

Immune and Inflammatory Glomerular Injury

Terry Cook

It is now over a century since the first animal model of glomerulonephritis was established by Lindemann [1] who injected rabbits with heterologous antiserum to rabbit kidney raised in guinea pigs. In recent years there has been increasing opposition to the use of animals for research with an escalation in damage to property and violent attacks on researchers by those opposed to such research. This has been a major problem in the UK and is an increasing problem in the US [2]. There is therefore a need for scientists who carry out animal research to demonstrate that the inevitable discomfort and harm which is suffered by experimental animals is reduced as far as possible by good experimental design and, most importantly, is justified by the benefits it brings in understanding and treating human disease. In this talk I will discuss models that have been used in studying immune and inflammatory glomerular injury and ask:

1. How representative they are of human disease
2. What are the key insights they have given into pathogenesis
3. How has this been translated into treatment
4. Are we in danger of being misled by reliance on animal models

Neutrophils and macrophages

It is clear that these cells play a major role in causing inflammatory glomerular damage leading to proteinuria and also to capillary wall rupture with crescent formation. Neutrophils are the key cell in heterologous nephrototoxic nephritis and macrophages are of central importance in the autologous phase. Macrophage depletion greatly attenuates injury in these models [6,7]. In anti-MPO induced glomerulonephritis in mice neutrophil depletion ameliorates disease [8] and the anti-MPO response has been shown to be directed against MPO on neutrophils and macrophages [9]. Macrophages in proliferative glomerulonephritis have an activated phenotype [3] and there is interesting work suggesting that they become programmed when they enter the glomerulus [10]. It is likely that the balance between different activation states in macrophages may determine whether there is progression or resolution of glomerular injury. Understanding the signals that attract and activate macrophages is of major importance.

Fc receptors

Fc receptors on circulating leukocytes play a central role in the induction of glomerular injury in models of nephrototoxic nephritis [11;12] and lupus nephritis [13]. The class of immunoglobulin deposited in glomeruli affects which Fc receptors are activated and determines severity of inflammation [14] and interaction between

I will discuss the following models which I think are most useful in understanding human glomerular disease:

Nephrotoxic nephritis

This model is induced by the injection of a heterologous antibody raised against a preparation of glomerular antigen. Classically there is acute injury produced by binding of the heterologous antibody to the glomerular basement membrane followed by a second phase of injury in which there is an autologous immune response to the heterologous antibody which then acts as a planted antigen. This second phase can be accelerated by pre-immunising the animals with heterologous immunoglobulin.

Typically the autologous phase is characterised by proliferative glomerulonephritis with crescent formation in susceptible animals. The heterologous phase closely resembles human anti-GBM disease but the autologous phase has more in common with an immune complex glomerulonephritis with the heterologous antibody acting as a planted antigen. Proliferative immune complex glomerulonephritis can also be produced by antigens planted by other mechanisms, for example, on account of their charge [3]

Heymann nephritis

This is a model of membranous glomerulonephritis originally induced in rats by immunisation with a suspension of renal cortex and adjuvant. Subsequently a passive

promoting inflammation. Complement is of central importance in the Heymann nephritis model of membranous gn and the alternative pathway has been shown to be critical in a mouse model of anti-MPO glomerulonephritis [17]. Alternative pathway activation has also been shown to be central in mouse models of dense deposit disease [18] and HUS. Antibodies directed at C5 have led to amelioration in models of lupus nephritis and dense deposit disease.

T cells

There is good evidence that as well as being important in controlling antibody synthesis T cells may have a direct effector role in glomerular injury in nephrotoxic nephritis in both mouse [19] and rat [20]. It is a challenge to define how important this mechanism may be in humans.

Genetics

Animal models have provided insights into genetic susceptibility to glomerular disease in models of lupus nephritis in the mouse [21] and in a model of crescentic glomerulonephritis in the rat [22]. In the latter model susceptibility was associated with a variation in copy number of the *Fcγr2* gene and we then showed that there was also variation in copy number of the human *FCGR3B* gene that was associated with susceptibility to lupus nephritis.

There are several models of SLE in mice which are characterised by the generation of autoantibodies and the development of glomerulonephritis with immune complex deposition. In my opinion the model that most closely resembles human disease is that produced by crossing NZB and NZW mice but other models include the MRL lpr mouse and the BXS B mouse. These models have been widely studied and have provided insights into genetic susceptibility to SLE, mechanisms by which tolerance is broken and have been used for elucidating the role of mediators of glomerular injury both by examining gene targeted animals and by administration of pharmacological agents.

Models of ANCA-mediated glomerulonephritis

A major step forward in our ability to model human glomerular disease was the demonstration by Jennette and co-workers that transfer to normal mice of splenocytes or serum from myeloperoxidase (MPO) deficient mice immunized with MPO led to a pauci-immune necrotising glomerulonephritis [4]. This was the first demonstration that anti-MPO antibodies were pathogenic and has led to further insights into the mechanisms of injury in ANCA-mediated glomerular inflammation.

Dense deposit disease and thrombotic microangiopathy

Pigs with a spontaneous deficiency of factor H and mice in which the factor H gene has been knocked out develop persistent activation of the alternative pathway of

complement inhibition. However, I think it is possible to speculate on where advances might come in future. I think promising areas for development of treatment include: inhibition of Fc receptor activation, modulation of macrophage activation, inhibition of complement, particularly the alternative pathway and C5 activation; inhibition of chemokines. It is also important to consider ways of targeting therapy specifically to the inflamed glomerulus and interesting strategies include the use of genetically modified macrophages [23] or of cytokines which are designed only to be activated at sites of inflammation [24].

