cells. Because tubular and glomerular epithelial cells share a common embryologic origin, we hypothesized that a similar pattern of gene expression might be present in the glomerulus. Our findings using the global test indicate that this may indeed be the case. This has interesting, be it speculative, implications. It is increasingly thought that proteinuria confers a toxic effect to the tubules, a phenomenon that has been regarded as one of the possible explanations of the link between glomerular damage and tubulo-interstitial injury (11). The similarity of proteinuria-induced gene expression pattern in tubules and glomerulus suggests that proteinuria has a comparable toxic effect on these tissue compartments. This could suggest that the increased passage of protein through the glomerular filtration barrier is harmful for podocytes. In support of this hypothesis is the observation that desmin protein expression in podocytes, which indicates podocyte stress, co-localized with albumin reabsorption droplets in the glomerulus.

Morigi et al previously found that exposure of cultured mouse podocytes to human serum albumin or IgG causes upregulation of endothelin 1 via activation of NF-κB and Ap1 (37). Also in the 5/6 nephrectomy model in rats, protein accumulation in podocytes was seen in conjunction with de novo podocyte desmin expression and an increased glomerular TGF-beta mRNA expression (12). In line with suggestions by these and other authors, the increased accumulation of proteins such as IgG and albumin in podocytes may initiate a self perpetuating process of podocyte damage and further proteinuria (38). In a recent study, Nagase et al (39) suggested that the proteinuria seen in Dahl SS rats might be caused by podocyte damage. Although we cannot disprove this hypothesis, our results would also be compatible with a pathogenetic pathway in which podocyte injury is a phenomenon secondary to proteinuria.

In conclusion, we have studied the expression of glomerular genes during the development of proteinuria in the Dahl rat. Our results warrant further investigation of the role of Hhip and Pmfbp1. We found an upregulation of dynamin mRNA and protein expression in rat and human proteinuric glomeruli, underscoring the importance of actin-regulating proteins in proteinuric diseases. Furthermore, our findings substantiate the hypothesis that the increased passage of proteins through the glomerular filtration barrier has a direct toxic effect on the glomerulus.

Acknowledgements

We would like to thank Paul Eilers and Jan Oosting for their help with the statistical analysis of the microarray data, and Jochen Reiser for his help with the dynamin experiment.

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Differentiation between chronic rejection and chronic cyclosporine toxicity by analysis of renal cortical mRNA

5

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Kidney Int. 2004 Nov;66(5):2038-46

Abstract

Background

In kidney transplantation, chronic allograft nephropathy (CAN) is the major cause of graft loss. Causes of CAN include chronic rejection and chronic cyclosporine A (CsA) nephrotoxicity. It is necessary to differentiate between these two entities in order to apply the appropriate therapeutic regimen for the individual patient, but this is hampered by the lack of discriminating functional and morphologic parameters. We investigated whether renal cortical mRNA levels for several matrix proteins can serve as discriminating parameters.

Methods

Patients with chronic rejection (n = 19) and chronic CsA toxicity (n = 17) were selected by clinical and histologic criteria. Protocol biopsies without histologic abnormalities, taken at 6 months after transplantation from patients receiving CsA, were used as controls (n = 6). Total RNA was extracted from the renal biopsy tissue, and mRNA levels of transforming growth factor-β (TGF-β) and the extracellular matrix (ECM) molecules collagen Iα1, IIIα1, IVα3, decorin, fibronectin, and laminin β2 were measured by real-time polymerase chain reaction (PCR).

Results

In both patient groups, the mean collagen IVα3 and fibronectin mRNA levels were significantly elevated compared to those in controls, whereas only in CsA toxicity were the laminin β2 and TGF-β mRNA levels significantly increased. The increase of laminin β2 and TGF-β mRNA levels was significantly higher in the CsA toxicity group than in the chronic rejection group ($P < 0.001$ and $P = 0.004$, respectively). Receiver-operating characteristic (ROC) curve analysis showed that with a 15.6-fold increase in laminin β2 mRNA expression as cut-off point, the presence of CsA toxicity could be predicted with 87% sensitivity and 88% specificity.

Conclusion

Renal laminin β2 and TGF-β mRNA levels can be used to differentiate between chronic rejection and chronic CsA toxicity in renal transplants. The method of mRNA quantification might be applicable as an additional diagnostic tool in clinical practice.

Introduction

Over the past decade, renal transplantation has become a very successful treatment modality for end-stage renal disease (ESRD). Due to improvement of immunosuppressive therapy, acute rejection episodes can be treated effectively, and the prevalence of early graft loss has diminished significantly (1). Long-term graft loss, however, currently forms a major problem in renal transplantation (1-3).

The term chronic allograft nephropathy (CAN) refers to the pathologic changes, including interstitial fibrosis, tubular atrophy, and fibrous intimal thickening, which are found in chronically dysfunctioning kidney transplants (2). Several risk factors for CAN have been recognized, such as the number and the severity of acute rejection episodes (4), ongoing chronic rejection, and excessive exposure to calcineurin inhibitors such as cyclosporine A (CsA) (2,5). Therefore, the lesions that occur in biopsies of patients with CAN may result from either one or a combination of these factors. Paradoxically, changes induced by chronic rejection are clinically and histopathologically hard to distinguish from those caused by the nephrotoxic effects of chronic exposure to CsA, meant to prevent chronic rejection. This makes it difficult to determine the optimal dose of the immunosuppressive regimen, in which the beneficial and nephrotoxic effects of CsA are balanced (6).

Some changes observed in routine light microscopy may help reveal the cause of chronic renal allograft dysfunction. These include peripheral nodular arteriolar hyalinosis, suggestive of chronic CsA toxicity, and transplant vasculopathy (intimal fibrosis, disruption of the lamina elastica in the presence of inflammation), suggestive of chronic rejection (7,8). However, these lesions are not decisively present in all patients with either of the diagnoses. Furthermore, due to the fact that peripheral nodular arteriolar hyalinosis and transplant vasculopathy appear focally in the tissue, sampling errors may obscure their presence.

In this study, we describe how differentiation between chronic rejection and chronic CsA toxicity may be improved with the aid of molecular techniques, based on the results of a quantitative analysis of the renal cortical mRNA levels of several extracellular matrix (ECM) components and the ECM-regulating molecule transforming growth factor-β (TGF-β) in two groups of patients suffering from either disease entity.

We focused on several molecules that make up the interstitial compartment of the kidney and are known to accumulate in renal fibrosis, including collagens I and III, and fibronectin, together with the ECM-regulating molecule TGF-β and its potential inhibitor decorin. In recent publications, attention has been drawn to expression of collagen IVα3 and laminin β2 in the discrimina-

tion between chronic rejection and CsA toxicity (9). These molecules are also the subject of the current study.

Methods

Patient populations

We reviewed all kidney transplant biopsies performed in our center over the past 20 years, taken because of renal function loss beyond 1 year after transplantation. We selected two groups of patients: the chronic rejection group and the chronic CsA toxicity group.

The chronic rejection group (n = 19), consisted of patients who received either prednisone and azathioprine (n = 6), or prednisone and CsA (n = 13). Of the 13 patients using CsA, seven received the Sandimmune formulation and six received the Neoral formulation. These patients, with an initially well-functioning kidney transplant, developed a progressive decline in renal function. A biopsy was taken 4.8 ± 3.8 years after transplantation, in which transplant vasculopathy (intimal fibrosis, intimal inflammation, and disruption of the lamina elastica), transplant glomerulopathy (characterized by double contours of the glomerular basement membrane (GBM)) or both were present as a histopathologic indication of chronic rejection, as defined by the Banff 97 classification (7). Biopsies with peripheral nodular arteriolar hyalinosis, a lesion suggestive of CsA toxicity, and biopsies with signs of de novo or recurrent native disease were excluded. Patients suffering from diabetes and patients with graft arterial stenosis were excluded.

The chronic CsA toxicity group (n = 17), consisted of patients with an initially well-functioning kidney transplant, who developed a progressive decline in renal function only after a switch was made from Sandimmune to Neoral; CsA formulations with a relatively low and a relatively high bioavailability, respectively. Before the switch, immunosuppression was aimed at CsA 24-hour trough levels of 100 g/L by administration of Sandimmune once daily. After the switch, Neoral was administered twice daily, aiming at a 12-hour trough level of 150 g/L. Thereby, the mean daily CsA dose was increased from 3.2 mg/kg to 3.5 mg/kg (10). Patients who developed a significant and progressive decrease in renal function after this switch, in the absence of other features that might explain the decline in renal function, were included. Renal biopsies, taken 7.1 ± 3.4 years after transplantation and 2.5 ± 1.2 years after the switch from Sandimmune to Neoral, showed peripheral nodular arteriolar hyalinosis in 16 of the 17 patients, histopathologically supporting the functional selection. Biopsies with histologic features suggestive of chronic rejection or de novo or recurrent glomerulonephritis were excluded. Patients suffering from diabetes and patients with graft arterial stenosis were excluded.

C4d staining was performed on all biopsy samples. None of the patients in the CsA toxicity group showed C4d depositions in their peritubular capillaries, while 26% of the patients in the chronic rejection showed diffuse C4d depositions in the peritubular capillaries.

Control group

As controls (n = 6), we used protocol transplant biopsies taken from patients at 6 months after transplantation with stable graft function at the time of biopsy. Apart from some cases showing signs of minor nonspecific age-related alterations, none of the biopsies showed any signs of rejection or drug toxicity. The glomeruli (at least ten present in the sections for evaluation) did not show any abnormalities. Patients in this control group all used CsA as immunosuppressive medication.

Clinical data

Clinical data included gender, patient age at time of biopsy, donor age, transplant-origin (cadaveric or living donor), number of acute rejection episodes, delayed graft function, time between transplantation and biopsy, time between switch and biopsy, mean arterial pressure (MAP), number of antihypertensive drugs used, use of angiotensin-converting enzyme (ACE) inhibitors, lowest serum creatinine, serum creatinine at biopsy, proteinuria at time of biopsy, and CsA trough levels. We estimated the best endogenous creatinine clearance and creatinine clearance at biopsy using the Cockcroft-Gault equation (11). The loss of renal function was defined as the best Cockcroft clearance minus the Cockcroft clearance at the time of biopsy.

mRNA isolation and cDNA synthesis

Four-micrometer cryostat sections of each biopsy were cut, air dried, and stored at -20°C until use for immunohistochemistry. One section was evaluated to localize the cortex, which was thereafter excised from the biopsy. RNA was subsequently extracted, as described previously (12). In brief, the tissue was lysed by rigorous mixing after suspension in 500 µL TRIzol® (Invitrogen Life Technologies, Carlsbad, CA, USA). After adding 100 µL chloroform, the solution was centrifuged at 15,000g for 15 minutes. The RNA was precipitated by addition of 5 µg of glycogen and 250 µL isopropanol. cDNA synthesis was performed using a reverse transcription (RT) kit (Omniscript Reverse Transcriptase) (Qiagen GmbH, Westburg B.V., The Netherlands).

Real-time polymerase chain reaction (PCR)

For several ECM molecules and TGF-β1, forward and reverse primers (Life Technologies BRL and Biosource International, Nivelles, Belgium) and TaqMan probes (Biosource International) were designed, using Primer Express® 1.5 software (PE Applied Biosystems, Foster City, CA, USA). To prevent amplification of genomic DNA, primers or probes were chosen spanning an exon-intron junction. Primers were located near the 3′ end of the mRNA. The 5′ ends of the Taqman probes were 6-carboxy-4,7,2′,7′-tetrachloro-fluorescein (TET)-labeled, except those for

glyceraldehyde-3-phosphate dehydrogenase (GAPDH), collagen IVα3, and decorin, which were 6-carboxy fluorescein (FAM)-labeled. The quencher dye at the 3' side of the probe was 6-carboxy-tetramethyl-rhodamine (TAMRA). The sequences of the primers and of the TaqMan probes are shown in Table 1.

Real-time PCR was performed using the ABI Prism 7700 sequence detector and software (PE Applied Biosystems) (13). Amplification cycles were 95°C for 10 minutes, followed by 40 cycles at 95°C for 30 seconds and at 60°C for 60 seconds. Kinetics of the reactions were determined using a standard curve. We used the ratio of the levels of the investigated molecule and GAPDH, a constitutively expressed gene, to correct for the amount of tissue used for RNA extraction and the efficiency of cDNA synthesis. To confirm the suitability of GAPDH as a housekeeping gene, we tested the correlation between the expression of two additional housekeeping genes (hypoxanthine phosphoribosyl transferase 1 (HPRT1) and β2-microglobulin (B2M)), and that of GAPDH in all samples.

Immunofluorescence for C4d

Immunofluorescence staining for C4d was performed on untreated slides using standard procedures as described before (14,15). As the primary antibody, mouse anti-C4d antibody (Quidel, San Diego, CA, USA), diluted to 2 ng/mL in phosphate-buffered saline (PBS) and supplemented with 1% bovine serum albumin (BSA), was used. The secondary antibody was fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG (Sigma Chemical Co., St. Louis, MO, USA), diluted 1:200 in BSA/PBS. The staining was evaluated independently by two of the authors (K.K. and M.E.), blinded for the diagnosis of the samples. C4d staining was observed in the peritubular capillaries (PTC) in a circumferential pattern. Sporadically, mesangial and GBM areas of the glomerulus stained positive. In accordance with scoring methods described in the literature on C4d deposition in renal allografts (16,17), biopsies were scored C4d-positive when more than 25% of the PTC showed an intense and circumferential staining as depicted in Figure 1. In most cases of diffuse positive samples all PTC were affected. In the few cases of discordant scoring, decision was reached by consensus.

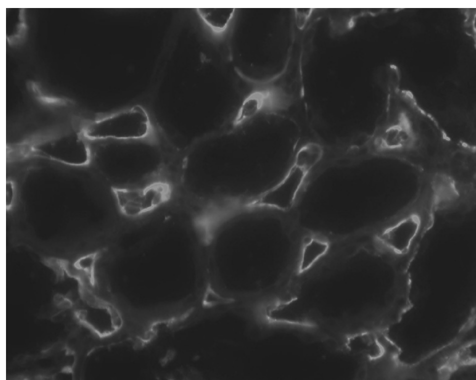


Figure 1. Immunofluorescence staining for C4d. In some chronic rejection samples diffuse circumferential staining of peritubular capillaries for C4d was observed (original magnification × 400).

Immunohistochemistry

For evaluation of protein expression in the tissue, immunohistochemistry was performed for laminin β2 and TGF-β. Four micrometer frozen sections were thawed, air dried, and incubated for 1 hour with mouse monoclonal anti-laminin β2 antibodies (Developmental Studies

Table 1. Primer and probe sequences

Molecule (a)	Forward primer	Reverse primer	TaqMan™ probe (b)
GAPDH	TGGTCACCAGGGCTGCTT	AGCTTCCCGTTCTCAGCCTT	5'-FAM-TCAACTACATGGTTTACATGTTCCAATAT-GATTCCACCAA-TAMRA-3'
B2M	TGCCGTGTGAACCATGTGA	CCAAATGCGGCATCTTCAA	5'-TET-TGATGCTGCTTACATGTCTCGATCCCACT-TAMRA-3'
HPRT1	TGACACTGGCAAAACAAT-GCA	GGTCCTTTTCACCAGCAAGCT	5'-TET-CTTGACCATCTTTGGATTATACTGCCTGAC-CA-TAMRA-3'
Collagen Iα1	CCTCAAGGGCTCCAACGAG	TCAATCACTGTCTTGCCCCA	5'-TET-ATGCCTGCACGAGTCACACCGGA-TAMRA-3'
Collagen IIIα1	GAGGATGGTTGCAC-GAAACA	TGTCATAGGGTGCAATATCTA-CAATAGG	5'-TET-TGAATATCGAACACGCAAGGCTGTGAGA-CT-TAMRA-3'
Collagen IVα3	AAGCCCACCACATGATTCT-GA	GCAGTTGTAGCCAGCCGTACT	5'-FAM-TCCAAGCACACTCCGCAGGCAGT-TAMRA-3'
Decorin	ACATCCGCATTGCT-GATACCA	AGTCCTTTGAGGCTAGCTG-CATC	5'-FAM-TCACCAGCATTCCTCAAGGTCTTCCTCC-TAMRA-3'
Fibronectin	GGAGAATTCAAGTGT-GACCCTCA	TGCCACTGTTCTCCTACGTGG	5'-TET-AGGCAACGTGTTACGATGATGGGAAGA-CAT-TAMRA-'3
Laminin β2	GGATGAGGCTCGGGACCT	CCCGTCCAACTGGGCTG	5'-TET-AGGAATTGGAAGGCACCTATGAG-GAAAATGA-TAMRA-3'
TGF-β1	CCCAGCATCTGCAAAGCTC	GTCAATGTACAGCTGCCGCA	5'-TET-ACACCAACTATTGCTTCAGCTCCACGGA-TAMRA-3'

a) GAPDH – glyceraldehyde-3-phosphate dehydrogenase; B2M – β2 microglobulin; HPRT1 – hypoxanthine phosphoribosyltransferase 1. (b) TET – 6-carboxy-4,7,2',7'-tetrachloro-fluorescein; FAM – 6-carboxy fluorescein; TAMRA – carboxy-tetramethylrhodamine.

Hybridoma Bank, Iowa City, IA, USA) diluted 1:16 in BSA/PBS, or rabbit polyclonal anti-TGF-β (Dako, Glostrup, Denmark), diluted 1:400 in BSA/PBS. The anti-TGF-β antibodies stain both the active and latent form of TGF-β. The slides were washed in PBS, and subsequently incubated with horseradish peroxidase (HRP)-conjugated antimouse Envision and HRP-conjugated antirabbit Envision, respectively (Dako). After 45 minutes of incubation, the slides were washed in PBS and the staining was developed with diaminobenzidine (DAB). The color was enhanced by incubating the slides in 0.5% $CuSO_4$ solution for 5 minutes. After counterstaining with hematoxylin, the slides were dehydrated and mounted. For each staining, all biopsy samples were stained in one session.

Digital image analysis

To quantify the amount of staining for TGF-β and laminin β2, images of the cortical part of the biopsies were taken at a 200 magnification using a Zeiss microscope equipped with a Sony DXC-950P 3 CCD color camera (Sony Corporation, Tokyo, Japan) and further analyzed using KS-400 image analysis software (version 3.0 for Windows) (Carl Zeiss Vision GmbH, Oberkochen, Germany). The cortical area stained was defined as the amount of staining within the color spectrum specific for the enhanced DAB staining, and above a fixed intensity threshold, as described previously (18). Recording and analysis of the images were performed with fixed settings.

Statistical analysis

Statistical analysis was performed using SPSS 10.0.7 for Windows software. We used log transformed (10log) mRNA levels for analysis. A one-way analysis of variance (ANOVA) with a Bonferroni post hoc correction was used for comparison of differences between groups. Using a

receiver-operating characteristic (ROC) curve, we determined the cut-off point of mRNA levels with the best combination of sensitivity and specificity that predicted the presence of CsA toxicity. Correlations between the mRNA data and the clinical characteristics of the patient groups were calculated using Pearson's correlation test. $P < 0.05$ was considered statistically significant.

