

Mouse Models of Diabetic Nephropathy

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Mice provide an experimental model of unparalleled flexibility for studying mammalian diseases. Inbred strains of mice exhibit substantial differences in their susceptibility to the renal complications of diabetes. Much remains to be established regarding the course of diabetic nephropathy (DN) in mice as well as defining those strains and/or mutants that are most susceptible to renal injury from diabetes. Through the use of the unique genetic reagents available in mice (including knockouts and transgenics), the validation of a mouse model reproducing human DN should significantly facilitate the understanding of the underlying genetic mechanisms that contribute to the development of DN. Establishment of an authentic mouse model of DN will undoubtedly facilitate testing of translational diagnostic and therapeutic interventions in mice before testing in humans.

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Diabetic nephropathy (DN) is the major single cause of ESRD in the United States (1), with costs for care of these patients projected to be \$12 billion/yr by 2010 (1). Despite the high prevalence of DN, only 20 to 40% of all diabetic patients are prone to developing kidney failure, and family-based studies suggest that a significant genetic component confers risk for DN (2–4). Studies of diabetic mice suggest that, like people, mice exhibit differential susceptibility to diabetes and renal and cardiovascular diseases (5–7). In mice, identification of a strain that is prone to disease provides the relevant discriminator rather than identification of an individual who is at risk for diabetic complications. However, in contrast to people, each inbred mouse strain represents a genetically homogeneous and readily replenished resource that is amenable to repeated experimental study.

Biomedical experimentation in mice affords significant advantages over experimentation in other species. These advantages include the development of diverse and unique genetic resources, including completion of a detailed map of murine genomic sequence that is freely available through the internet, as well as >450 inbred strains of mice that have been generated over the past century, with each strain genetically being homogeneous and offering a unique array of phenotypes (8,9). The availability of murine embryonic stem cells has also provided the ability to disrupt the expression and function of specific

preselected genes (10–12). Finally, repositories of mice that bear multiple mutations that alter function in each known gene are gradually being assembled (13–15). These unique resources have the potential to significantly facilitate studies into the pathogenesis of disease in mice. Through the use of the unique genetic reagents that are available in mice (including knockouts and transgenics), the identification of a robust mouse model of DN should significantly facilitate understanding of the underlying genetic mechanisms that contribute to the development of DN in people as well as in mice. Establishment of a valid mouse model of DN should also facilitate testing of translational diagnostic and therapeutic interventions in mice before testing in humans.

Working Definition of DN

In humans, DN manifests as a clinical syndrome that is composed of albuminuria, progressively declining GFR, and increased risk for cardiovascular disease (16,17). The occurrence of cardiovascular disease is an integral component of DN and underscores the systemic nature of this disorder, of which nephropathy is only one aspect. Another key aspect of DN in humans is that it is a late complication of diabetes, occurring progressively in susceptible people only after 15 to 25 yr of diabetes (18–20). Because of issues of cost and convenience, most studies of DN in mice have focused on the earlier harbingers of DN, including the development of albuminuria and histopathologic changes, and have not explicitly used renal insufficiency as an end point (21,22).

Diabetic albuminuria in humans is associated with the development of characteristic histopathologic features, including thickening of the glomerular basement membrane (GBM) and mesangial expansion. As albuminuria progresses and renal

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insufficiency ensues, glomerulosclerosis, arteriolar hyalinosis, and tubulointerstitial fibrosis develop (23). These pathologic features correlate well with GFR in humans with diabetes and kidney disease (24,25) and would be important features in a robust mouse model of DN.

Characterization of DN in Mice

The major deficiency in animal models of DN is the absence of renal failure. Whereas animal models of diabetic kidney disease exhibit albuminuria and some of the characteristic pathologic changes, reports of renal failure resulting from diabetes in mice or rats are lacking. Furthermore, associated increased risk for cardiovascular disease, neuropathy, and retinopathy have been poorly characterized. Whether the inability to detect renal failure in mice is simply a consequence of studying mice with established diabetes for an insufficient time period or the absence of renal failure reflects an intrinsic resistance to nephropathy of the strains studied thus far remains uncertain.