It is inevitable that there will be differences between the way experimental animals respond to glomerular injury and the way humans do that may lead to misleading conclusions from animal experiments and, of course, this is one of the arguments that those opposed to animal experimentation rely on. However, there is one specific example of how animal experiments may be misleading that I would like to explore and this is not due to differences between species but to a failure to use appropriate controls. In 1999 we published data on a mouse in which serum amyloid P component (SAP) had been genetically deleted and which developed autoimmunity [25]. We concluded that SAP protected against autoimmunity and this has been widely quoted. However, the knock out had been created using embryonic stem cells derived from the 129 strain of mouse which were then transferred to C57BL/6 mice. This means

From Bedside to Bench and Back Again: What Animal Models Teach Us
About Renal Disease and What They Don't

Paradigms in Diabetic Nephropathy (DN)
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1. Animal models of DN can be useful, even if imperfect, if they generate testable concepts in humans.
2. In order to be useful, these models:
 - a. Need to reflect components of the structural, functional and natural history, human conditions which are well understood.
 - b. Should recapitulate the order of the human disorder so that the highly disease specific processes involved in the genesis of the early lesions are not confused with the progression promoters associated with advanced injury, which share commonalities among multiple renal disorders.
3. An example of a concept that has been distorted, in the end, by the interpretations provided to animal models of DN (i.e., hyperfiltration/capillary hypertension/renin angiotensin system blockade) resulting in inappropriate extrapolation to humans will be provided.
4. A review of human diabetic nephropathy, natural history, and structural functional relationships and examples of useful animal models will be presented.

The Histology of Progressive Diabetic Nephropathy in Humans

The constellation of the renal structural lesions occurring in diabetes is unique, although many of these lesions can be individually observed in other renal disorders. The morphologic lesions in type 1 diabetes (T1DM), predominantly affect the glomeruli, with thickening of glomerular basement membrane (GBM) and mesangial expansion, although also the podocytes, renal tubules, interstitium and arterioles undergo substantial changes, especially at later stages of disease (1-5).

GBM thickening, the first measurable change, has been detected as early as 1.5 to 2.5 years after the onset of T1DM (6, 7). Thickening of tubular basement membrane (TBM) closely parallels that of GBM thickening (3). Mesangial expansion, predominantly due to an increase in mesangial matrix, develops later although an increase in the matrix component of the mesangium can be detected as early as 5-7 years after the onset of diabetes (8-11). While GBM thickening may develop steadily over time, mesangial expansion has a more asymptotic relationship with T1DM duration (Sienke J, Mauer M, unpublished observations). However, when renal insufficiency occurs, marked mesangial expansion and increased GBM width are present in virtually all T1DM patients (9-10). Diffuse mesangial expansion, commonly termed diffuse diabetic glomerulosclerosis, can be associated with nodular lesions consisting of areas of marked mesangial expansion forming large round fibrillar mesangial zones with palisading of mesangial

expansion, through its intimate relationship with filtration surface, better defines the clinical course of those destined to develop severe diabetic kidney disease. Although an increase in AER to the MA range is usually considered the first clinical expression of DN, some long-term T1DM patients have reduced GFR as initial indicator of renal disease (33). This situation has also been seen in T2DM patients (34).

As alluded to above, through much of the natural history of DN lesions develop in complete clinical silence. When persistent MA and proteinuria supervene, lesions are often far advanced and loss of GFR may then progress relatively rapidly toward ESRD. This typical clinical story is best described by non-linear analyses of structural-functional relationships (16). Using simple linear regression models, glomerular structural variables explained about 65% of AER and 35% of GFR variability among T1DM patients (17). However, using piecewise (spline) regression models, glomerular structural variables alone, GBM width, $V_v(\text{Mes}/\text{glom})$, and total filtration surface per glomerulus or TFS, explained 95% of variability in AER ranging from NA to proteinuria. These same glomerular structures, however, explained only 78% of GFR variability in this study, and this increased to 92% with the addition of indices of GTJA and interstitial expansion (16).

In summary, most of the AER and GFR changes in T1DM are explained by diabetic glomerulopathy lesions and these structural-functional relationships are largely driven by patients with more advanced lesions and clinical functional abnormalities while structure is highly variable (from virtually none to moderate severity) in patients without functional abnormalities. In the end, as in other slowly progressive renal diseases, clinical findings in DN may, at least in part, reflect the lesions outstripping of renal compensatory capacities and this may be mirrored in the non-linear analyses described above.

Reversibility of diabetic nephropathy lesions

Pancreas transplantation offers the opportunity to test the effects of long-term normoglycemia to prevent, halt or reverse DN lesions. GBM and TBM widths were decreased after 10-years of normoglycemia, returning to normal values in most patients (35). $V_v(\text{Mes}/\text{glom})$ and mesangial matrix fractional volume [Vv(MM/glom)] were also lower at 10 years than at baseline or 5 years (35). Light microscopic observations revealed a remarkable amelioration of glomerular structure, including the total disappearance of Kimmelstiel-Wilson nodular lesions and reopening of glomerular capillaries previously compressed by mesangial expansion (35). These findings call for further studies aimed at identifying the molecular and cellular mechanisms involved in these healing processes which could provide new directions in the treatment of DN.

