Changes in Glomerular Perm-Selectivity Induced by Angiotensin II Imply Podocyte Dysfunction and Slit Diaphragm Protein Rearrangement

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Molecular mechanisms governing the loss of glomerular membrane perm selectivity during progression of proteinuric kidney diseases are so far poorly defined. Discovery of the proteins of the podocyte slit diaphragm, including the nephrin-CD2AP-podocin complex, has represented a major breakthrough in understanding the crucial role of the glomerular epithelial layer in the pathogenesis of proteinuria in human congenital disorders. A number of studies have tried to address the role of nephrin in acquired proteinuric disorders with conflicting results. In human diabetic nephropathy a defect of nephrin gene and protein expression has been consistently reported, which translates in profound changes of filtration slit ultrastructural architecture. The exclusive effect of angiotensin II inhibitors of restoring deficient nephrin expression in proteinuric diseases underlines a close interaction between angiotensin II and podocyte proteins and indicates a fresh way to look at the renoprotective properties of these molecules.

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END-STAGE RENAL FAILURE is increasing worldwide at an alarming rate, fueled by steady rises in prevalence of underlying conditions such as diabetic nephropathy, primary or secondary glomerulonephritis, HIV nephropathy, and chronic allograft rejection. The consequent human and financial burden is becoming a staggering challenge to public health care systems as a result of the prohibitive costs of renal replacement therapy that could become unaffordable even in developed countries. Molecular mechanisms that lead to perturbation or loss of perm selectivity of the glomerular membrane during progression of proteinuric kidney diseases are so far poorly defined. There is increasing evidence of harmful secondary effects of the excessively filtered proteins across the glomerular capillary barrier on the kidney proximal tubule. The relationship between proteinuria and the rate of renal function decline in most glomerular disorders has been widely described in a number of clinical and experimental studies. Models of heavy proteinuria in rats are characterized by development of glomerulosclerosis, interstitial inflammation, and progressive tubulointerstitial fibrosis. Overreabsorption of proteins by the proximal tubules appears to be toxic both for the quantity and the type of proteins that activate inflammatory and fibrogenic factors leading to scarring. Gene expression profiling experiments allowed to identify more than 1000 genes that are upregulated in renal proximal tubules from mice with protein-overload proteinuria. Furthermore, in vitro studies showed that proximal tubular cell cultures exposed to protein overload produced more monocyte chemotactic protein-1 endothelin-1, RANTES as a result of nuclear factor (NFkB) activation. Protein overload proteinuria also induces tubular cell apoptosis that in the long term is responsible for disconnection of tubules from the glomerulus in rats with severe renal injury. Apoptosis also occurs in vitro as documented by a dose- and duration-dependent upregulation of the Fas-FADD-caspase 8 pathway by proximal tubular cells exposed to excess albumin.9

Besides proteinuria, angiotensin II (Ang II) has also emerged as a pivotal factor in the pathogenesis of renal injury, to the extent that infusion of Ang II in the rat causes proteinuria, and angiotensin-converting enzyme inhibitors (ACEi) prevent proteinuria by preserving the size-selective restriction to macromolecular probes both in animals and in people. Several nonhemodynamics effects of Ang II might also be important in renal disease progression, including mesangial cell proliferation, stimulation of both transforming growth factor β and plasminogen activator inhibitor expression, all resulting in increased synthesis of extracellular matrix; activation and infiltration of macrophages and induction of aldosterone production by adrenal glands. The clinical benefit of preventing renal deterioration by blocking the renin–angiotensin system is not entirely explained by antihypertensive action or the antiproteinuric effect.
of ACEi or Ang II receptor antagonists.\textsuperscript{15,16} Investigation of mechanism(s) underlying glomerular barrier dysfunction becomes crucial to identify both the glomerular cell target dysfunction and the intracellular responses downstream Ang II receptors.