In contrast to the absence of renal failure, several of the more short-term consequences, including the development of glomerular hyperfiltration, increased albuminuria, and some of the characteristic histopathologic changes, can be detected in animal models. Human DN proceeds through several distinct pathophysiologic stages, including an early stage of glomerular hyperfiltration, followed by the so-called silent phase in which GFR returns to normal (26). This is followed by the sequential development of microalbuminuria, dipstick-positive proteinuria, and then a progressive decline in GFR leading to ESRD (20,27,28). An optimistic view is that the detection of the early functional and histopathologic changes in mouse models of diabetes reflects inadequate duration of hyperglycemia before the study of these models.

Assessment of Hyperglycemia in Mice

In humans, the degree of hyperglycemia is a critical determinant of risk for developing DN (29,30). Similar studies have not been systematically performed in mice, but in small studies, nephropathy seems to correlate with the severity of hyperglycemia. Measurement of glucose control in mice is generally achieved by determination of fasting blood glucose or determination of glycosylated hemoglobin.

Fasting Blood Glucose

Because mice are nocturnal feeders, an overnight fast before measuring blood glucose usually translates into a more prolonged fast of approximately 24 h. This 24-h fast can activate several physiologic counterregulatory mechanisms that obscure the reliability of glucose readings, as a result of this significant distress. Because of this, fasting should be started on the morning of blood sampling. The National Institutes of Health (NIH) has established mouse metabolic phenotyping centers (www.mmmpc.org) that have developed a standard protocol that is composed of a fast between 7 a.m. and 1 p.m. with blood drawn at 1 p.m. This protocol has been adapted by the Animal Models of Diabetic Complications Consortium (AMDCC; www.amdcc.org), another NIH consortium whose

goal is to identify and establish mouse models of diabetic complications.

Glycosylated Hemoglobin (Including Hemoglobin A_{1c})

When carbohydrates become nonenzymatically linked to hemoglobin, these species can be separated from normal adult hemoglobin (designated HgbA₀) either by altered charge or by formation of a stable complex between the coplanar cis-diol groups of glycated hemoglobin in an affinity column and are collectively designated as HgbA₁ (31,32). These hemoglobins can be separated further into HgbA_{1a}, HgbA_{1b}, and HgbA_{1c}, among which HgbA_{1c} is the main glycated form in humans (33). HgbA_{1c} has been used widely to assess glucose control in people, and by extrapolation, it has been widely used to assess glucose control in diabetic mice. Studies in diabetic mice are consistent with the validity of the use of HgbA_{1c} to assess glucose control (34,35), although levels of the various glycosylated hemoglobin assays have not been correlated rigorously to fasting blood glucose values in mice.

Whereas HgbA_{1c} correlates well with glucose control in people (36), there seem to be some human populations that exhibit substantially different HgbA_{1c} values despite similar levels of glucose control (37). This observation has been attributed to differences in red cell life span rather than rates of glycation (38). Another intriguing possibility is that people with different endogenous rates of glycation exist despite similar levels of hyperglycemia and that “high glycaters” exhibit greater susceptibility to DN and retinopathy (39). Variability in red cell life span among strains of mice seems likely and may have a significant impact on interpretation of HgbA_{1c} levels (35,40,41).