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nuclei around the periphery of the nodule and compression of the associated glomerular capillaries (Kimmelstiel-Wilson nodules). Both mesangial expansion and GBM and TBM thickening are a consequence of extracellular matrix (ECM) accumulation, with increased deposition of types IV and VI collagen, laminin and fibronectin (12-13). In contrast, initial interstitial expansion is primarily due to an increase in the cellular component of this renal compartment (14); increase in fibrillar collagen is measurable only in patients with advanced disease (14).

Afferent and efferent arteriolar hyalinosis may be present within a few years after diabetes onset (5) and this vascular lesion contributes to ischemic global glomerular sclerosis. Similar lesions may occur in the glomerular subendothelial space (hyaline caps) and along the parietal surface of Bowman's capsule (capsular drops).

Abnormalities of the glomerular-tubular junction (GTJA) occur as late manifestations of the disease (15) predominantly in patients with proteinuria, and rarely at earlier stages (16). These manifest with focal adhesions, obstruction of the proximal tubular take-off from the glomerulus detachment of the tubule from the glomerulus (atubular glomerulus). These focal segmental glomerulosclerosis (FSGS) lesions have a marked predilection for the GTJ and are uncommon at other locations. The lesions at the GTJ are inversely correlated with GFR (15, 16) and probably contribute to the loss of renal function in proteinuric diabetic patients.

These various lesions of diabetic nephropathy progress at varying rates within and between T1DM patients, and, as discussed below, this is even more the case in type 2 diabetes (T2DM). For example, GBM width and $V_v(\text{Mes}/\text{glom})$ are significantly but not very precisely correlated with one another, with some patients having relatively marked GBM thickening without much mesangial expansion and others the contrary (9). Marked renal extracellular basement membrane accumulation resulting in extreme mesangial expansion and GBM thickening are present in the vast majority of T1DM patients who develop overt diabetic nephropathy (DN) manifesting as proteinuria, hypertension, and declining GFR (8, 17). Ultimately, focal and global glomerulosclerosis, tubular atrophy, interstitial expansion and GTJA facilitate this downward spiral. However, tubulo-interstitial lesions and GTJA contribute only ~10-15% to functional loss in T1DM patients whose GFR is above 40 ml/min/1.73m² (16). Tubulo-interstitial disease may be more important in the progression from moderate renal insufficiency to end-stage renal disease (ESRD) (18), but it is probably a mistake to extrapolate this to earlier stages of DN progression. The situation in T2DM is more complex. The real frequency of non-diabetic renal diseases among patients with T2DM and proteinuria is difficult to assess in studies of which patients biopsied for clinical reasons because of selection bias towards atypical cases (19-24).

Research renal biopsies in a large cohort of T2DM patients with microalbuminuria (MA) and proteinuria and described marked heterogeneity in renal structure among these patients; in fact, only a minority subset had DN patterns typical of those seen in T1DM patients; the remaining had mild or absent diabetic glomerulopathy with or without tubulo-interstitial, arteriolar and global glomerulosclerosis changes (25). Less than 10% of our proteinuria patients had non-diabetic renal diseases. Based on these observations, we proposed a classification system which included 3 major categories (25):

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Category C I: Normal or near normal renal structure. These patients (35% of MA and 15% of proteinuria) had normal renal biopsies or showed very mild glomerular, tubular, interstitial and/or vascular changes.

Category C II: Typical diabetic nephropathology. These patients (30% of MA and 50% of proteinuria) had established diabetic lesions with an approximately balanced severity of glomerular, tubulo-interstitial and arteriolar changes, a picture typical of that seen in most T1DM patients with obvious light microscopic DN changes.

Category C III: Atypical patterns of renal injury. These patients (35% of MA and proteinuria) had relatively mild diabetic glomerular changes considering disproportionately severe: (a) Tubular atrophy, TBM thickening and reduplication and interstitial fibrosis (tubulo-interstitial lesions). (b) Advanced glomerular arteriolar hyalinosis commonly associated with atherosclerosis of larger vessels. (c) Global glomerular sclerosis. In C III group these patterns were present in all possible combinations. More recently, examining the associations of albumin excretion rates (AER) and electron microscopic morphometrically quantitated DN lesions, we could mathematically define a spatial cluster of structural/functional relationships which contained the T1DM patients. About 1/3 of the T2DM fell outside of this cluster because of MA or proteinuria despite a paucity of diabetic glomerulopathy lesions (26). These objective data largely confirm the more subjective categorical classifications.

Thus, hyperglycaemia may cause different patterns of renal injury in T1DM compared to T2DM patients. Alternatively, the disproportionate tubulo-interstitial, glomerulosclerotic and vascular changes of T2DM could also be related to aging, atherosclerosis and systemic hypertension. The natural history of MA and proteinuria T2DM patients with minimal or no renal lesions is not yet well understood, however, GFR loss in the relatively short-term (about 3 years), is largely confined to T2DM research patients with mesangial expansion (27).