Results

Patient data

The clinical characteristics of the patients and the controls are listed in Table 2. Renal function at time of biopsy, donor age, patient age, number of acute rejection episodes, delayed graft function, the time interval between transplantation and biopsy, MAP, number of antihypertensive drugs used, and use of ACE inhibitors did not differ significantly between patient groups. There was significantly greater loss of renal function in the chronic rejection group than in the chronic

Table 2. Clinical characteristics of patients and controls

Group	Chronic rejection	Chronic CsA toxicity	Controls
N	19	17	6
Gender (female)	10 (53%)	4 (24%)	2 (33%)
Number of patients treated with CsA	13 (68%)	17 (100%)	6 (100%)
Patient age (years ± SD)	44 ± 14	49 ± 13	45 ± 6
Donor age (years ± SD)	35 ± 16	44 ± 10	47 ± 17
Living-donor kidney transplants (%)	5	35*	
Number of acute rejection episodes	0.8 ± 1.0	0.5 ± 0.6	
Delayed graft function (%)	21	18	
Time between transplantation and biopsy (years ± SD)	4.8 ± 3.8	7.1 ± 3.4	
Time between switch and biopsy (years ± SD)		2.5 ± 1.2	
Mean arterial pressure	108 ± 10	106 ± 8	
Number of anti-hypertensive drugs used (0/1/2/>3)	2/2/8/7	2/5/5/5	
Use of ACE-inhibitors (%)	21	29	
Best serum creatinine level (µmol/L ± SD)	113 ± 27	124 ± 25	
Serum creatinine at time of biopsy (µmol/L ± SD)	238 ± 83	201 ± 45	118 ± 25
Best creatinine clearance (mL/min ± SD)	74 ± 22*	61 ± 11	
Creatinine clearance at time of biopsy (mL/min ± SD)	36 ± 14	40 ± 15	68 ± 14
Loss of renal function (µmol/L ± SD)	38 ± 16†	21 ± 13	
Proteinuria at time of biopsy (g/24h ± SD)	2.9 ± 2.6	1.3 ± 1.5	
CsA trough levels at time of biopsy (µg/L ± SD)	110 ± 31	114 ± 30	

Creatinine clearance was estimated by the Cockcroft-Gault equation. Loss of renal function was defined as the difference between the best creatinine clearance and the creatinine clearance at the time of biopsy. * $P < 0.05$, † $P < 0.01$

Figure 2. Box and whisker plots of the log-transformed mRNA levels. The boxes contain 50% of the values. The upper and lower borders indicate the 25th and the 75th percentile, respectively. The upper and lower whiskers indicate the highest and lowest value, respectively. The black line in the box indicates the median and the (°) indicates an outlier. (A) Collagen Iα1. (B) Collagen IIIα1. (C) Collagen IVα3 [$P < 0.001$ chronic rejection (CR) and cyclosporine A (CsA) toxicity (CsAT) vs. controls]. (D) Decorin. (E) Fibronectin ($P < 0.001$ chronic rejection and CsA toxicity vs. controls. (F) Laminin β2 ($P < 0.001$ CsA toxicity vs. controls) ($P = 0.002$ chronic rejection vs. CsA toxicity). (G) Transforming growth factor-β (TGF-β) ($P = 0.004$ CsA toxicity vs. controls) ($P = 0.020$ chronic rejection vs. CsA toxicity). (H) TGF-β/decorin ratio ($P = 0.014$ chronic rejection vs. CsA toxicity).

CsA toxicity group (38 ± 16 mL/min and 21 ± 13 mL/min, respectively) ($P < 0.01$). There were significantly more patients with a living-donor kidney transplant in the CsA toxicity group, compared with the chronic rejection group (35% and 5%, respectively) ($P = 0.02$).

Cortical mRNA levels

GAPDH mRNA levels did not differ between groups (data not shown). Within the chronic rejection group, mean GAPDH expression did not significantly differ between CsA-using and non-CsA–using patients. There was a significant correlation between GAPDH mRNA and mRNA of the two other housekeeping molecules measured ($r = 0.74$ and $r = 0.92$ for B2M and HPRT1, respectively) ($P < 0.001$). Additionally, we tested comparisons between the chronic rejection and the CsA toxicity group for all transcripts using β2-microglobulin as the housekeeping molecule. This yielded the same results as when GAPDH was used. These findings support the suitability of GAPDH as a housekeeping molecule in the experiments.

The mean log transformed mRNA levels of collagen Iα1, collagen IIIα1, collagen IVα3, decorin, fibronectin, laminin β2, and TGF-β are shown in Table 3 and depicted in Figure 2. The mRNA levels of collagen IVα3 and fibronectin were higher in both patient groups compared to controls. The mRNA levels of laminin β2 and TGF-β were higher in the CsA toxicity groups compared to controls (Table 3, Figure 2f and h). The renal mRNA levels of collagen Iα1, IIIα1, and IVα3, decorin, and fibronectin were not significantly different between patients with chronic rejection and patients with CsA toxicity (Table 3, Figure 2a to e). The renal mRNA levels of laminin β2, TGF-β, and the ratio of TGF-β to decorin were significantly higher in patients with CsA toxicity than in patients with chronic rejection (Table 3, Figure 2f and g). After omitting patients who did not use CsA from the chronic rejection group, comparative analyses between groups yielded comparable results (Table 3). In addition, there were no differences in mRNA expression of all molecules analyzed between chronic rejection patients who used CsA and those who did not use CsA as immunosuppression

Table 3. Log-transformed mRNA expression levels

	Total chronic rejection (Ia)	Chronic rejection using CsA (Ib)	Chronic CsA toxicity (II)	Controls (III)
Collagen Iα1	0.3 ± 0.2	0.2 ± 0.3	0.2 ± 0.3	0.8 ± 0.2
Collagen IIIα1	0.3 ± 0.2	0.2 ± 0.2	0.3 ± 0.2	0.3 ± 0.1
Collagen IVα3	0.1 ± 0.2†	0.1 ± 0.2†	0.5 ± 0.1†	-1.9 ± 0.3
Decorin	0.0 ± 0.2	-0.2 ± 0.2	-0.1. ± 0.2	-0.4 ± 0.2
Fibronectin	-0.1 ± 0.2†	-0.3 ± 0.2†	-0.1 ± 0.2†	-1.6 ± 0.2
Laminin β2	0.6 ± 0.1‡	0.5 ± 0.2‡	1.1 ± 0.1†	-0.1 ± 0.3
TGF-β	0.8 ± 0.1**	0.8 ± 0.2**	1.3 ± 0.1†	0.5 ± 0.2
Ratio TGF-β / decorin	0.8 ± 0.2**	0.8 ± 0.2**	1.4 ± 0.1	0.9 ± 0.2

All values are mean ± SEM. * $P < 0.05$, I or II vs III; † $P < 0.01$, I or II vs III; ** $P < 0.05$, I vs II; ‡ $P < 0.01$, I vs II. Chronic rejection using CsA (1b) – mean log-transformed mRNA levels of the chronic rejection group after omission of patients using azathioprine as immunosuppression.

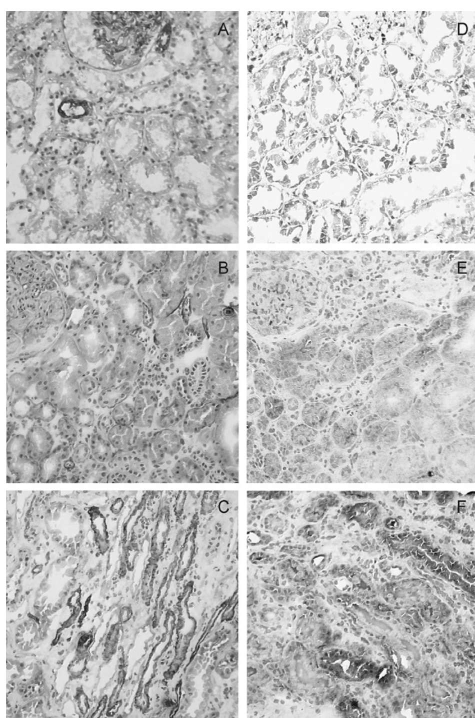


Figure 3. Immunohistochemical stainings for laminin β2 (A to C) and transforming growth factor-β (TGF-β) (D to F). In control tissue laminin β2 staining was observed in the glomerular basement membrane and in cortical vessels (A). In chronic rejection (B) and cyclosporine A (CsA) toxicity (C), expression of laminin β2 was observed in the tubular basement membrane. TGF-β staining was sporadically observed in glomeruli and tubuli of controls (D). In chronic rejection (E) and CsA toxicity (F), some tubuli showed a very intense staining for TGF-β.

(collagen Iα1, $P = 0.44$; collagen IIIα1, $P = 0.51$; collagen IVα3, $P = 0.57$; decorin, $P = 0.07$; fibronectin, $P = 0.10$; laminin β2, $P = 0.52$; TGF-β, $P = 0.53$). There were no significant differences between mRNA expression levels in cadaveric transplants and living-donor transplants within each patient group.

Immunohistochemistry

We performed immunohistochemical stainings to evaluate the tissue expression of laminin β2 and TGF-β at the protein level (Figure 3). In biopsies of controls, laminin β2 staining was observed in the GBM and in cortical vessels (Figure 3a). In chronic rejection as well as in CsA toxicity, sporadic expression of laminin β2 was seen in the tubular basement membrane (Figure 3b and c). TGF-β staining was sporadically observed in glomeruli and tubuli of controls (Figure 3d). In sections of biopsies from patients suffering from chronic rejection or CsA toxicity, some tubuli showed very intense staining for TGF-β (Figure 3e and f). There was no relation between this sporadic intensive staining and mRNA expression levels or clinical parameters.

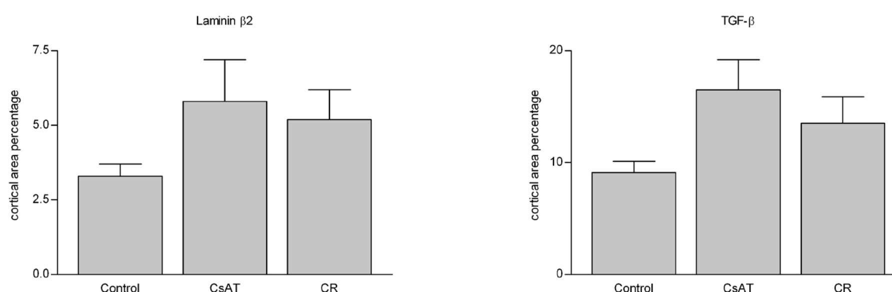


Figure 4. Quantification of immunohistochemical staining for laminin β2 and TGF-β. Bars represent mean cortical area percentage ± SEM. (A) The amount of laminin β2 staining is slightly higher in the cyclosporine A toxicity (CsAT) group compared to the chronic rejection (CR) group, but the difference between groups does not reach statistical significance. (B) TGF-β staining is increased in the CsA toxicity group compared to the chronic rejection group. There are no significant differences between groups.

In both patient groups, the amount of laminin β2 staining was increased compared to controls. The mean cortical area percentage was slightly higher in the CsA toxicity group than in the chronic rejection group (5.7 ± 1.5 and 5.3 ± 1.0, mean ± SEM) (Figure 4a). The differences between groups were not significant. The amount of TGF-β staining was higher in patient groups than in controls. The mean cortical area percentage was higher in the CsA toxicity group than in the chronic rejection group (15.8 ± 3.6 and 13.4 ± 2.2, mean ± SEM) (Figure 4b), but this difference did not reach statistical significance.

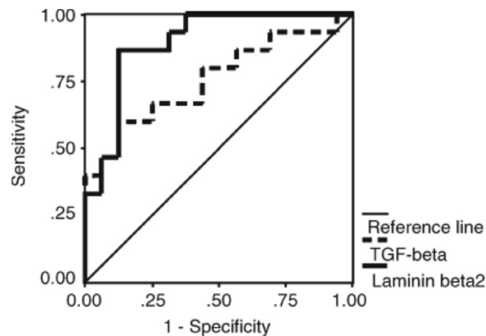


Figure 5. Receiver-operating characteristic (ROC) curve of laminin β2 and TGF-β. The fraction of true positive results (sensitivity) and false positive results (1-specificity) for laminin β2 (solid line) and TGF-β (dashed line) mRNA levels. The area under the curve indicates the accuracy of the test: 0.5 is the value expected by chance (diagonal line), 1.0 represents the ideal predictor. The area under the curves for laminin β2 and TGF-β are 0.896 and 0.758, respectively. Using a 15.6-fold increase in laminin β2 mRNA levels to those of normal controls as cut-off point, the sensitivity is 87% and the specificity 88% for the prediction of the presence of chronic cyclosporine A toxicity.

ROC curve analysis

ROC curve analysis Figure 5 showed that a 15.6-fold increase of laminin β2 mRNA levels compared to controls indicates the presence of CsA toxicity with 87% sensitivity and 88% specificity. The area under the curve was 0.90 ($P < 0.001$). Similarly, a ninefold increase of TGF-β mRNA levels predicts the presence of chronic CsA toxicity with 60% sensitivity and 88% specificity (area under the curve 0.76) ($P < 0.05$).

Correlations

No correlations were found between mRNA levels and age, donor age, number of acute rejection episodes, delayed graft function, time between transplantation and biopsy, the time between switch and biopsy, loss of renal function, MAP, and use of ACE inhibitors. Only in the chronic rejection group, TGF-β mRNA levels and the ratio of TGF-β to decorin mRNA levels correlated significantly with laminin β2 mRNA levels ($r = 0.56$ and $r = 0.68$, respectively) ($P < 0.05$).

Discussion

This study was designed to enhance the discrimination between chronic rejection and chronic CsA toxicity as a cause of CAN. With current clinical and histologic parameters this distinction is difficult to make because of the similarities in clinical presentation and aspecificity of the lesions

in needle biopsies of patients with CAN. We show that quantification of mRNA levels of laminin β2 and TGF-β can be used to distinguish chronic rejection from CsA toxicity, which will help in fine-tuning the immunosuppressive regimen. In this way, the beneficial effects of CsA are not abrogated by its nephrotoxic side effects (3).

In our center, we had the opportunity to define on functional criteria a patient group that suffered from chronic CsA toxicity, comprising patients who developed a progressive decline in renal function after a change in immunosuppressive medication was made leading to a higher CsA exposure (10). The appropriateness of these selection criteria was supported by histopathologic findings: peripheral nodular arteriolar hyalinosis, regarded as a finding suggestive of CsA toxicity, was present in 94% of these patients, and C4d deposition in PTCs, a feature frequently seen in chronic rejection (19), was absent in all patients of the CsA toxicity group. An additional selection criterion for the CsA toxicity group and a further proof of CsA toxicity might have been the recovery of renal function after stopping or reducing CsA administration. Unfortunately, these data were not available. In the chronic rejection group, 13 out of 19 patients also received CsA. However, since the CsA formulation in the majority of these patients had a relatively low bioavailability and administration was applied only once-daily, this group is less likely to have suffered from CsA toxicity. In addition, patients in this group had transplant vasculopathy or glomerulopathy, histologic features suggestive of chronic rejection, but they did not show histologic features consistent with CsA toxicity. Finally, although there was some heterogeneity in immunosuppressive medication in the chronic rejection group, no differences were found in mRNA data between patients using CsA and those who did not use CsA as immunosuppression, and analysis with only the group of chronic rejection patients using CsA as immunosuppression yielded the same results as the chronic rejection group as a whole. A striking difference in patient characteristics was that 35% of the CsA toxicity group, but only 5% of the transplants in the chronic rejection group were living donor transplants. This might support the opinion that injuries inflicted to cadaveric allografts before or during transplantation elicit a predisposition for the development of chronic rejection, as has been suggested before (2,20).

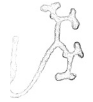
The ECM is a meshwork of proteins, in which remodeling continuously takes place by means of protein synthesis and degradation. Accumulation of ECM proteins reflects an imbalance of this dynamic process, resulting from an increase in protein synthesis, a decrease in protein degradation, or a combination of both. The synthesis of ECM components is enhanced by several profibrotic cytokines, including TGF-β. During chronic renal allograft dysfunction, this cytokine has been shown to be up-regulated at the mRNA and protein level in the grafts, sera, and peripheral blood mononuclear cells of patients taking CsA-based immunosuppression (21,22). TGF-β mRNA levels are up-regulated in cultured murine proximal tubular epithelial cells and fibroblasts after exposure to CsA (23). Moreover, in rats receiving CsA, administration of anti-TGF-β1 antibodies reduces the extent of histologic damage reminiscent of CsA toxicity (24). Decorin, a

low-molecular-weight proteoglycan, can bind and inactivate TGF- , thereby preventing its prosclerotic action (25). The TGF-β/decorin mRNA ratio may therefore be a better indicator of TGF-β activity than TGF-β mRNA levels alone. We found a significant increase of TGF-β mRNA levels and of the TGF-β/decorin ratio in CsA toxicity, supporting the notion that CsA has a stimulatory effect on TGF-β expression.

Laminin β2 is a normal component of the renal vasculature and the GBM (26). We observed an increase in laminin β2 mRNA expression in the CsA toxicity group compared to controls, the expression in the CsA toxicity group being also significantly higher than in the chronic rejection group. Laminin β2 mRNA expression was also higher in the CsA toxicity group when comparing it to the chronic rejection group with only those patients using CsA included. Only sparse information about factors stimulating laminin β2 expression is available. The results of our study suggest a direct or indirect stimulatory effect of CsA on laminin β2 expression, yet the underlying mechanism is unclear.

We performed immunohistochemistry combined with digital image analysis for laminin β2 and TGF-β to evaluate the expression of these molecules at the protein level. The pattern seen at the protein level resembled that at the mRNA level (ie, there was a tendency toward a higher expression of laminin β2 and TGF-β in the CsA toxicity group than in the chronic rejection group). However, differences in protein staining between groups did not reach statistical significance. Furthermore, a correlation between mRNA expression levels and protein deposition was absent. The observation that the extent of protein accumulation does not strictly coincide with mRNA levels has been described before (27). Additionally, in vivo accumulation of protein is not only determined by synthesis, but also by degradation of ECM products. Although we did not evaluate the mechanism of laminin β2 degradation, the notion that laminin β2 mRNA levels were increased in the CsA toxicity group compared to the chronic rejection group, while protein levels were not significantly different, might suggest that there is an increased degradation of laminin β2 in CsA toxicity. This might be due to the microvascular damage exerted by CsA (8).

Collagen Iα1 and collagen IIIα1 are components of the renal interstitium that are normally present in relatively small amounts (28). Accumulation of these molecules has been reported in a variety of chronic human kidney diseases (29) and CAN (30,31). There was no difference between the chronic rejection group and the CsA toxicity group in collagens I and III mRNA levels. We observed no differences in the mRNA levels between the patient groups and the control group. This might be explained by the possibility that the accumulation of collagens I and III in CAN is a result of an early increase in collagen synthesis that might have taken place before the overt damage seen in the tissues. Furthermore, it might be that the accumulation of collagens I and III in patients with CAN is due to an impaired degradation of these proteins, as has been suggested before (32).