**DETERMINANTS OF GLOMERULAR CAPILLARY WALL PERMEABILITY**

Under normal circumstances, the glomerular capillary wall functions as an efficient and selective barrier that allows a high flow rate of filtration for plasma water and small solutes, but almost completely retains macromolecules with the size of albumin or larger.\textsuperscript{17} Several experimental studies and clinical investigations in the past have focused on understanding how glomerular membrane perm-selective function is damaged or lost during development of proteinuric glomerular diseases. This is still an open question and of crucial importance, because a growing body of evidence suggests that abnormal filtration of plasma proteins through the glomerular capillary wall has an intrinsic toxicity on the proximal tubule and subsequently on the whole kidney, and seems to represent a key factor in renal disease progression.\textsuperscript{18,19} A mathematical approach to study glomerular capillary wall permeability suggests that in nephropathy, previously unexposed large nonselective pores are recruited in a new pathway permeable to macromolecules.\textsuperscript{20} The best-credited hypothesis is that the distortion of the epithelial layer with loss of podocyte–podocyte contacts and denudation of basement membrane could represent an exaggerated equivalent to the new nonselective pathway.\textsuperscript{20} Recent discovery of the molecular components of the slit diaphragm, specialized structure of podocyte–podocyte interaction, represented a major breakthrough in understanding the crucial role of epithelial layer of the glomerular barrier and the pathogenesis of proteinuria.\textsuperscript{21,22}

**SLIT DIAPHRAGM AND ASSOCIATED PROTEINS**

The slit diaphragm is a continuous structure that bridges the gap between the interdigitating foot processes of the adjacent podocytes. In 1974, Rodewald and Karnovsky proposed a “zipper” model, in which the slit diaphragm is composed by rod-like units extending from the podocyte foot processes to a linear central bar running parallel to the cell membranes.\textsuperscript{23} Although unique in structure and function, slit diaphragm shares certain components with other intercellular junctions, including zonula occludens-1 (ZO-1), a 225-kDa polypeptide\textsuperscript{24} specific for the tight junctions or zonula occludens and FAT and P-cadherin\textsuperscript{25} expressed in desmosomes and adherens junctions.

Nephrin, originally described as a peculiar protein of the slit diaphragm, was subsequently identified to be expressed in the brain, pancreas, and testis.\textsuperscript{26,27} Nephrin is a transmembrane molecule with an extracellular portion containing eight Ig motifs, characteristic of proteins participating in cell–cell interaction, and one fibronectin type III-like module. The predicted structure and biochemical properties of nephrin suggest that it could form dimers through homophilic interactions spanning the slit diaphragm.\textsuperscript{28} Nephrin has been proposed to form a zipper-like filter structure, which prevents molecules of the size of albumin and larger to penetrate the filter.\textsuperscript{21,28} The intracellular domain with nine tyrosine residues typical of signaling molecules incited studies that indicated Src family kinases as enzymes that phosphorylate nephrin initiating a signaling cascade and phosphorylation of other proteins.\textsuperscript{29,30}

Evidence concurs to indicate that nephrin is essential for the functional integrity of the glomerular filter. Mutations in the human nephrin gene (NPHS1) in severe congenital nephrotic syndrome of the Finnish type (CNF)\textsuperscript{31,32} result in the absence of the slit diaphragm, massive proteinuria in utero, and nephrotic syndrome at birth. Mice with targeted disruption of the nephrin gene develop heavy proteinuria, edema, and die within 24 hours. Histologic features are comparable to those seen in patients with CNF, including an enlarged Bowman’s space, dilated proximal and distal tubules, mesangial hypercellularity and, at the electron microscopic level, partial foot process effacement and absence of the slit diaphragm.\textsuperscript{26,33}