Morphometric analysis and structural-functional relationships

The critical lesion in T1DM is mesangial expansion, morphometrically termed mesangial fractional volume [$V_v(\text{Mes}/\text{glom})$] (the fraction of the cross-sectional area of the glomerular tuft made up by mesangium); this is the electron microscopically estimated structural parameter that best correlates with all functional parameters in T1DM (9, 17). Indeed, a highly significant inverse correlation exists between $V_v(\text{Mes}/\text{glom})$ and GFR (9, 15-17); when mesangium expands it restricts and distorts glomerular capillaries and diminishes capillary filtration surface (9), which is strongly directly related to $V_v(\text{Mes}/\text{glom})$ and inversely to GFR (28). $V_v(\text{Mes}/\text{glom})$ is also related to AER (9, 15-17, 29) and blood pressure levels (30). In contrast, GBM thickening is closely related to AER and less so to GFR or hypertension, suggesting that this lesion is a closer surrogate to the pathogenesis of albuminuria. Interstitial expansion and percentage of global sclerosis are also directly related to proteinuria, hypertension and inversely to GFR (4, 5, 9, 15, 16). Progression from normal albuminuria (NA) to MA and from MA to proteinuria is primarily related to progressive mesangial expansion (11) with no significant progression in interstitial fibrosis or GBM thickening over the 5 years of this study. These data may initially seem contradictory to recent studies describing that greater GBM width at baseline biopsy was predictive of AER after 5 or 6 years of follow-up (31, 32). However, given the linear course of GBM thickening vs. the non-linear trajectory of mesangial expansion, it is not surprising that GBM width, a strong correlate of AER, is a better predictor of DN risk while mesangial

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**Podocyte Injury: Lessons Learned From Animal Models
A Play in Five Acts**

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Just a decade ago, our knowledge of podocyte injury in animal models was rudimentary. We recognized 2 major paradigms of irreversible podocyte injury leading to focal segmental glomerulosclerosis (FSGS). The first is direct podocyte injury due to exposure to such cell toxins as puromycin aminonucleoside and adriamycin/daunorubicin (1). These toxic models fostered the concept of primary podocyte injury in the pathogenesis of foot process effacement and glomerulosclerosis. By contrast, renal ablation models pointed to glomerular hypertension (elevated glomerular capillary pressures and flow rates) as the primary pathophysiologic process in the course of adaptive responses to reduced number of functioning nephrons or other glomerular stress, in turn causing secondary podocyte injury (2). These basic paradigms form the conceptual dichotomy between "primary" and "secondary" (post-adaptive) FSGS. Only in the past few years has the molecular basis for these pathologic alterations been elucidated through a greater understanding of podocyte biology.

**ACT 1: SEEING IS BELIEVING
ULTRASTRUCTURAL STUDIES PROVIDE MECHANISTIC INSIGHTS**

Initial insights into the podocyte injury in proteinuric conditions came from meticulous 3-dimensional ultrastructural observations using scanning electron microscopy. Studies by Inokuchi et al. in puromycin nephropathy elucidated that foot process effacement consists of a distinctive and predictable change in podocyte shape (3). The foot processes, which are analogous to specialized lamellipodia in other biologic systems, spread and fan out, incriminating the actin cytoskeleton in the effacement process. This simple, but elegant, observation laid the groundwork for elucidation of specific actin-associated podocyte proteins that may be operant in foot process maintenance.

By contrast, seminal ultrastructural observations by Nagata and Kriz in an ablation model of uninephrectomy in the young rat showed remarkably different podocyte alterations (4). As the tuft hypertrophied in response to the reduced number of functioning nephrons, podocyte cell number did not increase. Podocyte cell bodies were forced to stretch to cover a much larger surface area and serve many more glomerular capillaries. Cell bodies hypertrophy and become attenuated into cytoplasmic sheets. Primary processes thin out and extend to remote capillaries. Filtrate is now delivered into the subcell body space, causing bulging of the cytoplasmic sheets and formation of pseudocysts, under which foot processes are largely preserved. The formation of denuded patches of GBM owing to podocyte detachment and the apposition of distended podocyte cell bodies to Bowman's capsule form the nidus of the segmental sclerotic lesion. In this model, podocyte insufficiency and maladaptive responses develop into irreversible structural lesions.

synaptotaxin, podocalyxin, GLEPP-1), express Ki-67 and enter the cell cycle (39, 41). A similar podocyte phenotype has been identified in primary collapsing glomerulopathy and human HIV-associated nephropathy, as well as an HIV transgenic model (39, 42). A decrease in the CDK inhibitor, p27 and increase in cyclin D1 underlie the proliferative phenotype (43, 44).

Evidence in human HIVAN supports a direct viral infection of renal parenchymal cells, rather than a systemic or indirect immune dysregulation by the HIV virus. Cohen et al in 1989 were the first to report the detection of HIV-1 in renal epithelial cells by DNA in situ hybridization (45). In 2000, Bruggeman et al. detected HIV-1 in renal epithelial cells of patients with HIVAN by RNA in situ hybridization (46). These findings were confirmed using DNA in situ hybridization and by application of riboprobes specific for both the nef and gag genes. Virus was identified in renal tubular cells, often involving many contiguous cells in individual tubular profiles, as well as podocytes, parietal epithelial cells, and some interstitial leukocytes (46). In one particularly illustrative case treated with highly active anti-retroviral therapy (HAART), virus persisted in the tubular epithelium as determined by RNA in situ hybridization, even after viral load in the peripheral blood had become undetectable and renal histology had improved (47). The ability of the kidney to serve as a reservoir for HIV-1 was later confirmed by Marras et al using laser capture microdissection to characterize the HIV-1 quasi-species present in tubular epithelium (48). Comparison of the envelope sequences from tubular epithelial cells and peripheral blood leukocytes in individual patients showed variations in the HIV-1 envelope sequences in tubular epithelium compared to blood, indicating that the kidney is able to support viral replication and quasi-species evolution as a separate compartment.