Collagen IVα3 is a component of both the GBM and the distal tubular basement membrane (TBM) (26). In a study by Abrass et al (9), de novo expression of collagen IVα3 protein was reported in the proximal TBM in chronic rejection, but not in CsA toxicity. In our study, we did not observe significant differences in collagen IVα3 mRNA expression levels between the two groups. Since we used total cortical tissue for mRNA analysis, it is possible that subtle differences of collagen IVα3 mRNA expression in the proximal tubulus epithelium between patient groups remained undetected. When we focused only on the chronic rejection group, the collagen IVα3 mRNA expression was higher in the C4d+ chronic rejection group than in the C4d– chronic rejection group (data not shown). C4d recently has gained much interest as a marker of humoral rejection, but there is only sparse information about the relation between C4d and accumulation of ECM (33). Future studies would be needed to decipher whether the relation found in our study is of pathogenic significance.

In both the chronic rejection and the chronic CsA toxicity group, we observed a significant increase in renal cortical fibronectin mRNA levels in comparison to controls. There were no differences in the mRNA levels of fibronectin between the chronic rejection and the CsA toxicity group. This is in line with previous studies showing an increase in fibronectin mRNA levels in a rat model of CsA toxicity (34), and in allograft rejection, both at the mRNA and the protein level (35,36).

One of the advantages of analyzing mRNA profiles may be that alterations in mRNA levels precede the development or aggravation of tissue damage (37). This holds promise for an earlier recognition of an unfavorable course after kidney transplantation (38,39). Furthermore, quantitative mRNA analysis can be performed rapidly, and requires only small amounts of renal tissue. In the future, diagnostic approaches using molecular analysis simultaneously with conventional strategies are likely to be implemented in clinical practice (40). We had the opportunity to compose merely on the basis of functional variables two patient groups that represent the extremes of a spectrum of causes leading to the development of CAN. Therefore, we studied the presence of markers that could discriminate between these two highly selected patient groups. An obvious prerequisite for implementation in clinical practice is to test the use of these markers in larger nonselected patient groups.

In conclusion, we measured human renal cortical mRNA expression levels of ECM components in two well-defined groups of patients suffering from either chronic rejection or chronic CsA toxicity. In both patient groups, the mean mRNA levels for collagen IVα3 and fibronectin were significantly elevated compared to those in controls, whereas in CsA toxicity the laminin β2 and TGF-β mRNA levels were also significantly increased. Most important, we showed that laminin β2 and TGF-β mRNA levels are significantly higher in patients with CsA toxicity than in patients with chronic rejection, and that measurement of these expression levels may help differentiate chronic CsA toxicity from chronic rejection.

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Early Interstitial Accumulation of Collagen Type I Discriminates Chronic Rejection from Chronic Cyclosporine Nephrotoxicity

6

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J Am Soc Nephrol. 2003 Aug;14(8):2142-9

Abstract

Little is known regarding the composition of the interstitial extracellular matrix of kidney allografts with deteriorating function. Collagen I, III, and IV, the collagen IVα3 chain, and the laminin β2 chain were investigated in biopsies of allografted kidneys with chronic cyclosporine A nephrotoxicity (CsAT) (n = 17), chronic rejection (CR) (n = 12), or chronic allograft nephropathy (CAN) (n = 19). α-smooth muscle actin expression was also examined. Normal native kidneys were used as control samples (n = 11). Biopsy samples were studied with routine light microscopy and immunostaining. The mean interstitial fibrosis scores were significantly higher for the CR and CAN groups, compared with the chronic CsAT group. The cortical tubulointerstitial areas of the CR and CAN groups, but not the chronic CsAT group, contained more collagen I than did normal control samples. Differences were noted even in biopsies with mild fibrosis. Accumulation of collagen III, IV, and IVα3 was increased in all patient groups. Collagen III accumulation was greater in the CR and CAN groups than in the chronic CsAT group. Receiver-operating characteristic curve analysis demonstrated that collagen I staining had the best discriminatory value in differentiating CR from chronic CsAT, with a sensitivity of 63% and a specificity of 94% at a cutoff value of 19%. Laminin β2 staining did not differentiate CR from CsAT. Increased α-smoothh muscle actin staining did not differ among the three groups. It was concluded that, during chronic CsAT, collagen III and IV were preferentially accumulated in the tubulointerstitium. Early increases in the deposition of collagen I, with collagen III and IV, were more specific for CR. CR seems to elicit a more pronounced fibrotic response than does chronic CsAT.

Introduction

Late loss of allograft function remains a major problem in renal transplantation (1). Kidney allograft failure is usually preceded by a process of chronic transplant dysfunction, which is characterized by a relatively slow but variable rate of decline in GFR, increasing proteinuria, and increases in BP. The pathologic changes in deteriorating grafts are often less specific. The term chronic allograft nephropathy (CAN) has been adopted to classify these changes, which include chronic obliterative vascular alterations, tubular atrophy, glomerulosclerosis, and interstitial fibrosis (2).

CAN includes both chronic rejection (CR) and chronic calcineurin inhibitor toxicity (2). The nature of vascular, glomerular, and interstitial changes in CAN biopsy specimens can sometimes help to define more specifically the cause of graft dysfunction. Concentric intimal thickening of arteries and arterioles, often accompanied by a moderate degree of mononuclear cell infiltration of the vessel walls, and duplication of the glomerular basement membrane are thought to indicate CR. Smooth muscle cell necrosis and peripheral nodular medial hyalinosis of arterioles are considered hallmarks of cyclosporine A (CsA) nephrotoxicity (CsAT) (3,4).

Extracellular matrix (ECM) accumulates in the cortical interstitium regardless of the cause of chronic graft dysfunction, as observed in native kidneys with chronic diseases (4,5). Surprisingly, the ECM composition of deteriorating allografted kidneys has rarely been studied. Determination of the ECM molecules that accumulate could enhance our understanding of the pathogenesis of graft dysfunction and could potentially help define the cause, if disease-specific changes are observed. In this study, we investigated whether the ECM composition differs between allografts that lose function because of CR and allografts with chronic CsAT. We studied the cortical interstitial ECM composition of kidney allografts of three groups of patients, ie, patients with chronic CsAT, patients with CR, and patients who were receiving CsA treatment but were most likely to experience CR.

Materials and Methods

Patient Selection

All kidney allograft biopsies that were performed in our center for clinical reasons > 1 year after transplantation, in the period between March 1, 1976, and March 1, 2001, were reviewed. Cases that had available frozen-tissue samples and that met the criteria defined below were included. Patients with graft artery stenosis or diabetes mellitus were excluded. In addition, biopsy samples with histologic signs of de novo or recurrent glomerulonephritis or acute rejection were excluded.

The group designated the chronic CsAT group (n = 17) consisted of patients who exhibited progressive declines in allograft function only after a switch was made from a CsA formulation with a lower bioavailability (Sandimmune; Sandoz, Basel, Switzerland) to one with a higher bioavailability (Neoral; Novartis, Basel, Switzerland) (6). A brief period of acute rejection in the early posttransplantation period was allowed and occurred in eight cases. Before the switch, the patients used Sandimmune once daily, aiming at a 24 hr trough level of 100 µg/liter, and demonstrated stable graft function for a mean of 5.1 ± 3.4 year. After conversion to twice-daily Neoral therapy, a higher target 12 hr trough level of 150 µg/liter was adopted. To reach this level, the mean CsA dose was increased from 3.2 to 3.5 mg/kg (6). Nephrotoxicity was not observed in the first months after conversion but became evident after 12 monts (6). Biopsies were obtained a mean of 2.4 ± 1.2 year after switching. None of the studied biopsies in this group exhibited signs of chronic allograft glomerulopathy. No positive staining for C4d in the peritubular capillaries (see below) was observed.

The patient group designated the CR group (n = 12) consisted of patients who exhibited progressive declines in renal allograft function with a calcineurin inhibitor-free immunosuppressive regimen. Immunosuppressive therapy consisted of prednisone and azathioprine. All of these patients initially demonstrated good allograft function (Table 1). Thirty-three percent exhibited C4d positivity in their peritubular capillaries.

A third group of patients received CsA but their allograft biopsies suggested CR as the cause of declines in allograft function. This group, designated the CAN group, consisted of patients with progressive declines in renal allograft function and biopsy findings suggesting CR, including arterial intimal fibrosis (n = 15) and/or chronic allograft glomerulopathy with glomerular basement membrane duplication (n = 4). No temporal relationship existed between the loss of function and a switch to Neoral for any of these patients. In this group, 21% of the biopsies were positive for C4d deposits in the peritubular capillaries. The control group (n = 11) consisted of specimens from normal kidneys obtained in autopsies and from normal kidneys that had not been used for transplantation for anatomic reasons.

Clinical and laboratory data were obtained in chart reviews. The collected data included posttransplantation time, allograft age (donor age plus time after transplantation), systolic and diastolic blood pressure, number of antihypertensive medications used, creatinine clearance (as estimated with the Cockroft-Gault formula), loss of allograft function, proteinuria, and CsA trough levels at the time of biopsy. The loss of allograft function was defined as the difference in the creatinine clearance measured before the worsening of allograft function, as assessed by the breakpoint in the regression lines of 1/creatinine clearance, and the creatinine clearance measured at the time of the index biopsy (7).

Light Microscopy

After routine staining, the biopsy specimens were coded and reevaluated with light microscopy. An experienced pathologist who was blinded with respect to the instituted immunosuppressive regimen scored the histopathologic changes. The Banff 97 classification system for histopathologic scoring of allografted kidneys was used (8).

Immunostaining

The ECM components that were studied were detected with an indirect immunoperoxidase technique. Staining for each molecule was performed in one session. The primary antibodies used were polyclonal mouse anti-human collagen I, anti-human collagen III, and anti-human collagen IV (Harlan Sera-Lab, Sussex, UK) and monoclonal mouse antibodies against the collagen IVα3 (Wieslab, Lund, Sweden) and laminin β2 (C4) (Developmental Studies Hybridoma Bank, Iowa City, IA) chains. The secondary antibody consisted of peroxidase-coupled rabbit anti-mouse IgG (Dako, Glostrup, Denmark). Cryostat sections (4 μm) were mounted on glass slides, dried for 1 h, and stored at -20°C until used. The tissue was fixed with 4% formalin for 10 min. After blocking of endogenous peroxidase by incubation for 30 min with 0.1% H_2O_2 in phosphate-buffered saline, the sections were incubated for 30 min with 5% normal rabbit serum. The sections were then incubated with the primary antibody for 1 h, followed by incubation with the secondary antibody for 30 min. The sections were incubated for 10 min in a filtered solution of 0.5 mg/ml diaminobenzidine and 0.02% H_2O_2 and were then incubated for 5 min with 0.05% $CuSO_4$ in saline solution. Counterstaining with hematoxylin was performed for 15 s. Between all steps, the slides were rinsed with phosphate-buffered saline.

Immunofluorescence staining for C4d was performed on untreated slides, as described previously (9). Mouse anti-C4d (Quidel, San Diego, CA) was used as the primary antibody (9). The secondary antibody was FITC-conjugated goat anti-mouse Ig (Sigma Chemical Co., St. Louis, MO). Staining was independently evaluated by two authors (KK and ME), who were blinded with respect to the diagnoses. A previously described scoring method was used (10).

Image Analysis

After immunostaining, the slides were coded and analyzed in a blinded manner. Digital image analysis was performed with a Zeiss microscope equipped with a full-color 3CCD camera (DXD 950p; Sony Corp., Tokyo, Japan) and KS-400 image analysis software, version 3.0 (Zeiss-Kontron, Eching, Germany). This method of digital image analysis has been validated and demonstrated to be highly reproducible (11,12). Images of the renal cortex of each biopsy specimen were obtained and analyzed with the aid of automated script protocols (macros) developed in our department. Recording and analyses of images were performed with fixed settings. The software allowed censoring of regions of noninterest. Larger vessels and glomeruli were omitted from the analyses. The deposition of each ECM component was determined in each biopsy sample by calculation of

the percentage of surface area stained by the indicator dye, relative to the total surface area. The degree of deposition was assessed by evaluation of the entire renal cortex, with a minimum of five microscopic fields per biopsy sample (at x200 magnification).

The biopsy specimens that were stained for the laminin β2 and collagen IVα3 chains were also directly evaluated for interstitial or tubular expression of those molecules, by two investigators (RB and KK) who were blinded with respect to the diagnoses. In the few cases of discordant scoring, decisions were reached by consensus.

Statistical Analyses

Statistical analyses were performed with SPSS for Windows software (SPSS, Inc., Chicago, IL). One-way ANOVA was used for comparisons of continuous variables with normal distributions. Either a Bonferroni or Games-Howel post hoc procedure was used when appropriate. The Mann-Whitney U test was used to compare continuous variables that were not distributed normally. The data are expressed as mean ± SD or mean ± SEM, as indicated. In a general linear model, values were adjusted for differences in the loss of allograft function and creatinine clearance at the time of biopsy. Correlations between the degree of deposition of individual ECM components and the clinical characteristics of the patients were evaluated with the Pearson correlation test. $P < 0.05$ was considered statistically significant. Using a receiver-operating characteristic curve, we determined the cutoff point of the percentage of staining for the various ECM molecules that predicted the presence of CR with the best combination of sensitivity and specificity.

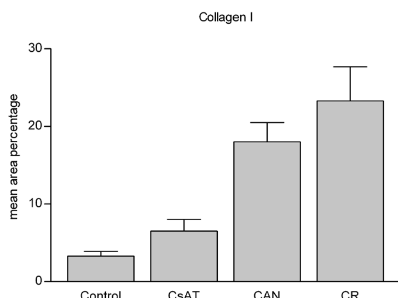
Results

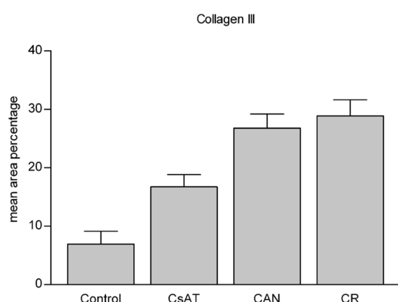
Patient Data

The clinical characteristics of the patients are presented in Table 1. The mean age of the allografted kidney was lower for the patients with CR, compared with the patients with chronic CsAT or CAN. The loss of creatinine clearance was greater for the patients with CR. The time after transplantation, BP values, number of antihypertensive drugs prescribed, and creatinine clearance at the time of biopsy were similar among the groups. No difference in CsA trough levels was observed between the groups receiving CsA.

Light-Microscopic Findings

The chronic histopathologic changes that were observed in the biopsy samples in routine light-microscopic analyses are summarized in Table 2. The mean interstitial fibrosis scores for the CR and CAN groups were significantly higher than that for the chronic CsAT group (CR group versus

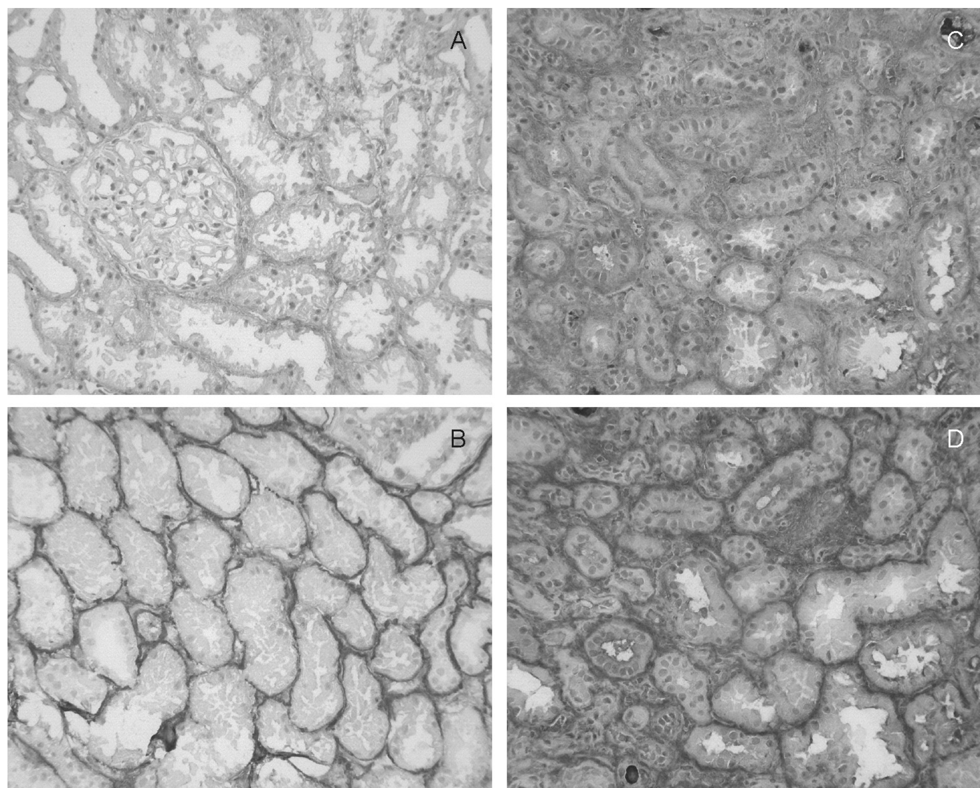




chronic CsAT group, P = 0.011; CAN group versus chronic CsAT group, P = 0.001). The tubular atrophy scores were higher for the CR and CAN groups, but the differences did not reach statistical significance (CR group versus chronic CsAT group, P = 0.422; CAN group versus chronic CsAT group, P = 0.159). The percentages of global glomerulosclerosis did not differ among the groups. The mean peripheral arteriolar hya-

Figure 1. Staining areas (mean ± SEM) for collagen I (A) and collagen III (B). CsAT, cyclosporine A nephrotoxicity; CAN, chronic allograft nephropathy; CR, chronic rejection. *P < 0.05, **P < 0.01.

Figure 2. Immunoperoxidase staining for cortical collagen I (A and C) and III (B and D). Representative images of a normal control sample (left) and a kidney allograft with CR (right) are shown.



line thickening score for the chronic CsAT group was higher than those for the CR and CAN groups, as expected from the case definitions.

ECM Components

First we studied the typical interstitial collagens, ie, collagen types I and III. The mean areas stained for these collagens are presented in Figure 1. The collagen I-stained areas were significantly increased in the CAN and CR groups, compared with the control group, but were not increased in the chronic CsAT group (mean area ± SEM for the CAN, CR, chronic CsAT, and control groups, 18.0 ± 2.5, 23.2 ± 4.4, 6.5 ± 1.5, and 3.3 ± 0.6%, respectively). No significant difference was observed between the CAN and CR groups.