Mutations in another gene, NPHS2 encoding for the 42-kDa integral podocyte membrane protein podocin, were recently identified as the cause of autosomal-recessive steroid-resistant nephrotic syndrome characterized by focal segmental glomerulosclerosis (FSGS).\textsuperscript{34} Podocin localizes to the podocyte foot process membrane at the insertion site of the slit diaphragm, where it binds to the cytoplasmic tail of nephrin as well as to two other podocyte proteins, CD-2-associated protein (CD2AP) and Nephl.\textsuperscript{35,36} Podocin is important for the stability of this complex, which is embedded in a special cell membrane domain, the lipid raft.\textsuperscript{37}
During development at the capillary loop stage, nephrin expression simultaneously appears into the foot processes together with the CD2AP. CD2AP is an 80-kD ubiquitously expressed protein interacting with CD2, a T cell membrane protein facilitating T cell adhesion to antigen-presenting cells.38 Targeted deletion of CD2AP in mice unexpectedly revealed the presence of proteinuria and a severe kidney pathology almost exclusively confined to the podocytes with loss of foot process integrity and obliteration of the spaces of foot processes, supporting a role of CD2AP in the specialized cell junction.39 In vitro experiments with an heterologous expression system allowed to demonstrate that CD2AP coimmunoprecipitated with the C-terminal cytoplasmic tail of nephrin and the actin-based cytoskeleton.40,41 More recent data have shown that mice with CD2AP haploinsufficiency had increased susceptibility to glomerular injury by nephrotoxic antibodies or immune complexes as well as patients with a mutation predicting to ablate expression of one CD2AP allele who are more inclined to develop focal segmental glomerulosclerosis.42 NEPH1, a molecule recently identified in podocyte areas, belongs to a family of three closely related proteins, NEPH1, NEPH2, and NEPH3. NEPH1 shows structure homology with nephrin, consisting of five extracellular immunoglobulin-like repeats by which probably interacts with nephrin forming the backbone of the slit diaphragm, a transmembrane domain and a cytoplasmic tail instrumental for the binding to the carboxyterminal domain of podocin and ZO-1.36,43,44 Nephrin and NEPH1 are both signaling molecules that can activate intracellular kinases able to induce a signaling cascade most probably important for the proper function of the glomerular filter.45

The actual structure of the slit diaphragm is, as yet, unresolved and very complex. The slit diaphragm proteins have an intimate relationship with the actin cytoskeleton of the foot process, schematically represented in Figure 1. Disruption or inefficient interplay of the slit diaphragm proteins with actin cytoskeleton has been proposed to present a final common pathway leading to foot process effacement in proteinuric diseases.46

ANG II AND CHANGES IN GLOMERULAR PERMEABILITY

A direct evidence of the effect of Ang II of modifying glomerular permeability rests on the seminal observation that infusion of Ang II in the isolated perfused kidney causes a dose-dependent increase in glomerular permeability to proteins, a phenomenon completely abrogated by pretreatment with a Ang II type 1 receptor antagonist.47 Further interest in this area has been generated by the observation that inhibition of ACE prevents or reduces urinary protein excretion, glomerular injury, and renal function deterioration in experimental animals12,48,49 and humans.50,51 Investigation of glomerular capillary wall permeability properties has suggested that in an experimental model characterized by massive proteinuria, such as passive Heymann nephritis, proteinuria depends on the recruitment of previously unexposed, large, nonselective pores, which constitute an escape pathway permeable to macromolecules.20 Studies by our group in male Munich Wistar Frömter (MWF) rats, which develop spontaneous proteinuria with age,52 have shown that Ang II inhibition or antagonism prevented proteinuria by preserving size-selective function of the glomerular capillary.12,53 This effect has also been demonstrated in human renal diseases.54-56 We have observed that in the MWF rats the elevation of the glomerular ultrafiltration coefficient induced by ACE inhibitors was not the consequence of increased glomerular-filtering surface area,57 but must be attributed primarily to an elevation of the hydraulic permeability of the glomerular capillary wall. Structural and theoretical evidence suggests that the hydraulic resistance of the glomerular capillary wall is attributed half to the glomerular basement membrane (GBM) and half to the slit diaphragm of the epithelial podocytes. However, studies using isolated GBM preparations have shown that the matrix network of the GBM is unlikely to be primary site of dysfunction in rats with proteinuria susceptible to the antiproteinuric action of ACE-inhibitors.58 Changes in glomerular podocyte morphology have been implicated in the pathogenesis of proteinuria in diabetic and nondiabetic nephropathies instead. Podocytes bear specific Ang II receptors that could initiate intracellular signaling responsible for redistribution of major proteins of the foot process and for the associated increases in transepithelial albumin permeability in the isolated perfused kidney.
CHANGES IN SLIT DIAPHRAGM PROTEIN EXPRESSION IN ACQUIRED PROTEINURIC CONDITIONS AND MODULATION BY ANG II BLOCKERS