Several animal models have provided insights into disease pathogenesis. One of the first models, Tg26, was established in transgenic mice containing a replication-defective HIV-1 construct that lacks the *gag* and *pol* genes and is expressed under the control of the long terminal repeat (LTR) viral promoter (49). In this model, which closely recapitulates the morphologic features of the human HIVAN, viral transgene was expressed in glomerular and tubular epithelial cells (50). Cross-transplantation of kidneys between Tg26 and W1 mice showed that renal transgene expression was required for the development of nephropathy (50). A similar model has been established in rats using the same transgene construct (51). Hanna et al. generated several different transgenic lines with mutations in one or more of the HIV genes, and found that nef was necessary and sufficient to produce the renal phenotype (52). In vitro studies in cultured podocytes suggest that nef-induced activation of Stat3 and Ras-MAPK1,2 via Src-dependent pathways is responsible for podocyte proliferation and dedifferentiation (53). Transgenic expression of nonstructural HIV-1 genes (*vif*, *vpr*, *nef*, spliced forms of *tar* and *rev*, but not *vpu*) selectively in podocytes using the nephrin promoter in mice with FVB/N genetic background results in podocyte injury, glomerulosclerosis and tubular microcyst formation (54). Podocyte specific expression of nef and vpr in a double-transgenic murine model recapitulated the severe morphologic and functional features of human HIVAN, suggesting a synergistic interaction of these proteins (55). In the Tg26 model of HIVAN, inhibition of podocyte proliferation using a CDK2 inhibitor reduced proteinuria and glomerulosclerosis (56).

In HIVAN and idiopathic collapsing glomerulopathy, the proliferating podocytes have an undifferentiated phenotype, leading to functional podocyte insufficiency, defective podocyte adhesion, and shedding of podocytes into the urine. Over the long-term, collapsing glomerulopathy is likely to lead to progressive podocyte depletion, as in other models of FSGS.

**ACT 2: GOING, GOING, GONE...
THE ROLE OF PODOCYTE DEPLETION**

In both toxic and adaptive models of FSGS, a central role for podocyte loss has been proposed. Podocyte depletion has been identified in many human glomerular diseases, including diabetic nephropathy, focal segmental glomerulosclerosis and IgA nephropathy (5, 6). In human disease, podocytes can be detected in the urine and the reduction in podocyte cell number correlates with the degree of proteinuria and severity of sclerosis (7).

This mechanism has been validated in several ingenious models of targeted podocyte cell death (8-10). Using a transgenic rat strain in which the human diphtheria toxin (DT) receptor is specifically expressed in podocytes driven by the podocin promoter, Wiggins and coworkers were able to produce different stages of glomerular injury depending on the percentage of podocytes depleted after injection of DT, consistent with a dose response (8). Over 40% podocyte depletion produced segmental and global glomerulosclerosis with high grade proteinuria and reduced renal function. In analogous experiments, Ichikawa and colleagues engineered a mouse model of glomerular sclerosis by selectively expressing human CD25 in podocytes (9,10). Injection of anti-Tac (Fv)-PE38 (LMB2) immunotoxin induced progressive proteinuria and glomerulosclerosis in a dose dependent fashion. By permanently labeling the podocyte lineage with lacZ, the investigators could determine their fate. The number of lacZ stained podocytes progressively declined as parietal epithelial cells avidly proliferated to cover the denuded tuft, resembling collapsing FSGS. These studies demonstrate the central role of podocyte depletion in the process of glomerular sclerosis. If the initial insult is of sufficient impact, there may be spreading of sclerosis to adjacent segments after the insult has been withdrawn. This process suggests a vicious cycle of local spreading of sclerosis, incriminating toxic substances secreted in a paracrine or autocrine fashion (such as TGFβ, Ang II, MIF) or reduction in survival factors (such as VEGF) (11).

**ACT 3: PROOF OF CONCEPT:
ANIMAL MODELS AND THE GENETIC BASIS OF FSGS**

Great advances have been made in our understanding of the genetic basis for MCD and FSGS. A number of critical podocyte proteins have been identified to be mutated or deficient in human forms of congenital nephrotic syndrome or inherited FSGS (12-24). Many of these proteins were identified first by positional cloning in affected families. Others were identified serendipitously while studying other disease systems. The critical role of these proteins in the mediation of FSGS was later validated in experimental models, including knock-out models or transgenic models expressing mutant proteins (25-31). The animal models provided proof of concept that deletion of a particular gene was sufficient to cause proteinuria or FSGS. A number of other podocyte proteins produce FSGS in null mice or conditional knock-outs, although a role in human disease has not yet been identified (32-35). The responsible genes encode proteins that are located in various subcellular domains of the podocyte, including membrane-associated (slit diaphragm, basal membrane), nuclear (transcription factors and chromatin bundling proteins), and cytosolic (associated with the actin cytoskeleton or cell energetics). These genes are listed in the table below. An asterisk marks the human disease genes that have been validated in animal models.