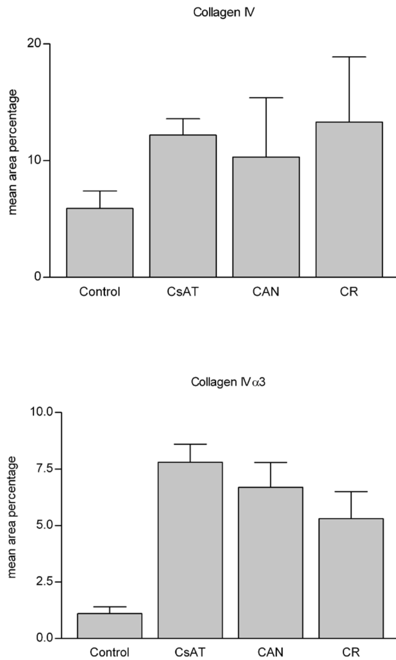


Figure 3. Staining areas (mean SEM) for collagen IV (A) and the collagen IVα3 chain (B). *P < 0.05, **P < 0.01.

The areas stained for collagen III were increased in all patient groups, compared with the control group (mean area ± SEM for the CAN, CR, chronic CsAT, and control groups, 26.8 ± 2.4, 28.9 ± 2.7, 16.7 ± 2.1, and 6.9 ± 2.2%, respectively). However, the areas of staining were significantly greater for the CAN and CR groups, compared with the chronic CsAT group. The staining areas did not differ significantly between the CAN and CR groups. Figure 2 presents representative

Table 1. Clinical characteristics of the patients studied

Clinical Characteristic	Patient Group		
	CsA toxicity (n = 17)	CAN (n = 19)	CR (n = 12)
Time after transplantation (yr)	7.1 ± 3.3	5.5 ± 4.1	5.1 ± 4.3
Allograft age (yr)	51 ± 9	42 ± 15	34 ± 12b
BP, systolic (mmHg)	148 ± 19	154 ± 19	157 ± 22
BP, diastolic (mmHg)	86 ± 5	88 ± 9.7	90 ± 12
No. of antihypertensive medications	1.8 ± 1.1	2 ± 1.2	1.9 ± 1.1
Creatinine clearance (ml/min)	38 ± 12	34 ± 15	32 ± 7
Loss of clearance (ml/min)	26 ± 10	39 ± 19	49 ± 23*
Proteinuria (g/24 h)	1.3 ± 1.5	1.9 ± 1.9	4.0 ± 3.3
CsA trough level (µg/ml)	114 ± 30	113 ± 31	

Values are mean ± SEM. CsA – cyclosporine A; CAN – chronic allograft nephropathy; CR – chronic rejection. * P < 0.05, compared with chronic CsA toxicity and CAN groups.

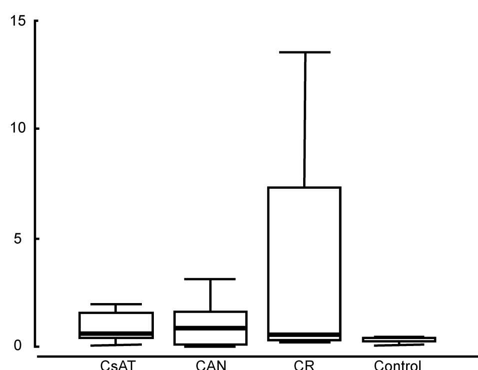


Figure 4. Box and whisker plots of the median staining areas for the laminin β2 chain. The boxes contain 50% of the values. The upper and lower borders indicate the 25th and 75th percentiles, respectively. The upper and lower whiskers indicate the highest and lowest values, respectively. The black lines in the boxes indicate the medians. No significant differences were observed among the groups (P values from the Mann-Whitney test: CsAT group versus CAN, CR, and control groups, 0.847, 0.925 and 0.056, respectively; CAN group versus CR and control groups, 0.868 and 0.172, respectively; CR group versus control group, 0.463).

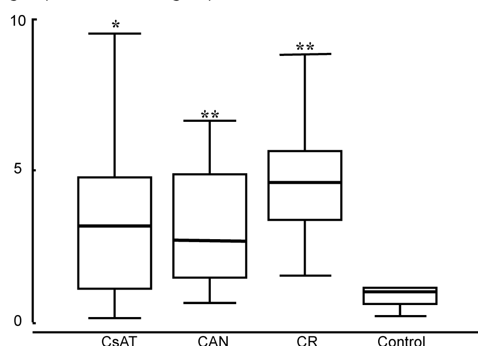


Figure 5. Box and whisker plots of the median staining areas for α-smooth muscle actin (SMA). *P < 0.05, **P < 0.01, compared with control group.

images of immunostaining for collagen I and III in an allografted kidney with CR.

Next we studied whether typical basement membrane collagen type IV expression was increased to the extent observed in native kidney disease (5). The mean areas of staining for collagen IV were significantly increased in all patient groups, compared with the control group, to the same degree (mean area ± SEM for the CAN, CR, chronic CsAT, and control groups, 10.3 ± 5.1, 13.3 ± 5.6, 12.2 ± 1.4, and 5.9 ± 1.5%, respectively) (Figure 3A). We also compared the staining areas for the three types of collagen in the three groups of patients. In the CAN and CR groups, the areas staining for collagen III were greater than those staining for collagen IV (CAN group, $P < 0.001$; CR group, $P < 0.01$) but did not differ significantly from those staining for collagen I. In the CsAT group, the area staining for collagen III was greater than that staining for collagen I ($P < 0.001$), but no significant difference was observed in comparison with the area staining for collagen IV ($P = 0.07$).

When we studied biopsies with mild interstitial fibrosis (< 25% interstitial expansion), we observed a difference in collagen I staining between the CsAT group and the CR and CAN groups combined (CsAT group, 6.5 ± 6%; CAN/CR group, 15.5 ± 8.7%; $P < 0.05$). In this subgroup with mild fibrosis, there was no difference in the time that had elapsed since transplantation (CAN/CR group) compared to the time that had elapsed since the Neoral switch (CsAT group). Collagen III and IV staining areas did not differ between the CsAT group and the CAN/CR subgroup. A meaningful comparison of biopsies with greater degrees of interstitial expansion was not possible because of the small numbers.

Because an earlier report suggested that immunostaining for the α3 chain of collagen IV and the β2 chain of laminin could differentiate CR from chronic CsAT (13), we also evaluated these

Table 2. Light-microscopic features of the allograft biopsies studied

Histological Feature	Patient Group		
	CSA Toxicity (n = 17)	CAN (n = 19)	CR (n = 12)
Interstitial fibrosis score	0.58 ± 0.21	1.76 ± 0.19†	1.65 ± 0.25*
Tubular atrophy score	0.69 ± 0.24	1.36 ± 0.22	1.29 ± 0.29
Arterial intimal thickening score	0.77 ± 0.21	1.48 ± 0.19	1.40 ± 0.25
Allograft glomerulopathy score	0.02 ± 0.17	0.25 ± 0.15	0.33 ± 0.19
Peripheral arteriolar hyalinosis score	1.92 ± 0.12‡	0 ± 0.11	0 ± 0.15
Global glomerulosclerosis (%)	28.0 ± 7.1	32.5 ± 6.4	24.4 ± 8.4

Values are mean ± SEM; Scored according to the Banff 97 classification system; * P < 0.05, compared with chronic CsA toxicity group; † P < 0.01, compared with chronic CsA toxicity group; ‡ P < 0.01, compared with CAN or CR group

molecules. The areas stained for the collagen IVα3 chain were increased in all patient groups, compared with the control group, to the same extent (mean area ± SEM for the CAN, CR, chronic CsAT, and control groups, 6.7 ± 1.1, 5.3 ± 1.2, 7.8 ± 0.8, and 1.1 ± 0.3%, respectively) (Figure 3B). The collagen IVα3 chain was expressed in a normal pattern, namely, at the distal tubular basement membrane (TBM), in all three patient groups. No interstitial staining or new expression at the proximal TBM was noted. In none of the groups did the areas staining for the laminin β2 chain change significantly, compared with the control group (Figure 4). However, we occasionally observed some new abnormal tubular expression in each of the groups (CsAT group, nine biopsy samples, 53%; CAN group, eight samples, 42%; CR group, six samples, 50%). The new expression was mostly confined to atrophic tubules or tubules surrounded by infiltrate, the proximal or distal morphologic features of which were difficult to assess. Less than 1% of tubules were affected in these samples, except for one sample in the CsAT group, three samples in the CAN group, and two samples in the CR group, in which 1 to 10% of tubules exhibited staining. No interstitial staining for the laminin β2 chain was observed, except for faint staining in some areas of periglomerular fibrosis.

We investigated whether the observed qualitative and quantitative differences in the interstitial ECM composition in kidneys with CR or chronic CsAT were correlated with the numbers of interstitial myofibroblasts present, as assessed with immunohistologic staining for α-smoothh muscle actin (SMA). Increases in SMA staining were observed for all patient groups, compared with the control group, but no differences were observed among the patient groups (Figure 5).

Correlations and Receiver-Operating Characteristic Curve Analysis

Among the three groups of patients, we observed no correlation between the areas of staining for the various molecules and the clinical variables of loss of allograft function, time after transplantation, allograft age, creatinine clearance, and proteinuria at the time of biopsy. A positive correlation (r = 0.379, P = 0.008) was observed between the interstitial fibrosis scores of all biopsy samples combined and the loss of creatinine clearance at the time of biopsy. The interstitial

fibrosis scores of all biopsy samples combined were also correlated with the areas of staining for collagen I and III (r = 0.499, P = 0.001, and r = 0.499, P = 0.002, respectively). A general linear model was used to adjust for differences in loss of function and creatinine clearance at the time of biopsy among the groups.

Receiver-operating characteristic curve analysis demonstrated that collagen I staining had the best discriminatory value in differentiating CR from chronic CsAT, with a sensitivity of 75% and a specificity of 88% at a cutoff value of 13% cortical interstitial staining and with values of 63% and 94%, respectively, at a cutoff value of 19% staining. The fraction of the area under the curve was 0.92 of the total area (P = 0.001). None of the ratios of the mean staining areas for the studied molecules proved to be useful for discrimination of CR from chronic CsAT.

Discussion

The aim of this study was to examine the composition of the cortical interstitial ECM of kidney allografts with either CR or chronic CsAT. We wondered whether quantitative and/or qualitative differences in ECM accumulation occur. Therefore, we quantitatively compared the deposition of several ECM molecules. We chose to study the molecules collagen I, III, and IV, because they are known to accumulate in the renal cortical interstitium during native kidney disease (5,14). The laminin β2 and collagen IVα3 chains were investigated because an earlier report suggested that de novo expression of these molecules at the proximal TBM could differentiate CR from chronic CsAT (13).

In this study, we observed quantitative and qualitative differences in the ECM composition of kidney allografts with CR or chronic CsAT. Our results demonstrated that collagen I, III, and IV accumulated in the renal cortical interstitium during CR. The most prominent increases were observed for collagen I and III. A similar pattern of ECM deposition was observed in the kidneys of patients who likely had CR but were receiving CsA. In the kidneys of patients with chronic CsAT, however, only collagen III and IV accumulated significantly.

Regulation of the renal interstitial ECM composition in vivo is complex and encompasses changes in ECM biosynthesis and degradation. In normal kidneys, collagen I and III are observed in the blood vessels and the interstitium, albeit in small amounts (Figure 2) (15). Collagen IV is a natural component of the glomerular basement membrane and TBM, and its α3 chain is normally observed in the basement membranes of glomeruli and distal tubules (15). Laminin is observed in

vessel walls and all basement membranes. The β2 chain, however, is observed only in glomeruli and vessel walls (16).

Quantitative data on the ECM composition of the cortical interstitium of human allografts with chronic CsAT or CR were lacking. A few studies reported increases in collagen III levels in grafts with chronic dysfunction but did not compare changes induced by CR and chronic CsAT (17,18). A number of in vitro studies have addressed the effects of CsA on the production of ECM molecules by renal cells. One study reported that CsA stimulated the production of pro-collagen I and IV in cultures of murine proximal tubular epithelial cells and the production of pro-collagen I in cultures of murine renal fibroblasts (19). Increases in the cortical expression of collagen I and IV were observed in vivo in a rat model of CsAT (20). The deposition of collagen III was not assessed in that model, however. Other data on collagen production were derived from a study on cultured human cells, which demonstrated an increase in collagen III production by renal fibroblasts when they were treated with CsA (21). Studies performed with a monkey renal fibroblast cell line (CV1) demonstrated that CsA stimulated the synthesis of type III collagen by a pathway leading to activation of the COL3A1 promoter and upregulation of COL3A1 mRNA (22). We observed the increased deposition of collagen III and IV in the interstitium of the cortex of human renal allografts as a result of chronic CsAT. In contrast to the results of the study performed in rats, we observed no significant increase in collagen I deposition. Although we did not observe a correlation between the time elapsed since the Neoral switch and the degree of collagen I deposition, we cannot exclude the possibility that, with time, collagen I would also accumulate during long-term chronic CsAT. The difference in collagen I staining between the CsAT and CAN/CR subgroups with mild fibrosis suggests that CR may stimulate collagen I accumulation earlier than does CsAT.

In our study, we observed greater expansion of the interstitial space in the CR group, compared with the chronic CsAT group, as well as more deposition of collagen type III. The difference in interstitial fibrosis could not be explained on the basis of differences in clinical variables, because no correlations with those variables were observed.

We wondered whether the differences in ECM deposition between CR and chronic CsAT could be explained on the basis of a difference in the numbers of myofibroblasts present. SMA is a marker of activated myofibroblasts, which are thought to play a major role in the deposition of scar tissue (23). Two earlier reports described increases in interstitial SMA staining in deteriorating human kidney allografts (17,18). In CR, SMA-positive cell numbers were reported to increase with increases in the interstitial area fraction and collagen III deposition (18). In our study, we did not observe differences in SMA staining among the CR, CAN, and chronic CsAT groups; all samples contained more SMA-positive cells than normal. To explain the greater degree of interstitial ECM deposition during CR, compared with chronic CsAT, a higher rate of collagen production by myofibroblasts or a lower rate of ECM degradation by tissue metalloproteinases could be

hypothesized. Data derived from a primate model of chronic cardiac rejection support the latter hypothesis, because it was demonstrated that the progression of myocardial fibrosis was associated with increased expression of tissue inhibitor of metalloproteinases 1 and 2 (24).

We also examined the α3 chain of collagen IV and the β2 chain of laminin. Our results are at variance with a report by Abrass et al (13), which suggested that a pattern of new expression of those molecules at the proximal TBM was specific for CR. In that study, CsAT resulted in the interstitial deposition of collagen I and III. Our study differs in the definitions of cases, the antibodies used, and the mode of analysis. The study by Abrass et al (13) examined patterns of distribution and provided no quantitative data on the investigated molecules. According to Abrass et al (13), collagen I and III are not normally observed in the cortical interstitial area and the collagen IVα3 and laminin β2 chains are not expressed at the TBM. However, we and others observed that collagen I and III are normally present in the renal interstitium, albeit in small amounts (5,15,25-27). We also observed that the collagen IVα3 chain is normally expressed at the distal TBM, as noted earlier (16,28). This discrepancy may be related to the different antibodies used. In the study by Abrass et al (13), allografted kidneys with rejection exhibited acute rejection; although the authors stated that their results also applied to allografts with CR, they did not systematically examine that idea and no data were presented. The new expression of the collagen IVα3 and laminin β2 chains at the proximal TBM observed by Abrass et al (13) might thus very well be related to tubulitis during acute rejection. In our study, we observed sporadic new expression of laminin β2 at the TBM in a portion of the biopsy samples in each study group, especially in areas of tubular damage. Staining for this molecule did not differentiate CR from chronic CsAT. We observed increased expression of the collagen IVα3 chain in the CR, CAN, and chronic CsAT groups but no abnormal distribution pattern. We observed a small nonsignificant increase in collagen I staining for the chronic CsAT group, compared with normal control values. It is possible that a small increase in collagen I expression by the patients with chronic CsAT, which was below our detection limit, could have resulted in the expression pattern observed in the study by Abrass et al (13).

In conclusion, we observed a more pronounced fibrotic response in the tubulointerstitium, involving collagen I and III, in allografts with CR, compared with those with chronic CsAT. Early increases in the cortical interstitial deposition of collagen I were more specific for CR. Staining for the collagen IVα3 and laminin β2 chains was not useful for the differentiation of CR from chronic CsAT.

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General discussion

Role of podocytes in proteinuria

Glomerular gene expression in proteinuria

Further perspectives on proteinuria

Chronic allograft dysfunction

Further perspectives on molecular diagnosis in chronic allograft dysfunction

7

The kidney is essential for the maintenance of the internal milieu of the body. Filtration of blood in the glomerulus is an important part of this process: plasma water is filtered, while most proteins and cells are retained. Damage to the glomerulus is often accompanied by proteinuria, the presence of abnormally high amounts of protein in the urine. Proteinuria presumably contributes to the progression of renal disease. At the tissue level, progressive loss of kidney function is mirrored by replacement of intact nephrons by scar tissue, a process called fibrosis. Once a critical amount of functional tissue is lost, the process of fibrosis is uncoupled from the initial cause of injury and becomes self-perpetuating.

Eventually, complete loss of kidney function will necessitate renal replacement therapy, for example through kidney transplantation. Also kidney grafts are subjected to injurious stimuli that drive the development of progressive loss of function. Peritransplant injuries, an immunologically hostile environment, and damage related to donor events contribute to development of progressive fibrosis in most renal allografts. Again, the pace of progression may be quickened by proteinuria.

In this thesis, we focused on two relatively distinct topics: the pathophysiology of proteinuria, and the recognition of causes of renal function loss in kidney transplants using a molecular approach. In this chapter, the results of the studies described in the previous chapters are summarized and placed in a broader perspective.

Proteinuria

A central but still incompletely answered question in renal physiology is how the glomerular filtration barrier works. Complementary to this is the question what the mechanisms of glomerular dysfunction are, in other words, what causes proteinuria. The different layers of the glomerular filtration barrier, the endothelium, the glomerular basement membrane (GBM), and the podocytes, are all assumed to contribute to the correct function of the glomerulus.