Experimental Models

Nondiabetic Nephropathies

Several monoclonal antibodies have been developed in the late 1980s to identify potential nephritogenic glomerular antigens by means of hybridoma technology. Among them, the monoclonal antibody 5-1-6 obtained from the immunization of a mouse with isolated rat glomeruli induced massive proteinuria when infused in the rat. Proteinuria was not accompanied by complement activation, recruitment of inflammatory cells, and ultrastructural changes in the glomerulus, except for the partial retraction of the epithelial foot processes. The antibody recognized an antigen localized on the outer surface of glomerular epithelial foot processes mainly around the slit diaphragms critically involved in the maintenance of the perm-selective function of the glomerulus. The antigen identity remained elusive for 10 years until the cloning of nephrin, which allowed the
or even preceded the onset of proteinuria. Abnormal nephrin expression was specifically linked to the formation of the slit diaphragms giving rise to proteinuria.

In this model, proteinuria and morphologic changes, including fusion and effacement of podocyte foot processes with the consequent slit diaphragm loss, were associated with the reduction in nephrin and podocin expression. Luimula et al. found the alpha splice variant of nephrin, lacking of the exon coding for the transmembrane domain of the protein, in the urine of PAN rats, suggesting that soluble nephrin variants might be important markers for proteinuric diseases.

Studies from our group in an accelerated model of passive Heymann nephritis (PHN), the experimental counterpart of human membranous nephropathy, have shown a time-dependent reduction of nephrin mRNA and protein expression in the glomeruli of proteinuric PHN rats. The redistribution and loss of nephrin from glomerular podocytes in PHN was an early event that coincided with or even preceded the onset of proteinuria. Nephrin partly dissociated from actin at the onset of podocyte injury in PHN. This was accompanied by a progressive loss of nephrin from podocyte foot processes and progressive disruption or displacement of the slit diaphragms giving rise to proteinuria. Abnormal nephrin expression was specific and not simply a consequence of podocyte damage to the extent that both the distribution and the amount of CD2AP and ZO-1, other podocyte proteins, did not undergo similar changes. Of interest was the observation that the remarkable renoprotective effect afforded by the ACE inhibitor or the angiotensin II type 1 receptor blocker paralleled a complete prevention of glomerular nephrin downregulation in accelerated PHN. This unprecedented finding indicated the crucial role of Ang II in conditioning the molecular network of the epithelial slit diaphragm and emphasized the protective effect of the drugs interfering with Ang II synthesis/activity on glomerular podocytes. Studies in the remnant kidney, a nonimmunologic model of renal injury peculiarly sensitive to renin–angiotensin system inhibition, confirmed the favorable effect of blocking Ang II type 1 receptors in restoring the reduced nephrin gene and protein expression. The most distinguished findings of this study were that a selective Ang II type 2 receptor antagonist attenuated the deficiency of nephrin expression despite less effectiveness in reducing proteinuria than Ang II type 1 receptor antagonist and, more importantly, the combined blockade of both receptors conferred additive renoprotective effect with respect to monotherapies.

Renoprotection afforded by ACE inhibitors or Ang II receptor antagonists could be the result of the direct blocking of the injurious effect of angiotensin II on the podocyte or be mediated by Ang II inhibitors’ capability of reducing intraglomerular hydraulic pressure and the consequent stretching of the podocytes. The latter possibility is consistent with the in vitro observation of the remarkable cytoskeletal reorganization induced in podocytes in response to mechanical stress.

The antiproteinuric effect of Ang II blockers is partly related to the attenuation of ZO-1 expression as documented in the genetic model of renal injury, the MWF rats. Altered pattern of ZO-1 distribution characterized by a discontinuous staining at light microscopy and an abnormal presence of clusters of gold particles in podocyte foot processes cytoplasm was observed within the podocytes of MWF rats well before the development of glomerulosclerosis but not in controls. ZO-1 abnormality was restored by ACE inhibitors, which also prevented proteinuria. ZO-1 serves as adaptor protein that links peripheral actin bundles to P cadherin/catenin complex at the slit diaphragm. It might be possible that Ang II could induce actin cytoskeleton reorganization leading to ZO-1 redistribution, loss of albumin perm selectivity, and consequent podocyte permeability dysfunction.