**ACT 5: THE MISSING LINK
HOW PODOCYTE INJURY PROMOTES SCLEROSIS**

Conventional wisdom suggests that foot process effacement, if reversed can lead to restoration of glomerular architecture (as in steroid responsive MCD). The failure of reparative mechanisms promotes persistent proteinuria and the development of glomerulosclerosis. Precisely how podocyte injury promotes glomerulosclerosis is poorly understood, but podocyte loss is emerging as a central pathomechanism. Evidence from animal models suggests that critical perturbations in the balance between pro-apoptotic and anti-apoptotic factors promote podocyte depletion and progressive glomerulosclerosis (Reviewed in 38). For example, toxins such as puromycin and adriamycin induce podocyte production of ROS, leading to podocyte DNA damage, apoptosis, and GBM protein peroxidation. Mechanical stretch can promote podocyte hypertrophy and apoptosis (57). Excessive protein trafficking through the podocyte itself generates ER stress and podocyte injury (58). Many of the pro-apoptotic factors listed below (such as Ang II and TGFβ) also possess proclerotic properties, providing a link to sclerosis.

The TGFβ1 transgenic mouse is a particularly valuable model that has shed mechanistic insights into the inter-relationship between podocyte apoptosis and glomerulosclerosis (59). Podocytes expressing TGFβ1 undergo apoptosis associated with marked upregulation of Smad7. TGFβ1 and Smad7 promote podocyte apoptosis through different mechanisms. TGFβ1 induces apoptosis by activation of mitogen-activating protein (MAP) kinase p38 and elastic activator caspase-3, whereas TGFβ-inducible Smad7 inhibits signaling by the cell survival factor NF-κB. In this model, podocyte depletion through apoptotic pathways leads to progressive FSGS.

Balance of Factors Influencing Podocyte Depletion (adapted from Shankland, ref 38)	
Podocyte pro-apoptotic factors	Pro-survival (anti-apoptotic) factors
Angiotensin II	Cyclin 1
AT1 Receptor	Nephrin
TGF-β	CD2AP
Cyclosporine	Decamethasone
Smad7	Bcl-2
ROS	Cell-cell contact
Detachment	VEGF
↓ p21	Collagen via Ras-ERK signaling
↓ p27	Focal adhesion kinase
Stress-tension	Hepatocyte growth factor
bFGF	Insulin-like growth factor
Lytic C5b-9	
p53	
Hyperglycemia	

A unifying concept in all these models is the central role of the actin cytoskeleton in coordinating cell signaling from the various membrane compartments. Interference with cell signaling may promote the stereotypic response of foot process effacement common to all these conditions (36). This hypothesis is supported by evidence that the slit diaphragm is a mechanosensor that serves as a platform for signal transduction (37).

HUMAN GENE PRODUCTS	GENE	INHERITANCE	CHROMOSOME
Slit Diaphragm proteins			
Nephrin*	NPHS1	AR	19q13.1
Podocin*	NPHS2	AR	1q25-31
CD2 associated protein*	CD2AP	AD	6p12
Transient Receptor Catoin 6	TRPC6	AD	11q21-22
Cytosolic proteins			
Alpha-actinin 4*	ACTN4	AD	19q13
Phospholipase C ε1	PLCE1	AR	10q23-24
Basal Membrane proteins			
Laminin β2*	LAMB2	AR (Pierson syndrome)	3p21
Beta 4 integrin*	ITGB4	AR (Epidermolysis bullosa)	17q11
Nuclear Proteins			
Wilms' tumor 1	WT1	AD (DMS, Frasier syndrome)	11p13
Chromatin bundling protein	SMARCAL1	AD (Schinke syndrome)	2q34-36
Mitochondrial Products			
Mitochondrial tRNAleu	mtDNA-A3243G	Maternal	mtDNA

**MOUSE GENE MUTANTS
(Role in human disease unknown)**

Slit Diaphragm Proteins
Neph1
Fyn
FAT1
Actin Associated Proteins
Nck 1/2

**ACT 4: MORE IS NOT NECESSARILY BETTER:
PODOCYTE PROLIFERATION AND DYSREGULATION IN COLLAPSING FSGS**

The mature podocyte is a post-mitotic cell. Podocytes can undergo DNA synthesis to a limited degree but do not proliferate because they arrest in the G2/M phase of the cell cycle (38). Findings in human collapsing FSGS suggest that podocytes may exhibit rare replicative capacity under select conditions, producing the glomerular pseudocysts typical of this variant (39). This has been difficult to prove without lineage specific markers, and there is increasing evidence that parietal epithelial cells contribute to the glomerular epithelial cell proliferation (40). In collapsing FSGS, the podocytes downregulate their mature podocyte markers (WT-1,

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RENAL FIBROSIS AND PROGRESSIVE RENAL INJURY – WHAT WE CAN LEARN FROM ANIMAL MODELS

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Introduction

Human chronic kidney disease (CKD) is characterized by progressive azotemia, culminating in uremia, and often accompanied by proteinuria and hypertension. Morphologically, this functional deterioration is due to lesions affecting ultimately all compartments of the kidney, including glomeruli, vessels, tubules and interstitium. In addition to these chronic sclerotic changes, there may also be disease-specific findings that are evident even at advanced stages. A common vicious cycle has been proposed for the usually inexorable progression of human CKD, where remaining nephrons not destroyed by the initially disease process undergo ultimately maladaptive changes, furthering progressive injury. Thus, the varying human conditions resulting in CKD may have many non-specific scarring changes in addition to specific lesions related to the initial disease process. This relentless progression of CKD with loss of GFR is a key characteristic that should be reproduced in experimental animal models, when the goal is to test interventions and their efficacy in altering the course of CKD.