Role of podocytes in proteinuria

In recent years there has been much interest in the role of the podocyte in the development of proteinuria. Indeed, podocytes show conspicuous changes during proteinuria: their normal complex shape is simplified through foot process effacement, and their delicate cytoskeleton is condensed at the basal part of the cell (1). This first morphological clue towards a role of podocyte changes in the development of proteinuria was further substantiated by the discovery of the genetic base of several congenital or hereditary forms of the nephrotic syndrome. Genes involved in the development of these diseases were found to be expressed exclusively in podocytes. This

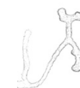
led to the hypothesis that these genes and the proteins that they code for might also be involved in the development of acquired forms of proteinuria (2,3). Initial studies seemed to affirm this hypothesis: in acquired primary proteinuric diseases such as minimal change disease, FSGS, and membranous nephropathy, expression of the podocyte-protein nephrin was decreased (4-6). Animal studies also pointed towards a decrease in podocyte-associated proteins in proteinuria (7,8). However, most of these studies described the expression of single molecules, and only studied either mRNA or protein level. We wanted to further investigate the expression of a group of podocyte-associated molecules and relate the expression to ultrastructural and clinical parameters. This was the subject of a study described in chapter 2. We studied the glomerular mRNA and protein expression of several podocyte-associated molecules in patients with acquired nephrotic syndrome, in comparison to that of patients with a non-glomerular type of kidney disease and healthy controls. In line with previous studies, we indeed found a decrease in podocyte proteins in proteinuric diseases. At the same time, our results suggested that the loss of protein expression is not the actual cause of proteinuria: comparison of protein expression levels with the podocyte ultrastructure made clear that the decreased protein staining could be explained by the loss of the specialized podocyte architecture. Also, mRNA synthesis was not impaired, but was even increased in most diseases, suggesting a compensatory reaction of the podocyte to proteinuria.

A shortcoming of the study presented in chapter 2 may be that support for the hypothesis, that the podocytes show a compensatory reaction to proteinuric damage, is merely derived from correlations rather than from mechanistic studies. Furthermore, most cases of human acquired proteinuric diseases already showed high levels of proteinuria at the time the renal biopsy was taken. This limited the evaluation of changes in podocyte ultrastructure and mRNA and protein expression in time. To gain insight in the processes at play in the podocyte during the development of proteinuria, we used a rat model of proteinuria development, the Dahl salt-sensitive (Dahl SS) rat. These animals spontaneously develop proteinuria starting from week 4 to 6 of age, and later on show progression to glomerulosclerosis. We were interested in the early phases of proteinuria, and studied these rats at different time points between their 2nd and 10th week of life. The results of the studies in rats, outlined in chapter 3, were in line with the concept raised from the study in patients, ie, the changes in expression of podocyte-associated molecules do not precede proteinuria. Instead, despite clear disturbance in glomerular permselectivity as measured by urinary albumin and protein excretion at ten weeks of age, expression of most podocyte-associated molecules was still normal at that time, except for some segmental loss of nephrin staining intensity. Podocyte ultrastructural changes, most importantly foot process effacement, also notably lagged behind the development of proteinuria.

The one exception to this general pattern of undisturbed protein expression of studied podocyte-associated molecules was the expression of the glycoprotein podoplanin. We observed a segmental loss of podoplanin expression already at four weeks of age – the first time point at

which proteinuria could be detected. Others have already related the expression of podoplanin to proteinuria. In fact, the name podoplanin has been derived from the fact that loss of this protein occurs simultaneously with foot process effacement (pes planus (lat.) – flat feet). Also, injection of anti-podoplanin antibodies in rats induces proteinuria (9-11). In our study, the decrease in expression of podoplanin in proteinuric rats compared to non-proteinuric rats was only seen at the protein level. There were no genetic differences between Dahl SS and SHR rats in the podoplanin gene that could explain proteinuria, and podoplanin mRNA expression was even increased in Dahl SS rats compared to SHR rats. Furthermore, glomeruli from proteinuric Dahl SS rats with and without podoplanin loss showed similar podoplanin mRNA expression levels.

Next to expression in the glomerular podocyte, podoplanin is found in several other cells, including alveolar cells, osteoblasts, skeletal muscle, and lympathic endothelial cells (12). Also, a number of tumors show expression of podoplanin. Several investigators have described that podoplanin is coupled to ezrin, which connects podoplanin to the actin cytoskeleton (13,14). Ectopic expression podoplanin in cultured cells induces the formation of filopodia (actin-supported cell extensions), a finding that would explain the migratory behavior of podoplanin-positive tumor cells (15). Podoplanin exerted this actin-remodeling effect via downregulation of the activity of RhoA, a member of the Rho family of GTPases that are involved in the (dis)assembly of actin filaments. Whether podoplanin has a similar role in podocytes has not been established yet. We did not find differences in ezrin mRNA expression, but this does not rule out a difference in ezrin-podoplanin interaction at the level of post-translational protein modifications. With respect to actin-remodeling properties of podoplanin, we did find a trend towards upregulation of RhoA mRNA expression in podoplanin-negative glomeruli. This suggests a link between podoplanin expression and actin cytoskeleton regulation. In analogy to the role of podoplanin in filopodia formation via RhoA downregulation, we hypothesize that a decrease of podoplanin protein expression in the podocytes facilitates RhoA-mediated foot process effacement and actin cytoskeleton condensation. Further evidence that may confirm or disprove such theories will have to come from in-vivo manipulation of podoplanin, for example through genetic approaches. Podoplanin knock-out mice die of respiratory failure soon after birth (16). Kidney defects or maldevelopment in these mice have not been studied. Investigations into the role of podoplanin in the podocyte will benefit from mouse models with a podocyte-specific podoplanin deletion. The Cre-loxP system for podocyte-specific gene manipulation is available (17,18), but mice with podoplanin-floxed alleles have not been generated yet. It would be of interest to see whether such podocyte-specific podoplanin deficient mice develop a normal glomerulus, and whether they show proteinuria or increased disease susceptibility.



Glomerular gene expression in proteinuria

Besides our study of the expression of well-known podocyte-associated molecules, we also evaluated proteinuria-associated changes in glomerular mRNA expression through a more genome-wide approach. The results of these experiments are described in chapter 4. We used microarray chips to which we hybridized RNA isolated from glomeruli of 4- and 6-week-old Dahl SS and SHR rats. These time points were chosen because they flank the period in which the first onset of proteinuria is seen.

For the evaluation of the array results we used several approaches. In recent years, the labs of Kreutz et al (19-21) and Garrett et al (22,23) have identified quantitative trait loci (QTLs) that are linked to the development of albuminuria in the Dahl SS rat. We evaluated which of the differentially expressed genes between the two rat strains localized to these genome regions. Secondly, we used pathway analysis software to evaluate which pathways were represented in the list of differentially expressed genes. Furthermore, we compiled a list of genes known to be differentially regulated upon protein loading in renal proximal tubular epithelial cells, and evaluated the glomerular expression of these genes using the global test (24).

We identified around 500 genes that were differentially regulated between the two strains, regardless of the time point. Of these, 115 genes were located on the previously defined QTLs. We validated the expression of hedgehog interacting protein (Hip, 5.2 times upregulated), and polyamine modulating factor binding protein 1 (Pmfbp1, -2.8 times downregulated). Both genes are located on the albuminuria QTL on rat chromosome 19.

Pathway analysis indicated an overrepresentation of cytoskeleton-associated genes in the list of differentially expressed genes. This may be explained by the fact that proteinuria is known to occur simultaneously with changes in the cytoskeletal organization of podocytes. During the first 8 weeks of life of the rats we did not observe widespread differences in the ultrastructure of the podocyte foot processes, meaning that already early in the development of proteinuria there is a change in podocyte cytoskeleton regulation that is not reflected in morphological alterations. We studied the GTPase dynamin in more detail. Recent studies by the Reiser group have indicated a role for dynamin in the organization of the podocyte actin cytoskeleton (25). In their studies, a cathepsin L-dependent cleavage of dynamin resulted in proteinuria. Our results contrast their findings, as we found an increase in dynamin mRNA expression in proteinuric rats, as well as an increased dynamin protein expression in proteinuric patients. The models in which dynamin was studied differ considerably between our study and that from the group of Reiser. We used a model of spontaneous development of proteinuria, while in the animals studied by Reiser and colleagues, proteinuria was induced by LPS or puromycin aminonucleoside. This may indicate that the regulation of dynamin in diverse proteinuric conditions is different. Similar to the concept

discussed in chapter 2, the upregulation of dynamin may reflect a compensatory response to the development of proteinuria.

Furthermore, we found that a group of genes that is differentially expressed upon protein loading in proximal tubular epithelial cells is also differentially regulated in glomeruli of proteinuric versus non-proteinuric rats. This finding substantiates the hypothesis that increased glomerular protein filtration has a toxic effect on the podocyte. In support of this, a double staining of albumin and desmin (a podocyte stress marker) showed a colocalization of albumin resorption droplets and desmin accumulation in the podocytes.

Further perspectives on proteinuria

Development of proteinuria

In trying to delineate the pathogenesis of proteinuria, the problem is that regardless which component of the glomerular filtration barrier is damaged the result is proteinuria. This shows that the different components of the glomerular filtration barrier are all indispensable, or are functionally interlinked. This functional relationship is obvious. For example, podocytes and endothelial cells produce the GBM they rest on; malfunction of either of these cell types may lead to a disturbed glomerular filtration that is difficult to trace back to a single cause. Adding more complexity to the situation is the fact that the filter may only work properly if not only the static components are correctly assembled, but also the glomerulus is perfused under physiologic conditions, as this may lead to the in vivo formation of pressure and charge gradients that have a role in glomerular permselectivity. This complex interrelationship between different static and functional components of the filtration barrier makes it difficult to establish their respective roles in glomerular filtration in health and disease. Thus, the question at which site glomerular filtration takes place, posed more than 30 years ago by Farquhar in a review on the topic (26), is at present still valid. Still, while important parts of the pathogenesis of proteinuria remain uncharted, some pathways can be delineated. The perspectives on the different components of the glomerular filtration barrier will be discussed below.

Interplay between proteinuria and foot process effacement

Podocytes are involved in every type of glomerular proteinuria. Clearly, in congenital proteinuric diseases podocyte damage is often the cause of proteinuria (see chapter 1). However, the studies in this thesis suggest a secondary role for podocytes in acquired proteinuric diseases: Foot process effacement only developed after the occurrence of proteinuria (chapter 3); podocyte protein expression remained normal in the initial stages of proteinuria (chapter 3), changes in expression of most podocyte-associated proteins (except that of podoplanin in the Dahl SS rat) seemed to be related to the extent of foot process effacement (chapter 2, 3). Also, a selected number of genes

that are expressed in tubular epithelial cells as a consequence of proteinuria also show up in the glomerular gene expression profile in proteinuric rats (chapter 4).

Several studies in the literature support the above findings. Some authors have argued that foot process effacement affects the process of glomerular filtration in such a way that proteinuria occurs (27), ie, foot process effacement is the cause of proteinuria. However, most recent studies are compatible with foot process effacement as a result of proteinuria. Jarad et al (28) found that proteinuria preceded podocyte foot process effacement in a podocyte specific laminin β2 knockout mouse model. Kalluri described three different mouse models that show proteinuria without initial foot process effacement (29). Van den Berg et al have found that foot process effacement is not related to the amount of proteinuria in minimal change disease: patients in remission showed a foot process width that was comparable to that in patients with overt proteinuria (30).

Possible causes and consequences of foot process effacement

The exact cause of foot process effacement remains unclear. Also, the role of this process in glomerular function is not completely understood. Dysfunctional podocyte proteins, changes in GBM composition, protein overload, and (lack of) stimulation by growth factors and cytokines have all been related to the development of foot process effacement. Clearly, this is a final common pathway of podocyte damage.

From a teleological point of view, several interesting hypotheses have been raised to explain what goal foot process effacement could serve: Reiser et al found that podocytes express the co-stimulatory molecule CD80. Increased expression of CD80 was found in conjunction with the development of proteinuria. This suggested to them that podocytes might have a role in immunological danger signaling, and display a stereotypical reaction to such 'danger signals'. In a review on this novel aspect of podocyte function, Reiser and Mundel suggested that foot process effacement and the resulting increase in glomerular permeability could have a role in increasing the speed with which noxious molecules are cleared from the blood (31,32). However, it is questionable whether this theory is valid in situations where, as outlined above, foot process effacement follows the development of proteinuria.

Another interesting theory (33) suggests that foot process effacement is a way for the glomerulus to counteract the increased intracapillary pressure. The different shape of the podocyte, with the reinforced cytoskeleton, would then increase stability at the expense of permselectivity.

The role of the GBM and the endothelium

The glomerular basement membrane supports the endothelial cells and podocytes. The GBM per se seems to have only a minor role in glomerular filtration, although it is indispensible for the correct function of podocytes and endothelial cells. For example, patients with Pierson's syndrome, caused by a mutation in the laminin β2 gene, are proteinuric and show foot process effacement.

As discussed in chapter 1, the glomerular filtration barrier is size- and charge selective (34). With regard to the charge selectivity, several recent studies have excluded an important contribution of heparan sulphate proteoglycans (HSPGs) in the GBM to charge selectivity (35-37).

If HSPGs do not contribute to the charge selectivity, what role do they serve? In embyrogenesis, HSPGs have a role in distribution and gradient formation of signaling molecules such as hedgehogs. One could speculate that in a similar fashion HSPGs in the GBM have a role in the interaction between endothelial cells and podocytes. Indeed, such interaction may be important in glomerular filtration: Collino et al have recently shown that stimulation of endothelial cells with plasma of women with pre-eclampsia releases factors that subsequently influence podocyte homeostasis (38).

Recent studies have shown that the endothelial fenestrations, traditionally thought to be too large to contribute to ultrafiltration, may be filled with proteoglycans that could contribute to the charge and size selectivity of the glomerulus. With this in mind, it is to be expected that the glomerular endothelium has a more important role in glomerular function and dysfunction than hitherto acknowledged. A pivotal role of endothelial cells would provide a plausible explanation for the proteinuria that is seen in diseases with systemic endothelial dysfunction, such as diabetes and pre-eclampsia. Furthermore, it would explain the observation that proteinuria is a risk factor for the development of cardiovascular diseases, as both diseases can be traced back to endothelial dysfunction (39).

If indeed the endothelium is an important first barrier to protein, this would mean that in a normal situation the GBM and podocytes are not exposed to plasma-concentrations of protein. Loss of endothelial function would lead to a higher concentration of protein in the GBM and thus at the basal cell membrane of the podocyte foot processes. From this perspective, our observation of a proteinuria-related gene response in the glomerulus (chapter 4) would be compatible with the hypothesis that size selectivity of the glomerulus is located in the endothelium. Indeed, when we directly stained frozen slides of Dahl SS and SHR rats with anti-rat-IgG antibodies, we found an increased distribution of rat IgG in the GBM and podocytes of the proteinuric Dahl SS rat (results not shown). This is in line with observations in humans with proteinuric kidney diseases (40). In this regard, it is of interest that the Dahl SS rat later develops hypertension, which has been regarded as another symptom of endothelial dysfunction (41). Thus, future studies should focus on the role of the endothelium in the glomerular filtration barrier. There have been reports of genes expressed specifically in glomerular endothelium (42). This offers opportunities to generate transgenic mouse models that can be used to dissect the role of the endothelium in glomerular filtration.

Consequences of proteinuria

While the cause of proteinuria remains unclear, the consequences of proteinuria are more clear, at least from an epidemiologic point of view: proteinuria is associated with the progression of renal disease. This raises the question whether proteinuria solely reflects the damage inflicted upon the nephron, which leads to further progression of renal disease, or that proteinuria forms a part of the pathogenetic process that underlies progression of renal disease.

As discussed in chapter 1, reports from the literature on this topic are controversial: some studies bring support for the possibility that proteinuria causes tubulointerstitial inflammation and fibrosis, others dispute such a link. In either scenario, impaired glomerular function precedes the development of progressive renal disease. Our findings (chapter 4) are compatible with the hypothesis that proteinuria is part of the pathogenetic process that leads to further loss of glomerular function, as judged from gene-expression patterns and histological studies. Others have described TGF-β and endothelin upregulation in podocytes in response to proteinuria (43,44), also pointing towards a deleterious role of proteinuria in glomerular function.

Once a critical amount of cells and tissue are damaged, loss of glomerular function becomes self-perpetuative. This vicious cycle seems to be present at two levels in the kidney: At the tissue level, loss of nephrons increases the workload for the remaining nephrons, leading to hyperfiltration and further damage. At the cellular level, loss of podocytes increases the demands placed on the remaining cells. Wiggins et al described a sequence of podocyte hypertrophy, adaptation, and decompensation in rats with a relative shortage of podocytes (45). The stress placed on podocytes as a result of increased protein trafficking through the glomerulus may be the driving force for this process.

Transplantation

Chronic allograft dysfunction

There has been a clear progress in renal transplant survival rate over the years. Especially the first year graft-survival rate has increased tremendously through improvements in immunosuppressive medication, and monitoring of its administration. Nowadays, failure of renal grafts on the long term is the most limiting factor in transplantation.

Multiple causes may underlie the development of chronic allograft dysfunction, but the eventual course is common with respect to clinical and histopathological presentation: there is a slow decline in renal function, mirrored at the histopathological level by the presence of vascular damage, tubular atrophy, and interstitial fibrosis. Different causes of chronic allograft dysfunction often coexist, thus forming a spectrum of injurious stimuli ranging from the toxicity of excess immunosuppressive medications to an ongoing activity of the immune system. An important question is how to distinguish the separate causes in patients that present with non-specific clinical and histopathological features. The studies described in chapters 5 and 6 aimed to address this question using molecular techniques. In chapter 5, we studied the mRNA expression levels of growth factors and extracellular matrix (ECM) proteins that are important in the development of interstitial fibrosis. In chapter 6, we used an immunohistochemical analysis of the composition of interstitial fibrosis. These markers were studied using biopsies from renal allografts of patients from two well-defined groups, each representing one side of the spectrum of different causes of chronic allograft dysfunction: the chronic rejection group and the chronic cyclosporine A toxicity group.

The composition of the ECM is continuously modified through synthesis and degradation. Previous experiments have shown that pathophysiological changes could be observed more readily at the level of synthesis, through measurement of mRNA expression levels of molecules known to be involved in the disease process. As described in chapter 5, differences in mRNA expression of laminin β2 and TGF-β could differentiate between chronic rejection and chronic cyclosporine A toxicity as a cause of chronic allograft dysfunction. The mRNA expression levels of these proteins were higher in the cyclosporine toxicity group than the chronic rejection group. Analysis of laminin β2 and TGF-β mRNA expression levels allows recognition of the actual cause of allograft dysfunction with a specificity and a sensitivity of over 85 percent.

In chapter 6, we have shown that the accumulation of collagen I in interstitial fibrotic lesions was more pronounced in patients with chronic rejection than that in patients with chronic cyclosporine toxicity. Collagen III and IV accumulated in the interstitial area to similar extent in either disease. Others have found that an ectopic production of certain collagen and laminin chains

could help differentiate chronic rejection and cyclosporine A toxicity (46). In our studies, we could not confirm that such abnormal expression patterns can distinguish between different disease entities.

Besides showing that molecular techniques can have additive value in dissecting the cause of clinically non-specific disease processes, the studies in chapter 5 and 6 may give insight in patho-physiological processes that play a role in transplant rejection. For example, from the results of the studies described in chapter 6, we propose that chronic rejection seems to stimulate the accumulation of collagen I more pronounced in comparison to chronic cyclosporine toxicity. However, the mRNA expression of collagen I was similar in both diseases, suggesting a role for an impaired collagen I degradation process in chronic rejection. The increased laminin β2 mRNA expression in chronic cyclosporine A toxicity might point to a stimulatory effect of cyclosporine A production on the synthesis of this transcript. Since vascular damage may stimulate laminin β2 production, the increase in laminin β2 expression could also reflect a more pronounced vascular damage in cyclosporine A toxicity compared to chronic rejection. It should be stated, however, that such pathophysiological hypotheses are based only on correlative evidence.