**Diabetes**

Little is known about the pathogenesis of persistent proteinuria associated with diabetic nephropathy, the leading cause of end-stage renal disease worldwide. The numerous studies so far reported did not contribute to shed light on the role of nephrin in the pathogenesis of proteinuria in diabetes. The first report documented that irrespective of the cause of the disease, either genetic or chemical, renal nephrin gene expression increased as early as 6 to 8 weeks in nonobese diabetic mice and in streptozotocin diabetic rats, both of which
already developed proteinuria, concomitant with the presence in the urine of soluble nephrin. A subsequent study from the same group showed that in a preproteinuric state, nephrin expression was unchanged, whereas it was reduced with the development of glomerular injury in diabetic rats. The trend of nephrin expression was somewhat puzzling and did not allow to conclude that changes in nephrin might be a determinant of the loss of glomerular filtration function in experimental diabetic nephropathy. As for chronic nondiabetic nephropathies, the treatment with the ACE inhibitor and Ang II type 1 receptor antagonist, irbesartan, was effective in normalizing nephrin levels, which can be taken to suggest that modulation of nephrin expression is, at least in part, Ang II-dependent. This interpretation is further reinforced by findings that the blood pressure effect of calcium channel blockers does not limit proteinuria or restore nephrin expression. By contrast, the vasopeptidase inhibitor omapatrilat, which remarkably attenuated albuminuria, has been found effective in normalizing nephrin expression in diabetic spontaneously hypertensive rats. One can speculate that the activity of Ang II to lower proteinuria depends on its capacity to restore podocyte cytoskeleton dysfunction and its consequence on slit diaphragm architecture. Recent data strengthen such view by showing that Ang II induces actin cytoskeleton reorganization, leading to ZO-1 redistribution and loss of albumin perm selectivity across podocyte monolayer as well as in isolated perfused kidney through activation of Src kinases (Daniela Macconi, personal communication, 2003).

Human Studies

Nondiabetic Nephropathies

Studies on nephrin expression in acquired human nephroses led to controversial results as a result of the lack of simultaneous assessment of nephrin mRNA and protein expression. The issue appears so controversial that some authors reported different findings in different studies. A very preliminary report based on an exiguous number of patients showed that glomerular nephrin mRNA evaluated by reverse transcriptase–polymerase chain reaction in three adult patients with minimal-change nephrotic syndrome (MCN) and one with membranous nephropathy (MN) was significantly decreased. Conversely, in situ hybridization and immunohistochemistry experiments revealed no changes in the expression of nephrin mRNA and protein in childhood kidney disease with proteinuria as MCN, FSGS, and MN, with an abnormal pattern of protein staining in IgA nephropathy. Consistent with data in children were the results in adult patients of normal nephrin staining in MCN and FSGS and a selective reduction in IgA nephropathy. This view has been challenged by further documenting loss of nephrin staining inversely correlating with proteinuria in patients with nephrotic syndrome. Discrepancies of these results prompted us to evaluate with a more systematic approach nephrin mRNA and the corresponding protein using antibodies recognizing the extracellular and the intracellular portion of the molecule in a consistent number of nephrotic patients. Glomerular expression of nephrin mRNA and protein was not different in patients with MCN and FSGS in comparison with control subjects who had undergone nephrectomy for kidney adenocarcinoma. By contrast, a remarkable reduction or even absence of nephrin mRNA and staining intensity for extracellular nephrin domain was found in IgA nephropathy patients in the face of a normal expression of the intracellular nephrin domain. Finding of a comparable CD2AP expression in all acquired nephroses and in controls suggested that nephrin changes in IgA nephropathy patients were specific and not shared by other podocyte proteins. Finally, we assessed whether nephrin abnormalities observed in IgA nephropathy actually translated in reorganization of the filtration slit fine architecture by transmission electron microscopy studies. Despite slit pores having a normal frequency and width, a marked reduction in the percentage of electron dense slit diaphragms was observed in IgA nephropathy (Fig 2C, D), but not in patients with MCN in respect to control subjects (Fig 2A, B), reflecting nephrin changes showed by immunohistochemistry.