Selected Animal Models Relevant to Human Disease

Other speakers will focus on immune and inflammatory glomerular injury and various injuries of the podocyte and details of diabetic nephropathy. In this review, I will briefly mention some aspects of diabetic nephropathy, and then focus on non-immune progressive disease models that manifest with predominantly tubulointerstitial fibrosis or glomerulosclerosis, including hypertensive and non-hypertensive models.

Diabetic Nephropathy

As will be discussed in detail elsewhere, human diabetic nephropathy is clinically characterized by progression from microalbuminuria to proteinuria and slow progression to ESRD. The key morphological findings, which should be present in a useful animal model of this process, include mesangial matrix expansion, with or without Kimmelstiel-Wilson nodules, GBM thickening, arteriolar hyalinization, and importantly tubulointerstitial fibrosis. These lesions should then result in renal dysfunction that is progressive with accompanying proteinuria to mirror human disease. Multiple models of diabetic nephropathy in rodents have been investigated, most with only minor changes of mild mesangial expansion and occasional sclerosis. These include destruction of pancreatic beta cells by streptozotocin injection to model type 1 diabetes, or genetic strains such as obese Zucker rats or the leptin receptor deficient *ob/ob* mice as models of type 2 diabetes. However, these rodent models have been lacking in some of the key characteristics of human diabetic nephropathy, most notably sclerosis, tubulointerstitial fibrosis and progressive uremia, and thus the NIH funded a multi-center consortium, the Animal Models of Diabetic Complications Consortium (AMDDC). The goal of the AMDDC was to create a better mouse model for study of diabetes and its complications.

Briefly, we examined response of multiple mice strains to injected streptozotocin, and found great variability both in hyperglycemic response, microalbuminuria and renal morphology, with most severe lesions being seen in KK/H^{lj} mice. Of note, C57BL/6J mice, a common background strain for numerous transgenic and knockout mice, were quite resistant to injury. Numerous manipulations of superimposed genetic abnormalities have been investigated in these various strains. Our preliminary data of AT1a $-/-$ mice show worse diabetic nephropathy than in wild type, and our more recent preliminary data indicate that even endothelial cell-specific knockout of the AT1a receptor results in paradoxically worse injury. Recent data

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HIV-Associated Nephropathy (HIVAN)

Finally, excellent animal models exist for human HIVAN. The characteristic morphological findings of collapsing lesions of the glomerular tuft with overlying podocyte proliferation, due to dedifferentiated podocytes and foot process effacement with accompanying tubulointerstitial fibrosis, inflammation and tubular injury are well-mirrored in several models of HIV nephropathy where mice have been made transgenic for HIV structural genes, either in all cells, or specifically in the podocytes. Of interest, injury in response to HIV transgene expression is also highly strain dependent, perhaps mirroring the difference in various human ethnic groups to HIVAN as a consequence of HIV infection. Specific mice transgenic for only single HIV structural genes have further shed light on which component of the HIV gene is necessary and sufficient to cause the HIVAN.

Summary

In summary, although many animal models exist, a rodent model that faithfully reproduces key elements of the most frequent and compelling cause of CKD in humans, namely diabetic nephropathy, is still lacking. Excellent models exist for short-term study of mechanisms of tubulointerstitial fibrosis. The varying models of primary or secondary FSGS vary in their fidelity to capture properties of human disease. Importantly and increasingly recognized is the strain dependence of severity of injury, making appropriate key controls essential for interpretation of data and extrapolation to human conditions.

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indicate that superimposing deficiency of endothelial cell nitric oxide synthase (eNOS) on the db/db mice also worsens injury. These mice had arteriolar hyaline, GBM thickening, mesangial expansion and occasional nodules. Importantly, these mice also showed dramatic albuminuria and decreased GFR to less than half of that seen in eNOS (+/+) db/db mice. Thus, these mice provide a robust and useful model of diabetic nephropathy.

Tubulointerstitial Fibrosis

Tubulointerstitial fibrosis is well modeled by inducing unilateral ureteral obstruction, which is effective in all background strains of mice tested and in rats. Although the mode of initial injury does not mirror common causes of tubulointerstitial fibrosis in humans, the model has numerous advantages. These include that the contralateral non-obstructed kidney can serve as an internal control, that animals do not develop hypertension or progressive uremia such that these systemic factors do not obfuscate basic intrarenal mechanisms of progressive fibrosis, and the lack of strain dependence of injury. In addition, the onset of injury is fairly rapid, with early changes of fibrosis occurring within several days, being well established by five rapid, and quite advanced by 10-14 days. We and others have used this model extensively to examine mechanisms of tubulointerstitial fibrosis in various transgenic mice and knockout mice with added pharmacological intervention. For instance, in one such series of studies, we investigated the dependence of tubulointerstitial injury on TGF β , using $\beta6^{-/-}$ mice. The heterodimeric integrin $\alpha_6\beta_6$ is expressed in epithelia in skin, lung and in the kidney. It can cleave TGF β from latency-associated peptide, and thus serves as one mechanism for local activation of TGF β . Interestingly, $\beta6^{-/-}$ mice were completely protected from fibrosis induced by UUO. However, superimposing exposure to angiotensin or aldosterone, commonly associated with renal fibrosis in numerous models, restored fibrosis to these mice. This fibrosis was not associated with activation of TGF β by other means, as seen by lack of phosphoSmad 2, but was associated with increased plasminogen activator inhibitor-1 (PAI-1). In other studies, separate manipulation of parenchymal versus bone marrow-derived cells has been done in this and also other models by creating chimeric mice using bone marrow transplant, allowing identification of injury related to each of these cell populations.