An interesting finding in these studies was that increased mRNA expression does not consistently coincide with protein accumulation. Besides differences in protein degradation, changes in mRNA processing and regulation as well as variations in the efficiency of mRNA to protein translation may account for these differences. It has been shown that even in unicellular eukaryotic organisms such as yeast there is no direct correlation between mRNA expression and protein abundance (47). This again underscores the complexity of regulation of processes such as fibrosis, and indicates that some caution in extrapolating result between different levels of organization (i.e. mRNA, protein, cellular, and tissue level) is warranted.

The studies described in chapters 5 and 6 were performed in two well-selected populations of chronic cyclosporine A toxicity and chronic rejection, representing both ends of the spectrum of chronic allograft dysfunction. Although this was helpful in defining a molecular profile that could distinguish both causes, the strict selection of patients also forms the main limitation of the study. As stated before, in many patients, different causes of allograft dysfunction coexist, and therefore we cannot draw conclusions with regard to the usefulness of these markers in non-selected patient groups. In the future, the markers studied by us and by others should be tested in larger, unselected patient groups.

Further perspectives on molecular diagnosis of chronic allograft dysfunction

What is to be expected from future research of molecular markers in chronic allograft dysfunction? The search for markers that can identify specific causes of allograft dysfunction, or distinguish progressors from non-progressors in an early phase of the disease, will be instrumental in better treatment of patients with a renal transplant. Also, further knowledge of the precise pathogenesis of allograft dysfunction will be needed to improve the transplant survival rate. Use of epidemiologic data may be helpful in selecting which pathogenetic processes to study. These topics will be discussed below.

Microarray studies would seem to be a logical next step in the search for markers of specific causes and mechanisms of chronic allograft dysfunction. Several microarray studies have already been reported, which have offered important new information with regard to pathophysiological processes in acute rejection (48,49). For example, the role of humoral rejection became more apparent in the microarray-based analysis of acute rejection biopsies. However, microarray-assisted investigations in chronic allograft dysfunction have so far not brought up new pathophysiological concepts. Hotchkiss et al (50) studied expression profiles in biopsies from patients with chronic allograft dysfunction with and without arteriolar hyalinosis (suggesting a different pathogenesis), but could not find a different expression profile, most likely because the disease had already passed to a chronic phase or final common pathway, in which specific causes are obscured by a general process of renal deterioration. This shows that molecular markers that identify specific causes of allograft dysfunction, or distinguish progressors from non-progressors should be found early in the disease process, when specific therapies can still be installed. This leads to another problem: a relatively long follow-up time is required to determine early markers that reliably predict the long-term outcome. During this long follow-up time, immunosuppressive regimens may change, and as a result the markers found in these long-term studies may not be applicable for the next 'generation' of patients. For example, the studies described in chapters 5 and 6 included only a few patients that used tacrolimus, while at the moment most transplant centers have switched from cyclosporine to tacrolimus as a calcineurin inhibitor. Thus, frequent changes in immunosuppressive regimes will impede the evaluation of long-term effects of medication, and even more, the evaluation of the usefulness of early molecular markers for long-term prediction of transplant course.

Instead of focusing on the specific characteristics of processes leading to chronic allograft dysfunction, another fruitful strategy could be to identify the pathophysiological processes that chronic allograft dysfunction shares with other chronic kidney diseases. Again using microarray, Donauer et al found that the expression profiles of native end-stage renal disease kidneys were similar to those of transplanted kidneys with chronic allograft dysfunction (51). Also, risk factor

analysis makes clear that not only immunological mechanisms but also non-immunological injuries are of importance for long-term graft survival. Donor age, for example, is one of the most important risk factors, accounting for approximately 30 percent of the variation in long-term graft survival (52). Together, these microarray experiments and epidemiological data may indicate that the pathophysiologic mechanisms that drive the deterioration of kidney grafts are similar to those at play in progressive disease of native kidneys. A more extensive comparison of both would enhance the insight in the nature of disease progression.

Other lines of investigation

Because the kidney is transplanted into an immunologically hostile environment, one is inclined to think that the processes leading to transplant failure are predominantly immunological in nature. The notion that native and transplanted kidneys follow similar final pathways to renal function loss shows that this is not necessarily the case, and also illustrates the importance of the use of epidemiologic data. In this regard, it is interesting to note that there are considerable differences in the reported kidney allograft half-life between continents. In the USA, reported 10-year renal allograft survival rates amount to about 40 percent, while in France, 10-year survival rates of 61 percent were reported (53). Also the allograft survival in the Netherlands is considerably longer than that in the USA (M. Mallat and Y. Sijpkens, personal communication). It would be interesting to compare on an international level the risk factors of chronic allograft dysfunction in order to explain this divergence of long-term survival rates.

As outlined previously, proteinuria is a risk factor for progression of renal disease in both native and transplanted kidneys. Because proteinuria, in contrast to risk factors such as donor age, may be to some extent modifiable, it would be interesting to study the role of proteinuria in the progression of allograft dysfunction. Post-transplant proteinuria may be caused by certain immunosuppressive drugs (54), and also in the setting of post transplant glomerulopathy. The latter is a specific pathophysiological process that is thought to mainly involve humoral rejection (see chapter 1), primarily resulting in endothelial damage (55). The proteinuria that is seen in most cases of chronic allograft dysfunction is not necessarily caused by immunological mechanisms. Instead, more general mechanisms that are also involved in native glomerular disease and ageing may be at play. The glomerular damage seen with increased age is thought to be a podocyte disease: as a result of several stress factors, podocytes are lost and cannot be replaced. This is suggested to be the starting point of a final common pathway to glomerulosclerosis, and via pathways lined out in the introductory chapter on nephron loss (56). Prevention of age-related podocyte damage may in part be accomplished by reducing hypertension, preferably with angiotensin II blockers that have a kidney- or podocyte-protective effect. Similar to the 'accelerated senescence' concept proposed by Halloran to explain pathophysiologic processes in transplanted kidneys, glomerular aging may also progress faster in transplantation. Attempts to limit other podocyte-damaging

factors, for example through blood pressure control using angiotensin II blockers, may thus prove beneficial in native and transplanted kidneys alike.

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8 Nederlandse samenvatting

Door het afvoeren van afvalstoffen houdt de nier de samenstelling van het bloed, en daarmee het vocht dat alle cellen van het lichaam omspoelt, constant. Dit proces vindt plaats in de ongeveer een miljoen functionele eenheden van de nier, de nefronen. Het eerste onderdeel van een nefron is de glomerulus, een kluwen van haarvaatjes. De wand van deze haarvaatjes functioneert voor het bloed dat er doorheen stroomt als een filter: water, zouten en kleine moleculen passeren de bloedvatwand probleemloos, terwijl cellen en eiwitten in het bloedvat achterblijven. Per dag wordt er door alle nefronen gezamenlijk ongeveer 180 liter ultrafiltraat of pre-urine geproduceerd. Dit ultrafiltraat stroomt vervolgens door naar het tweede onderdeel van het nefron, de tubulus. In de tubulus wordt het grootste gedeelte van het ultrafiltraat heropgenomen. Door variatie in de mate van opname en door actieve uitscheiding van stoffen reguleert de tubulus de precieze samenstelling van de urine, en daarmee ook die van het bloed.

Een belangrijk probleem dat bij veel nierziekten optreedt is proteïnurie, het verlies van eiwit in de urine. Waar normaal gesproken het filter van de nier zeer slecht doorgankelijk is voor eiwitten, is bij patiënten met proteïnurie deze selectiviteit verminderd aanwezig en worden er belangrijke hoeveelheden eiwit in de urine uitgescheiden. Het eerste deel van dit proefschrift gaat over de oorzaak en kenmerken van proteïnurie, met een speciale focus op de rol van een van de celtypes in de glomerulus, namelijk de podocyt.

Het optreden van proteïnurie is gecorreleerd aan het verlies van nierfunctie. Vergaand verlies van nierfunctie maakt nierfunctievervangende therapie of niertransplantatie noodzakelijk. De levensduur van een niertransplantaat is echter eveneens beperkt. Een belangrijk aspect in deze beperking van levensduur is dat zowel activiteit van het immuunsysteem als immuunsuppressie schadelijk kunnen zijn voor de nier, zonder dat deze eenvoudig van elkaar zijn te onderscheiden. Het tweede deel van het proefschrift is erop gericht met behulp van moleculaire technieken een onderscheid te maken tussen verslechtering van nierfunctie ten gevolge van een tekort of juist een overdosering van immuunsuppressie.

Proteïnurie

Om de vraag te beantwoorden hoe het komt dat het glomerulaire filter niet goed functioneert, dat wil zeggen, hoe proteïnurie ontstaat, volgt hier eerst een beschrijving van de normale bouw en werking van de glomerulus. Het glomerulaire filter bestaat uit drie onderdelen. Van binnen naar buiten bezien allereerst het endotheel, vervolgens de glomerulaire basaalmembraan en tenslotte de glomerulaire epitheelcel of podocyt. Glomerulair endotheel is voorzien van fenestraties en is in zijn geheel bedekt met een glycocalyx, een negatief geladen oppervlak van proteoglycanen. Deze glycocalyx draagt mogelijk bij aan de ladingsselectiviteit van glomerulaire filtratie.

De glomerulaire basaalmembraan ondersteunt de cellulaire componenten van het filter, maar heeft zelf waarschijnlijk slechts een beperkte rol in het filtratieproces. De podocyt tenslotte is de epitheelcel van de glomerulus en vormt de buitenbekleding van de capillairen.

Het is gebleken dat beschadiging van elk van de onderdelen van het filter afzonderlijk al leidt tot een slechtere filtratiefunctie en proteïnurie. In de afgelopen jaren heeft vooral de podocyt sterk in de belangstelling gestaan. De eerste twee studies beschreven in dit proefschrift concentreren zich op de rol van de podocyt in proteïnurie.

De podocyt en proteïnurie

De podocyt is een sterk gedifferentieerde cel met een opvallende architectuur: het cellichaam bevindt zich in de urineruimte en is met de capillairwand verbonden via talloze uitlopers, 'voet-pootjes' of pedikels genaamd (foot processes). Pedikels van aangrenzende podocyten zijn in elkaar vervlochten als de vingers van een hand. Tussen de pedikels is een membraantje uitges-pannen, het filtratiespleetdiafragma. Al sinds de jaren '50 is bekend dat de podocyt opvallende veranderingen ondergaat als er proteïnurie bestaat: de fraai vormgegeven pedikels platten af en de podocyt verandert van een complex vertakte cel in een simpele platte epitheelcel. Onduidelijk was hoe dit proces van obliteratie van epitheelcelpedikels zich verhoudt tot het ontstaan van proteïnurie.

In de afgelopen tien jaar is de moleculaire biologie van de podocyt meer opgehelderd. De meest baanbrekende ontdekking was de beschrijving van nefrine, het eiwit dat een belangrijk bestand-deel vormt van het filtratiespleetdiafragma. Patiënten die geen nefrine maken (zoals patiënten met het congenitaal nefrotisch syndroom van het Finse type) hebben al voor de geboorte ern-stige proteïnurie en moeten veelal in hun eerste levensjaar een niertransplantatie ondergaan om in leven te blijven. Sinds de ontdekking van nefrine is een hele reeks van podocyt-specifieke eiwitten ontdekt, die van groot belang zijn voor het correct functioneren van de cel (zie figuur 3 van hoofdstuk 1). Het ontbreken van deze eiwitten, vaak in het kader van zeldzame erfelijke aandoeningen, is gerelateerd aan proteïnurie, nierfunctie verlies en uiteindelijk nierfalen.

Terwijl de zeldzame erfelijke aandoeningen die gepaard gaan met proteïnurie een grote rol heb-ben gespeeld bij de opheldering van de podocyt biologie, komt proteïnurie het meest voor in het kader van op latere leeftijd verworven aandoeningen. De vraag die wij ons in dit proefschrift hebben gesteld is of deze podocyt-specifieke eiwitten, evenals in aangeboren nierziekten, een belangrijke rol spelen in verworven vormen van proteïnurie.

In de studies beschreven in hoofdstuk 2 hebben wij geanalyseerd hoe een aantal podocyt-spec-ifieke eiwitten tot expressie komen in verworven nierziekten. Hiervoor hebben wij biopten van patiënten met nierziekten bestudeerd, waarbij wij ons de vraag stelden hoe tijdens proteïnurie de podocyt verandert op morfologisch niveau en op eiwit- en mRNA niveau. Het bleek dat de

expressie van podocyt-specifieke eiwitten in de glomerulus was verminderd, terwijl de expressie van mRNA coderend voor diezelfde eiwitten veelal was toegenomen. Beide processen waren gerelateerd aan de mate van obliteratie van epitheelcelpedikels. Deze combinatie van veranderingen wijst mogelijk op een secundaire rol van de bestudeerde podocyt-specifieke eiwitten in het ontstaan van proteïnurie in verworven nierziekten.

Het gebruik van menselijk materiaal brengt als belangrijke beperking met zich mee dat biopten pas genomen worden op het moment dat een nierziekte zich al heeft gemanifesteerd. Daardoor kan het verloop van podocyt-veranderingen in de tijd niet gevolgd worden. Om beter inzicht te krijgen in dit tijdsverloop hebben wij gebruik gemaakt van een diermodel, namelijk de Dahl salt-sensitive (Dahl SS) rat. Ratten van deze stam ontwikkelen vanaf ongeveer vier tot zes weken na geboorte spontaan proteïnurie, wat uiteindelijk uitmondt in het verlies van nierfunctie. In hoofdstuk 3 zijn de resultaten beschreven van een analyse van de regulatie van podocyt-specifieke eiwitten gedurende de ontwikkeling van proteïnurie in de Dahl SS rat. De resultaten van deze studie kwamen overeen met de bevindingen uit hoofdstuk 2: ondanks het bestaan van proteïnurie was er geen vroege verandering in expressie van de meeste podocyt-specifieke eiwitten op mRNA en eiwit niveau. Verder bleek ook dat de ontwikkeling van obliteratie van epitheelcelpedikels niet synchroon loopt met de ontwikkeling van proteïnurie: het eerste moment waarop wij significante obliteratie waarnamen was tien weken na de geboorte, terwijl wij proteïnurie al na vier tot zes weken konden aantonen.

De enige uitzondering op het algemene patroon van onveranderde eiwitexpressie tijdens de ontwikkeling van proteïnurie was de expressie van het glycoproteine podoplanine. Dit eiwit bleek tegelijkertijd met het ontstaan van proteïnurie in segmenten van de glomerulus sterk verminderd tot expressie te komen. Deze veranderingen waren progressief: op latere leeftijd was de afname van podoplanine eiwitexpressie meer uitgesproken. Wij konden de veranderingen in podoplanine eiwitexpressie niet verklaren door een verandering in mRNA expressie, noch door een genetisch verschil tussen de stammen.

Uit andere studies naar de rol van podoplanine is bekend dat expressie van dit eiwit leidt tot vorming van zogenaamde filopodia, celuitlopers die een zekere biologische gelijkenis vertonen met glomerulaire epitheelcelpedikels. De moleculaire mechanismen die bij de vorming van filopodia een rol spelen zijn gedeeltelijk opgehelderd. Wij hebben geconstateerd dat de processen die betrokken zijn bij vorming van filopodia juist tegenovergesteld zijn aan de processen die plaatsvinden in de glomerulus van de proteïnurische Dahl SS rat. Dit wijst er mogelijk op dat de podocyt via vermindering van podoplanine-expressie beschikt over een mechanisme om obliteratie van epitheelcelpedikels te bewerkstelligen.

Glomerulaire genexpressie in proteïnurie

In hoofdstuk 4 wordt de nadruk verlegd van het onderzoeken van bekende genen en eiwitten naar het vinden van nieuwe genen die betrokken zijn bij het ontstaan van proteïnurie. Voor deze studie hebben wij gebruik gemaakt van een microarray techniek. Met deze techniek kan in een experiment de expressie van zo'n 30.000 genen in kaart worden gebracht. Wij hebben deze techniek toegepast op dezelfde rattenstammen als die bestudeerd werden voor de experimenten beschreven in hoofdstuk 3. Voor de analyse gebruikten wij geïsoleerde glomeruli verkregen op tijdstippen juist voor en juist na de ontwikkeling van proteïnurie.

Uit de microarray analyses bleek, dat zo'n 500 genen verschillend tot expressie komen tussen de beide stammen. Ongeveer een kwart van deze genen ligt op chromosoom regio's die al eerder in verband waren gebracht met de ontwikkeling van proteïnurie in de Dahl SS rat. Een aantal van de genen die verschillend tot expressie komen hebben wij verder onderzocht. Naast dat wij ons gericht hebben op individuele genen is het ook mogelijk groepen van genen, waarvan bekend is dat die een bepaalde samenhang hebben, te analyseren. Uit pathway analyses kwam naar voren dat al vroeg in de ontwikkeling van proteïnurie genen betrokken bij het cytoskelet verschillend tot expressie komen tussen beide stammen. Hoewel dus pas relatief laat na de ontwikkeling van proteïnurie herkenbare ultrastructurele veranderingen optreden in de podocyt, is al vroeg in de ontwikkeling van proteïnurie de moleculaire aanzet tot verandering van het cytoskelet waarneembaar.

Uit de literatuur is bekend dat in tubulus epitheelcellen een profibrotische en proinflammatoire reactie opgewekt wordt wanneer deze cellen worden blootgesteld aan hoge concentraties eiwit. Wij vroegen ons af of een dergelijke reactie ook in de glomerulus waarneembaar is. Uit de literatuur hebben wij een groep genen samengesteld die in tubulusepitheel onder invloed van proteïnurie veranderd tot expressie komen. Gebruik makend van de zogenaamde 'global test' konden wij aantonen dat het expressiepatroon van deze groep genen ook in de glomerulus op een zelfde manier verandert. Dit wijst er mogelijk op dat de verhoogde passage van eiwitten door de glomerulus schade toebrengt aan de glomerulus en een eerste stap is in de verdere teloorgang van de glomerulus.

Proteïnurie is gecorreleerd met achteruitgang van nierfunctie en kan uiteindelijk leiden tot een verlies van nierfunctie die dialyse of niertransplantatie noodzakelijk maakt. De chronische problemen bij niertransplantatie vormden het tweede onderdeel van dit proefschrift.

Transplantatie

In de nu ongeveer 40-jarige geschiedenis van niertransplantatie is de levensduur van getransplanteerde nieren sterk verbeterd. Deze vooruitgang is met name toe te schrijven aan verbeterde immuunsuppressie waardoor afstoting in de eerste fase na transplantatie beter te behandelen is. Daarmee zijn de problemen rond niertransplantatie in de loop der jaren verschoven van de acute naar de chronische fase: op langere termijn gaat de nierfunctie van een niertransplantaat onvermijdelijk achteruit. De gemiddelde overleving van getransplanteerde nieren ligt in Nederland rond twaalf jaar, in de Verenigde Staten rond acht jaar. Maar waar er nog altijd verbetering wordt behaald in de transplantaatoverleving na een jaar, blijft de lange termijn overleving al jarenlang ongeveer gelijk.