Diabetes

Studies addressing nephrin expression in human diabetic nephropathy gave more consistent results than those in nondiabetic proteinuric nephropathies. Downregulation of nephrin in the kidney in both type I and type II diabetes together with a shift from the normal linear to a granular pattern of protein immunostaining was observed. A re-
cent study from ours has shown that diabetic patients with nephropathy had remarkably less renal expression of extracellular nephrin. Changes in nephrin gene and protein were associated with podocyte ultrastructural abnormalities, including reduced presence of electron dense filamentous structure within the slit diaphragms, an unprecedented finding in human diabetes. The slit diaphragm change secondary to selective reduction in extracellular nephrin could possibly contribute to the glomerular permeability defect of diabetes. Nephrin loss would eventually lead to perturbed function of interacting slit diaphragm molecules, resulting in further increases in proteinuria. On the other hand, primary defective expression of slit diaphragm-specific molecules, including CD2AP and podocin that serve as anchors for nephrin to the cytoskeleton, could be responsible for nephrin loss. Lack of CD2AP prevents nephrin binding and the formation of interdigitating processes and slit diaphragms in vivo. However, preserved CD2AP and podocin expression in diabetic glomeruli makes it unlikely the contribution of these molecules to impaired perm selectivity in human diabetes. Impaired nephrin extracellular domain expression in diabetes could be the result of changes in cytoskeleton distribution and cleavage of the protein induced by Ang II challenge as observed in human cultured podocytes. That Ang II could modulate nephrin expression is supported by in vivo data of a normalization of nephrin expression by ACE inhibitor in glomeruli from type II diabetic patients. Progression of diabetic nephropathy is largely dependent on a complex interplay of genetic and metabolic factors. Among the latter, hyperglycemia seems an independent risk factor to the extent that the levels of glycosylated hemoglobin strongly predict the renal function decline. Reduction of nephrin expression on the surface of cultured podocytes after exposure to glycated albumin would support the notion that glycemia-dependent modifications could have a role in the
abnormal pattern of nephrin expression in diabetes. All of these in vivo and in vitro data would indicate that nephrin abnormalities in diabetes could be the result of an acquired defect induced by the diabetic milieu rather than by a genetic background. In this regard, a recent cross-sectional study in 996 type I diabetic patients did not show any association between polymorphisms of the coding region of the nephrin gene and progression of diabetic nephropathy or development of renal dysfunction. Linkage with candidate regions for nephrin has also been excluded in type II diabetic patients with nephropathy.

**CONCLUSIONS**

The importance of the podocytes in glomerular physiology and pathology has accumulated rapidly in the last few years. The crucial role of the slit diaphragm in the development of proteinuria is now secure, and the molecular composition of this vital structure is gradually defined. A number of evidence suggested that the nephrin–CD2AP–podocin complex is a crucial functional unit that anchors the slit diaphragm to the actin cytoskeleton and is required for proper glomerular filtration. Nephrin has a key role in the function of the glomerular filtration barrier based on data from patients with congenital nephroses. Contradictory results are available on nephrin involvement in the pathogenesis of proteinuria in acquired nephritic disorders of humans as a result of heterogeneity of the patients studied as well as of the methodologic approach to assess nephrin gene and protein expression. By contrast, studies in experimental and human diabetic nephropathy consistently indicate that among slit diaphragm proteins, nephrin is selectively reduced, which would translate in changes of filtration slit architecture. Results presented here support this interpretation. The peculiar ability of Ang II inhibitors to restore deficient nephrin expression in podocytes disclose a novel molecular target for renoprotection. Understanding the molecular mechanisms underlying such phenomenon will open exciting perspectives for therapeutic approaches to retard or even prevent diabetic patients from ever progressing to end-stage renal disease. This will undoubtedly represent a major challenge for renal medicine in the next coming years.

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