Glomerulosclerosis

Radiation Nephropathy

Multiple models of glomerulosclerosis with progressive uremia exist. The radiation nephropathy model is well established in the rat, but the C57BL/6J mouse strain is resistant. This model mirrors sclerosis that follows initial endothelial/thrombotic microangiopathy injury in humans, such as might occur not only after radiation, but also in chronic HUS. The model is non-hypertensive with gradual development of proteinuria. Morphologically, there is early endothelial injury with late sclerosis developing by week 12.

Puromycin Aminonucleoside and Adriamycin Nephropathies

Other models of progressive glomerulosclerosis have more in common with human idiopathic FSGS. These models are characterized by nephrotic range proteinuria and progression to ESRD. Most of these models have been developed in rats, with strain resistance in most mice to the selective epithelial cell toxins. Depending upon dosing and route of administration, e.g., i.v. or i.p., varying time courses of injury may be seen after injection with puromycin aminonucleoside (PAN). With single i.v. injection, there is an early abrupt onset of nephrotic syndrome that reaches its peak around day 8-10, with apparent resolution followed by long-term slow development of gradual proteinuria associated with FSGS lesions. Morphologically, there is early foot process effacement, which is maintained as sclerosis develops. Adriamycin causes a similar pattern of injury, but can only be administered

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intravenously. Unfortunately, most mice strains are resistant to either of these models. Balb/c mice are, however, susceptible to adriamycin. Thus, the course and initial abrupt onset of nephrotic syndrome mirror well many aspects of human idiopathic FSGS. A major drawback is the lack of widespread susceptibility in most mice background strains.

Remnant Kidney Model

Other commonly used models of segmental sclerosis mirror well the secondary sclerosis that occurs in many human CKD conditions. These models have progressive, usually non-nephrotic, proteinuria with progression to ESRD. There is development of hypertension, and proteinuria with sclerosis over 8-12 weeks in the widely studied rat remnant kidney model. Several variations of induction of this model exist, including polectomy, cauterization and ligation. The most hypertensive and rapidly progressive injury occurs with ligation of branches of the left kidney, along with right uninephrectomy, to produce a total of 5/6 nephrectomy. Although most rat strains are susceptible to induction of sclerosis by this model, most mice strains are resistant, including C57BL/6J. The 129Sv mouse strain is susceptible. Technically, induction of the model may be difficult in the mouse to achieve uniform ablation of renal tissue. We have therefore developed a combination approach to optimize development of hypertension and injury, with ligation associated with cauterization of some of the left kidney mass to obtain more uniform ablation of renal tissue. In the susceptible 129Sv mouse early sclerosis is evident by 6-8 weeks, with well-developed and widespread sclerosis with associated tubulointerstitial fibrosis by week 12. Changes are quite similar to those seen in human disease, with accompanying proportional vascular lesions and tubulointerstitial lesions. Of note, in some rat strains, including the Munich-Wistar rat, there are surface glomeruli, which have been widely used for direct puncturing and measurement of pressures and hemodynamic factors that could be involved in glomerulosclerosis.

Although these remnant kidney models in mice and rats do mirror many aspects of human disease, it is of interest that effects on sclerosis in the rat in our experiments have not correlated directly to the effects on proteinuria. In contrast, in the mouse, effects on sclerosis did correlate with effects on proteinuria. Whether these observations mirror the relatively short period of follow-up after intervention, with longer time required to normalize proteinuria and restore podocyte foot processes, is under investigation.

Podocyte-Specific Injury and Sclerosis

Specific injuries related to the podocyte will be discussed in detail elsewhere in this symposium. I will here briefly discuss specific use of mice models where the injury is very specifically directed only to the podocyte. In studies led by Ichikawa, we created mice transgenic for a toxin receptor only on the podocyte. When mice were injected with exogenous toxin, only cells expressing this receptor, i.e., only the podocytes were injured. This model, depending upon dose of toxin used, results in rapid proteinuria and development of sclerosis with dedifferentiation of the podocytes. Sclerosis developed by five weeks after injection of toxin. A similar model developed by the group of Wiggins et al. in the rat has shown the importance of podocyte depletion in inducing progressive sclerosis. Of interest, this concept has also been used to create chimeric mice where only some podocytes express the hCD25 receptor. These chimeric mice developed equally severe injury as mice with all podocytes expressing the receptor, supporting that injury may spread from podocyte to podocyte within the glomerulus. As there subsequently also is injury to the mesangial cell and endothelial cells, this model also allows study of mechanism of transmission of injury from the initially injured cell to other glomerular cells.

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In conclusion, there are a number of reproducible animal models of

glomerulonephritis which, I believe, have led to insights into pathogenesis that are

highly likely to be of relevance to human disease. However, it is disappointing that

there is very little to show for all the work that has been done on these models in

terms of rational development of new therapies.

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