De achteruitgang van getransplanteerde nieren op lange termijn wordt geweten aan meerdere factoren: beschadigingen opgelopen rondom de operatie (ischemie, reperfusieschade), laaggradige maar steeds aanwezige activiteit van het immuunsysteem en toxische effecten van immuunsuppressieve medicatie. Al deze mechanismen kunnen, vaak gelijktijdig, bijdragen aan verlies van functioneel nierweefsel, en daarmee verlies van nierfunctie. Opmerkelijk is dat deze oorzaken, hoe verschillend ze ook zijn, leiden tot een ziektebeeld met vergelijkbare klinische en histologische kenmerken: de nierfunctie, meestal gemeten als kreatinineklaring, loopt langzaam terug en in biopten van getransplanteerde nieren wordt een beeld van tubulusatrofie en ophoping van extracellulaire matrix gezien – een proces van verbindweefseling van nefronen.

Moleculaire analyse van lange termijn nierfunctieverlies

De doelstelling van de studies beschreven in hoofdstuk 5 en 6 was om met moleculaire analyses beter onderscheid te kunnen maken tussen de verschillende oorzaken die ten grondslag liggen aan lange termijn dysfunctie van niertransplantaten. Daarbij hebben wij gebruik gemaakt van twee goed gedefinieerde groepen van patiënten met chronische transplantaat dysfunctie. Een groep bestond uit patiënten met bewezen nierschade ten gevolge van toxische effecten van immuunsuppressieve medicatie (de cyclosporine toxiciteit groep), de andere groep bestond uit patiënten met nierschade ten gevolge van een chronische afweerreactie (de chronische rejectie groep).

Bij de selectie van markers hebben wij ons gericht op de extracellulaire matrix. De extracellulaire matrix verandert voortdurend van samenstelling doordat haar componenten steeds worden aangemaakt en afgebroken. Uit vorige studies is gebleken dat het kwantificeren van de aanmaak van de extracellulaire matrix een voorspellende waarde kan hebben voor de mate van fibrosering. In hoofdstuk 5 hebben wij onderzocht of de mate van mRNA expressie van extracellulaire matrix

genen kan helpen onderscheid te maken tussen patiënten met chronische rejectie en die met cyclosporine toxiciteit. Wij hebben gevonden dat de mRNA expressie van laminine β2 en TGF-β verhoogd is in nierweefsel van patiënten met cyclosporine toxiciteit ten opzichte van dat van patiënten met chronische rejectie. De verschillen in mRNA expressie maken een onderscheid tussen beide ziekteprocessen mogelijk met een sensitiviteit en specificiteit van meer dan 85 procent.

In hoofdstuk 6 hebben wij met behulp van immunohistochemie en digitale beeldanalyse de moleculaire samenstelling van de renale extracellulaire matrix bestudeerd. Wij hebben gevonden dat een aantal componenten van de extracellulaire matrix, te weten collagenen III en IV, in gelijke mate voorkomen in chronische rejectie en cyclosporine toxiciteit. De expressie van collageen I was echter hoger in chronische rejectie ten opzichte van cyclosporine toxiciteit, in een mate die een redelijk betrouwbaar onderscheid tussen beide ziekteprocessen mogelijk maakte.

Naast dat deze studies behulpzaam zouden kunnen zijn in het onderscheiden van ziekteprocessen, kunnen ze ook gebruikt worden om meer inzicht te krijgen in de ontstaanswijze van deze aandoeningen. Chronische rejectie gaat gepaard met een sterkere ophoping van collageen I in de extracellulaire matrix. Tegelijkertijd was de mRNA expressie van collageen I niet verhoogd in biopten van patiënten met chronische rejectie. Dat zou erop kunnen wijzen, dat de afbraak van deze collageen keten verminderd plaatsvindt. Laminine β2 mRNA komt verhoogd tot expressie bij cyclosporine toxiciteit. Aangezien laminine in de vaatwand voorkomt zou deze verhoogde expressie kunnen passen bij een vaatwandbeschadiging ten gevolgen van cyclosporine, hetgeen in overeenstemming is met het bestaan van arteriolaire hyalinose als histologisch kenmerk van cyclosporine toxiciteit. Niettemin moet worden opgemerkt dat dergelijke hypothesen berusten op correlaties, niet op een direct bewijs.

Een belangrijke beperking van de studies in hoofdstuk 5 en 6 wordt gevormd door de manier waarop de patiëntengroepen zijn geselecteerd. Zoals eerder aangegeven kan verlies van nierfunctie na transplantatie veroorzaakt worden door een groot aantal mechanismen die meestal gelijktijdig optreden. Uit het spectrum van patiënten met een chronisch verlies van nierfunctie na transplantatie zijn alleen de uitersten – de groep patiënten met chronische afstotingen en die met juist een toxisch overschot aan immuunsuppressie – geselecteerd. De patiënten van wie de nierfunctie is achteruitgegaan door een combinatie van deze en andere factoren zijn in de analyses van hoofdstuk 5 en 6 buiten beschouwing gelaten. Om te zien of de gevonden moleculaire markers ook daadwerkelijk toepasbaar zijn in de patiënten die zich niet in de uitersten van het spectrum bevinden zullen de markers eerst getest moeten worden in een grotere en ongeselecteerde patiëntenpopulatie.

Toekomst van onderzoek in chronische transplantaat dysfunctie

In de hierboven beschreven studies hebben wij gezocht naar markers die kunnen bijdragen in het onderscheiden van specifieke ziekteprocessen. Om dit onderscheid te kunnen vertalen in een betere manier van behandeling is het noodzakelijk dat deze markers relatief vroeg in het ziekteproces worden geanalyseerd. Daarin schuilt mogelijk een toekomstig probleem: omdat de ontwikkelingen in immuunsuppressieve medicatie nog altijd doorgaan wisselt de medicatie waaraan patiënten in de eerste fase na transplantatie zijn blootgesteld met enige regelmaat. Patiënten die nu in de chronische fase na niertransplantatie zijn hebben daarom vaak een andere vorm van immuunsuppressie gehad dan de huidige generatie patiënten met een niertransplantaat. Het is daarom de vraag of de markers gevonden in de huidige chronische patiënten populatie toepasbaar zijn bij toekomstige patiënten.

Verder is het van belang te constateren dat de processen die leiden tot achteruitgang van nierfunctie na transplantatie niet allemaal van immunologische aard zijn. Niet-immunologische factoren zijn evenzeer van belang en bijvoorbeeld donorleeftijd verklaart al zo'n 30 procent van de variatie in het beloop na transplantatie. De mechanismen waardoor nierfunctieverlies optreedt in niertransplantaten zijn dan ook niet wezenlijk verschillend van die mechanismen waardoor in 'natieve' nieren de nierfunctie verloren gaat. Een centraal proces is de zogenaamde hyperfiltratie: zodra, onverschillig door welke reden, een kritisch aantal nefronen verloren is gegaan, neemt de belasting van de overgebleven nefronen zodanig toe (hyperfiltratie), dat dit op zichzelf schadelijk is en leidt tot een verder verlies van nefronen. Zo vormt een specifiek ziekteproces het beginpunt van een stereotiep proces van verder nierfunctieverlies dat is losgekoppeld van de oorspronkelijke oorzaak.

Een van de factoren die samenhangen met progressie van nierziekten is proteïnurie. Anders dan andere risico- of progressiefactoren (zoals bijvoorbeeld leeftijd of, in transplantatie, HLA-mismatching) is proteïnurie beïnvloedbaar. Een betere kennis van de pathofysiologische mechanismen van proteïnurie kan dus betekenis hebben voor de behandeling van nierziekten in zowel natieve als getransplanteerde nieren.

Appendices

Color Figures

Curriculum vitae

Publications

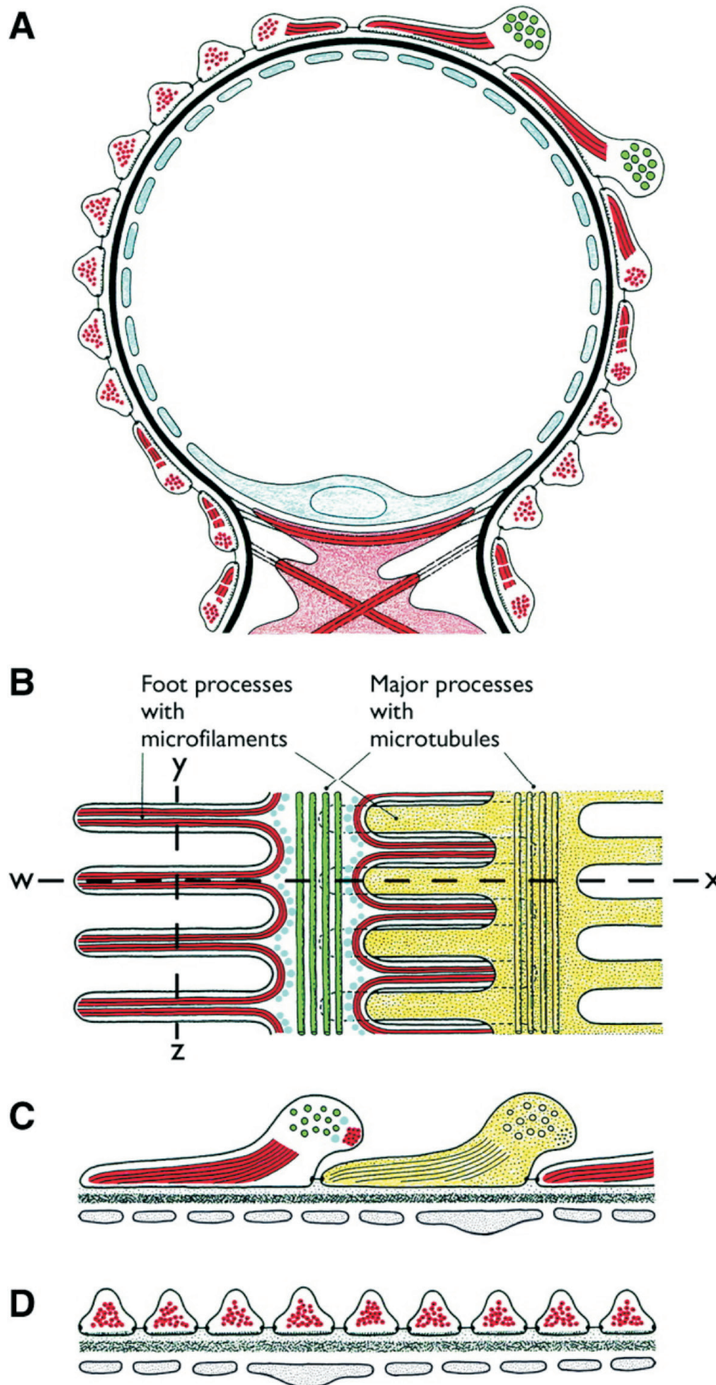


Figure 2. Organization of the podocyte cytoskeleton. A cross section through a capillary loop shows that the capillary is surrounded by podocyte foot processes. At the base of the capillary, contractile mesangial cell filaments are attached (a). A schematic view from above shows that the foot processes sprout perpendicular to the major processes. The cytoskeleton of the major processes is composed of microtubuli, to which the actin-based cytoskeleton of the foot processes is attached (b). In (c) and (d) the lateral view capillary wall is depicted, corresponding to the w-x and y-z line in (b), respectively. Adapted from (14), used with permission from The American Physiological Society and the author.

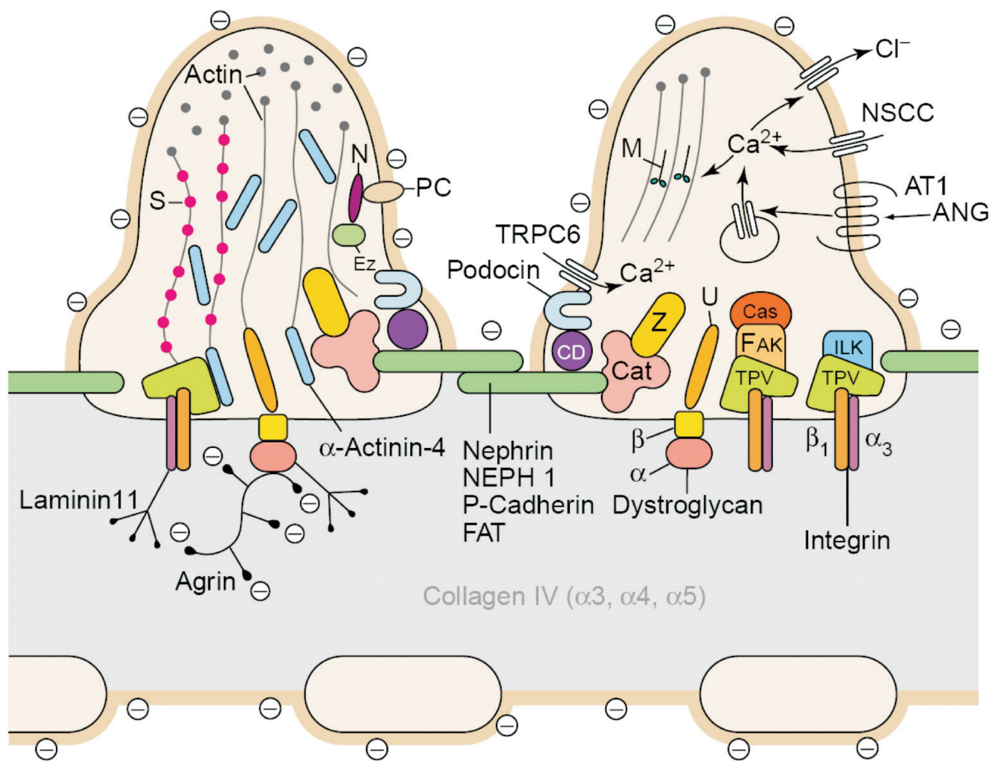


Figure 3. Molecular organization of the podocyte foot process. Two podocyte foot processes with the bridging slit diaphragm are depicted, resting on the glomerular basement membrane (GBM). The central part of the foot process is the actin cytoskeleton (indicated in the left foot process with grey dots), which is reinforced by synaptopodin and α-actinin-4, and has contractile properties as a result of myosin fibers (M). Connected to the cytoskeleton are several molecules that reside in the negatively charged apical membrane, including podocalyxin (PC) via NHERF2 (N) and ezrin (Ez), and podoplanin (not depicted). At the basal membrane, the actin cytoskeleton is connected to the GBM via dystroglycan (linking utrophin (U) to agrin), and the integrin-complex (integrin, talin-paxillin-vinculin (TPV), integrin linked kinase (ILK), focal adhesion kinase (FAK)). The slit diaphragm is composed of nephrin, NEPH-proteins, P-cadherin, and FAT, and the slit diaphragm domain contains several molecules that play a role in the anchoring and signaling of the slit diaphragm (TRPC6, podocin, CD2AP (CD), β-catenin (cat), and ZO-1 (Z)). The podocyte has several receptors, including the angiotensin II receptor AT1. See text for further details. Adapted from (505) and (506), with permission from Elsevier, Lippincott Williams & Wilkins, and the author.

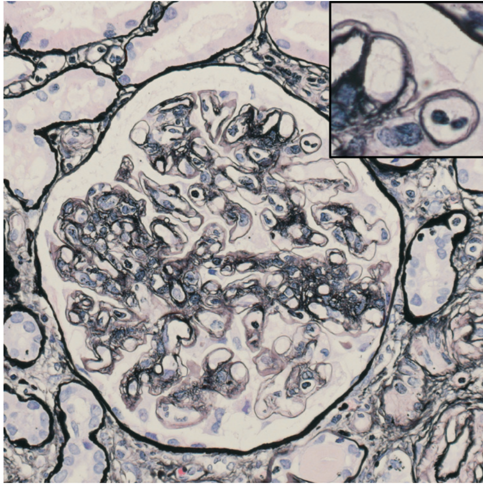


Figure 4. Transplant glomerulopathy. Transplant glomerulopathy is seen in chronic rejection. This glomerular lesion is characterized by a duplication of the glomerular basement membrane (inset).

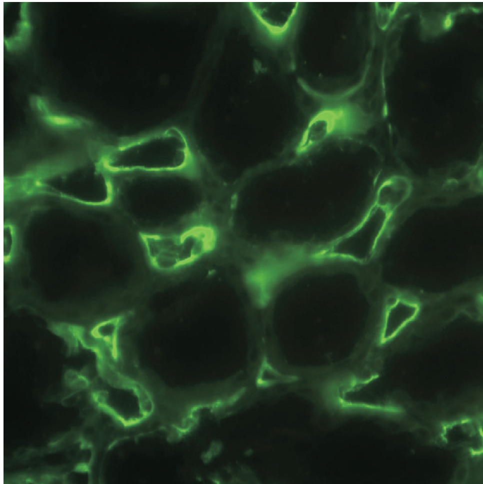


Figure 5. C4d deposition. In chronic humoral rejection, the complement split product C4d remains covalently bound to endothelial cells. This picture shows widespread deposition of C4d in peritubular capillaries.

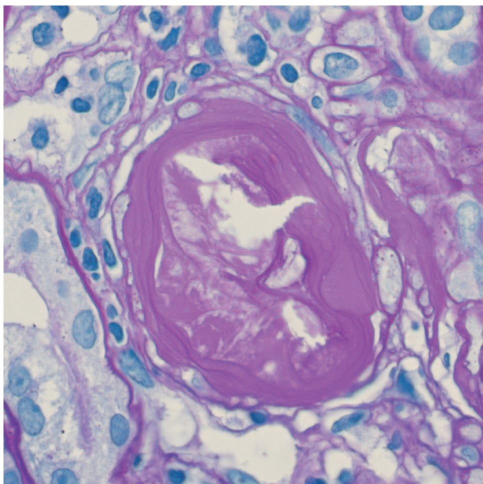


Figure 6. Arteriolar hyalinosis. Nodular depositions of hyaline material are seen in the media of arterioles, indicating the presence of chronic CNI toxicity.

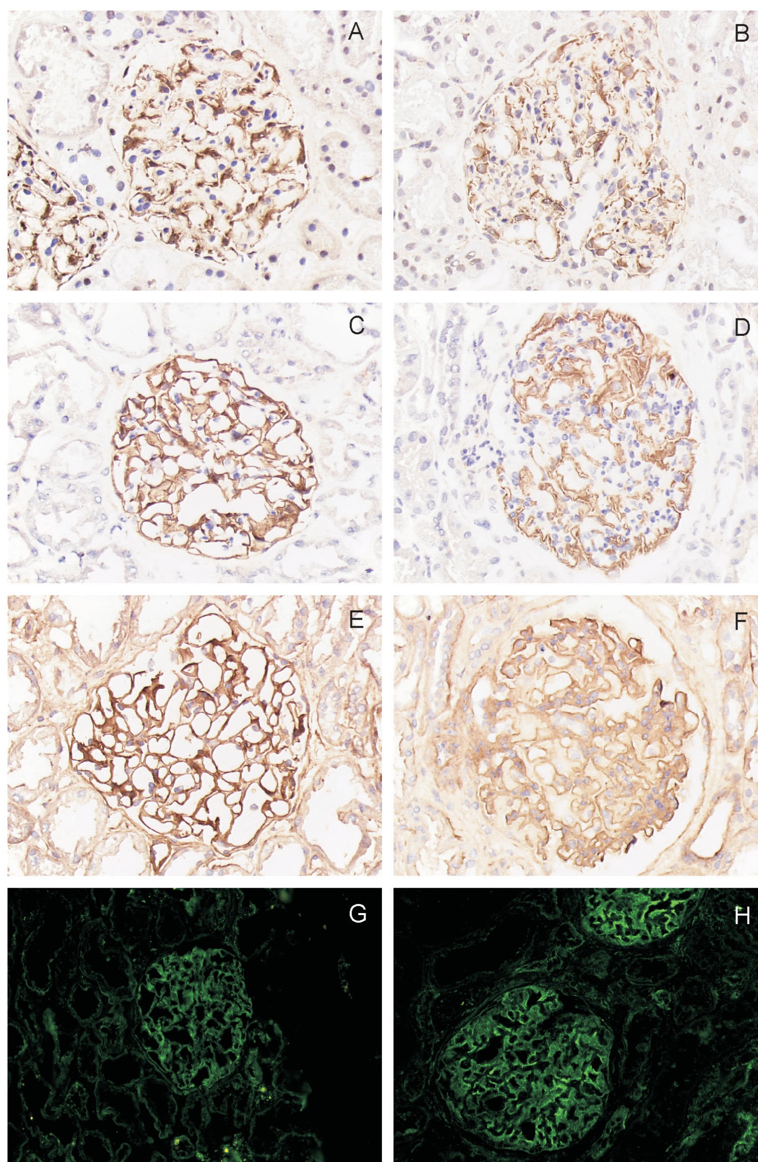


Figure 2. Staining pattern of nephrin, podocin, podocalyxin, and CD2-associated protein (CD2AP) in normal and diseased human kidney sections. Nephrin (A), podocin (B), and podocalyxin (C) show a podocyte-like staining pattern in normal glomeruli as visualized by an immunohistochemical diaminobenzidine staining. The staining pattern of nephrin is more dispersed than that of podocin and podocalyxin, which show a fine glomerular basement membrane (GBM)-like line along the capillary loops of the glomerulus. CD2AP, visualized with immunofluorescence, shows a GBM-like staining pattern (D). In diseased situations, the staining for nephrin (E; minimal change disease), podocin (F; focal segmental glomerulosclerosis [FSGS]), and podocalyxin (G; FSGS) is less intense, and nephrin and podocin stainings show a more granular staining pattern. CD2AP staining shows no clear differences between control (D) and diseased tissue (H; diabetic nephropathy). Magnification x 400.

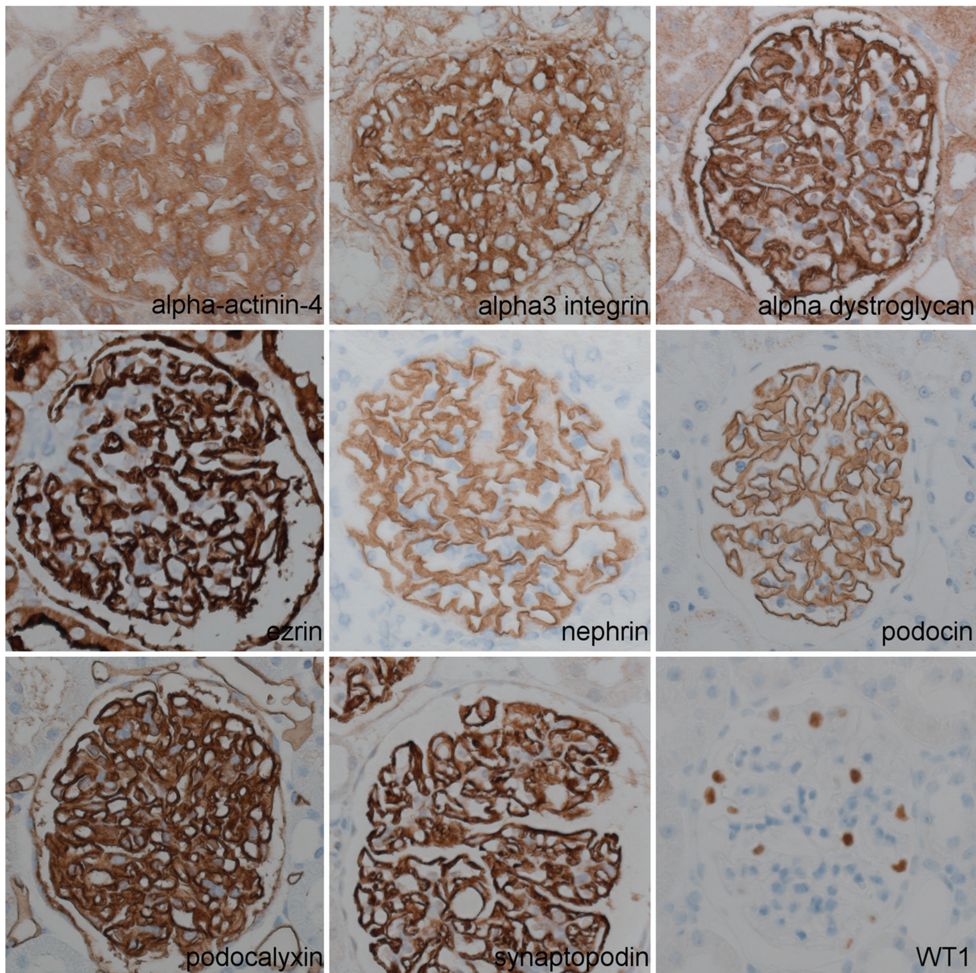


Figure 4. Immunohistochemistry of podocyte proteins. Despite marked proteinuria, 10-week-old Dahl SS rats showed normal expression of α-actinin-4, α3 integrin, α-dystroglycan, ezrin, nephrin, podocin, podocalyxin, synaptopodin, and WT1. Original magnification: x 400.

Figure 5. Podoplanin protein expression in Dahl SS glomeruli was progressively lost in a focal and segmental fashion. Loss of podoplanin protein expression was first seen in 4-week-old Dahl SS rats and increased thereafter. In contrast, podoplanin protein expression remained normal in SHR rats throughout the time course of the study. The upper row of images in A shows podoplanin staining in Dahl SS rats at the indicated time points and in a 10-week-old SHR rat. The lower row of images in A shows desmin staining in Dahl SS rats at the indicated time points and in a 10-week-old SHR rat. Starting at 6 weeks, desmin expression was visible in the extramesangial areas of Dahl SS glomeruli. Expression level increased with age. No change in desmin expression was observed in aging SHR rats. Sequential sections of the kidney of a 10-week-old Dahl SS rat stained with anti-rat podoplanin antibodies, anti-podocin antibodies, and with PAS show that loss of podoplanin is not related to morphological alterations observed by light microscopy or to alterations in podocin expression (B). Nephrin expression was diminished sporadically in segmental parts of glomeruli, but only in 10-week-old Dahl SS rats (C). Original magnification: x 400.

A

Dahl SS

SHR

2 wks

4 wks

6 wks

8 wks

10 wks

10 wks

podoplanin

desmin

B

podoplanin

podocin

PAS

C

nephrin

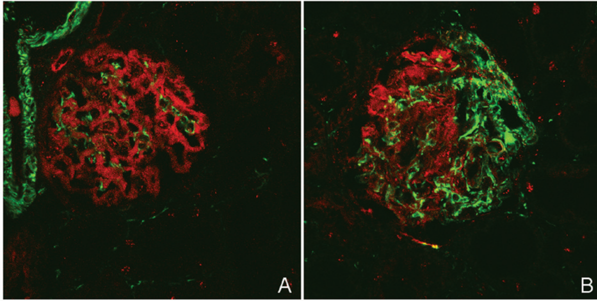


Figure 7. Podoplanin (red) and desmin (green) were costained in kidney sections from 8-week-old Dahl SS rats. Normal glomeruli show desmin expression in renal blood vessels and glomerular mesangium and podoplanin expression in podocytes. Desmin and podoplanin did not colocalize (A). In glomeruli that showed segmental loss of podoplanin expression, increased desmin expression was observed in the podoplanin-negative areas (B). Original magnification: x 630.

Chapter 4

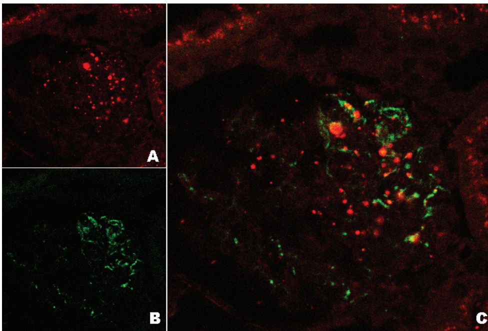
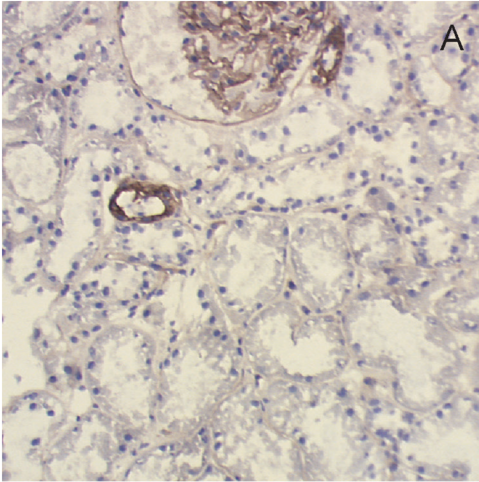
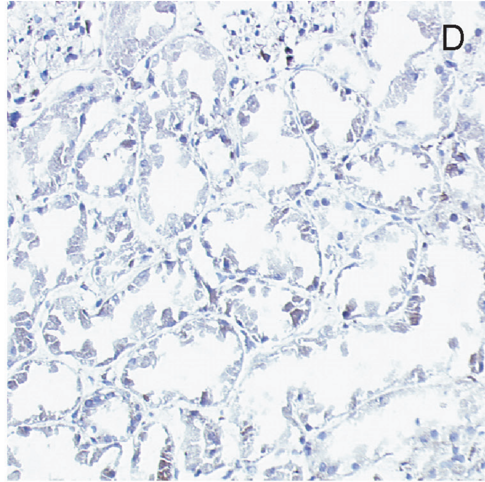


Figure 4. Co-immunostaining of desmin (green) and albumin (red) in a section of an 8-week-old proteinuric Dahl SS rat. Increased desmin expression is seen mostly in areas with extensive albumin accumulation. Desmin (A), albumin (B), merge (C).

Chapter 5

Figure 3. Immunohistochemical stainings for laminin β2 (A to C) and transforming growth factor-β (TGF-β) (D to F). In control tissue laminin β2 staining was observed in the glomerular basement membrane and in cortical vessels (A). In chronic rejection (B) and cyclosporine A (CsA) toxicity (C), expression of laminin β2 was observed in the tubular basement membrane. TGF-β staining was sporadically observed in glomeruli and tubuli of controls (D). In chronic rejection (E) and CsA toxicity (F), some tubuli showed a very intense staining for TGF-β.


A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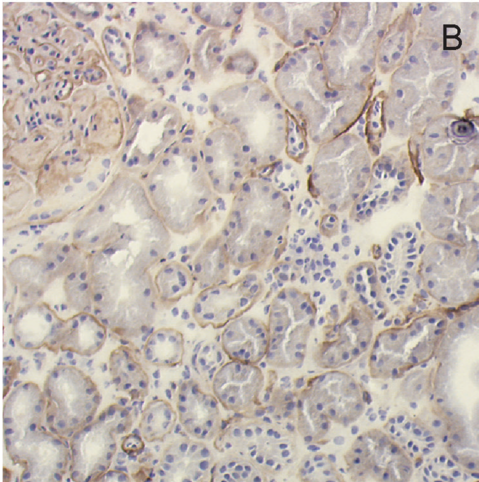
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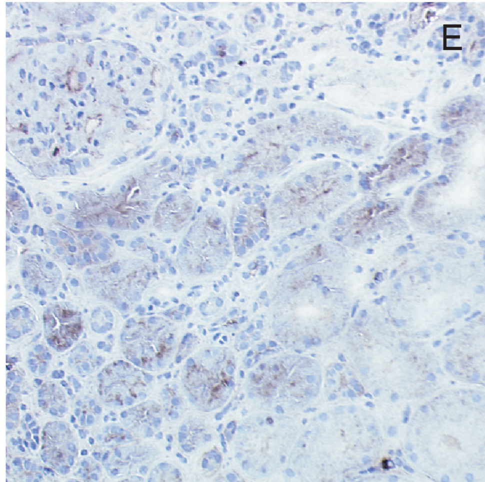
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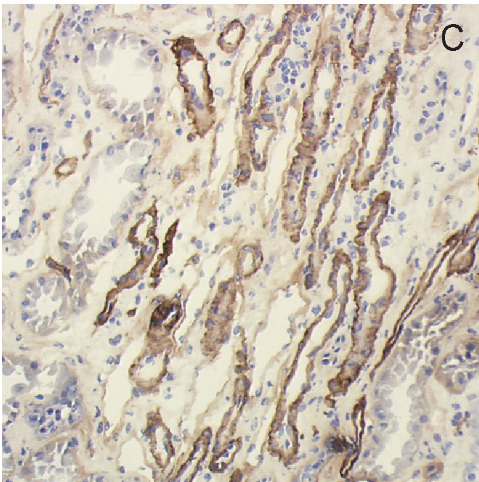
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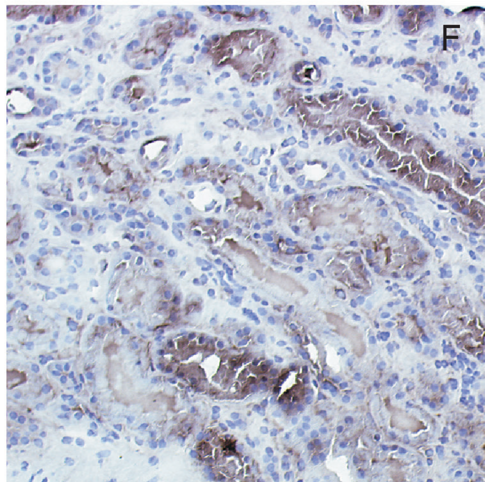
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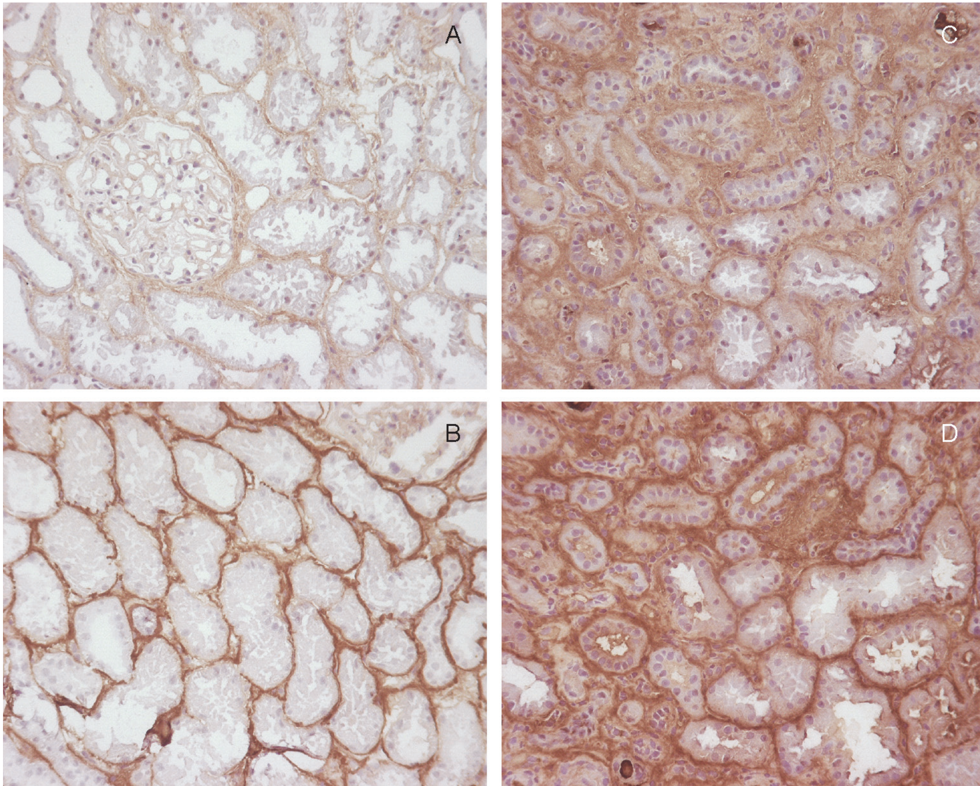

Figure 2. Immunoperoxidase staining for cortical collagen I (A and C) and III (B and D). Representative images of a normal control sample (left) and a kidney allograft with CR (right) are shown.

Curriculum vitae

De auteur van dit proefschrift werd in 1979 geboren in 's Hertogenbosch. Hij doorliep het Voortgezet Wetenschappelijk Onderwijs aan het Greijdanus college te Zwolle, waar hij in 1997 eindexamen deed. Datzelfde jaar ving hij de studie Geneeskunde aan, een jaar later behaalde hij de propedeuse (cum laude), in 2001 voltooide hij het doctoraal examen en in 2004 behaalde hij het artsexamen. In 2005 legde hij het doctoraal examen Biomedische Wetenschappen af. Voortbouwend op onderzoek begonnen tijdens zijn studie werd van 2004 tot 2007 het onderzoek verricht waarvan de resultaten zijn beschreven in dit proefschrift. Dit vond plaats op de afdeling Pathologie van het Leids Universitair Medisch Centrum (hoofd prof. dr. G.J. Fleuren) onder leiding van prof. dr. J.A. Bruijn, dr. E. de Heer en dr. M. Eikmans, in samenwerking met de afdeling Pharmakologie und Toxikologie van de Freie Universität Berlin onder leiding van prof. dr. Reinhold Kreutz. In 2007 begon de auteur met de specialisatie Tropengeneeskunde, achtereenvolgens in het Groene Hart Ziekenhuis te Gouda (opleider dr. H. van Huisseling) en in het Havenziekenhuis te Rotterdam (opleider dr. K.H.A. van Eeghem).

Hij is getrouwd met Gerdien van der Horst, samen hebben zij een zoon, Loek.

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