Assessment of Renal Function in Mice

GFR

A serious issue impeding the evaluation of renal function in mice has been the lack of a simple, reproducible method to estimate GFR in conscious mice. As in humans, blood urea nitrogen levels are extremely sensitive to extracellular fluid volume depletion and may be artificially elevated in the individual with diabetes from volume depletion resulting from the osmotic diuresis of hyperglycemia. The use of serum creatinine as a tool to evaluate renal function in mice has also been called into question since Meyer *et al.* (42) reported that creatinine levels in plasma of mice measured by the Jaffé alkaline picrate method yielded significantly higher levels (three to five times) than those measured by HPLC. These studies concluded that the Jaffé method greatly overestimates the plasma creatinine levels, and attributed this increase to noncreatinine chromagens. Other studies have found similar assay-dependent variability in the accuracy of creatinine as a measure of renal function in mice (43,44) and in humans (45).

The impact of these cross-reacting chromagens on apparent levels of plasma creatinine may be significantly affected by ketoacidosis (*e.g.*, as occurs in diabetes) (46,47). Because absolute creatinine levels as measured by HPLC are substantially lower in mice (0.128 ± 0.026 to 0.207 ± 0.012 mg/dl) than in humans (48,49), the impact of these chromagens on apparent creatinine values may be significantly greater in normal mice

than in normal humans. In mice with normal renal function, the picric acid–based method routinely overestimates HPLC-determined serum creatinine by two- to fivefold, yielding apparent serum creatinine values of 0.6 to 1.0 mg/dl (48,49). Conversely, enzymatic creatininase methods coupled to a peroxidase indicator more closely agree with HPLC methods, providing another potentially useful method for determination of serum creatinine in mice (44). Despite the availability of this information regarding the inaccuracy of serum creatinine measured by the Jaffé reaction, the assay remains widely used by the biomedical research community to assess renal function in mice. Thus, artifactual increases in serum creatinine likely contribute to reports of decreased creatinine clearance in many studies that examine DN in mice.

With the increasing use of mice as an experimental model of diabetic kidney disease, awareness of these deficiencies must be heightened. Several approaches have been adopted to re-address this problem. New methods to accurately measure plasma creatinine measurements by HPLC have been established and have confirmed significant overestimation of plasma creatinine using picrate-based methods. When compared with inulin clearance, creatinine clearance determinations using the Jaffé reaction underestimates GFR by >50% (48,49).

Measurement of GFR using inulin clearance, the traditional gold-standard measurement, has also been adapted for use in mice. Clearance of FITC-labeled inulin has been measured in both anesthetized and conscious mice (50,51). Serial measurements of inulin clearance can be determined in conscious mice over a period of months using an intravenous bolus FITC inulin and measuring the decay rate of inulin in plasma (50). Using these techniques, substantial differences in GFR between mice of different strains and sexes have been reported (50,52,53). Consideration of the effects of surgical preparation and/or anesthesia is appropriate when GFR determination is performed in anesthetized mice. It remains to be determined how well HPLC-determined creatinine clearance correlates with FITC inulin clearance in individual mice. Nevertheless, previously published studies in which GFR has been estimated in mice using plasma creatinine values determined by picrate-based methods should be reevaluated carefully in light of the aforementioned problems.

BP

There are several proven methods for monitoring BP in mice that can be applied to the study of diabetic mouse models. Indirect measurements using tail-cuff manometry systems are simple and noninvasive. Their reliability and reproducibility have been well documented across a number of experimental systems (54). These systems measure BP in conscious mice and are inexpensive and adaptable to most laboratory settings. However, they lack the precision and the sensitivity of systems that directly measure intra-arterial pressure. This approach is also complicated by physiologically apparent stress in tested mice that can affect the level of BP, but this can be minimized by training mice adequately beforehand. In our experience, tail-cuff manometry is a useful approach to screen for alterations of BP in mouse models, realizing that very modest

differences may be missed because of the relative insensitivity of this method. Using this approach in studies under the auspices of the AMDCC, we have found that BP tend to be reduced in most strains of streptozotocin (STZ)-treated mice when significant hyperglycemia is present (54A). Tail-cuff measurements have also been reported by others in models of diet-induced diabetes and does not seem to be dramatically affected by diabetes (55) and genetically altered mice that are treated with STZ (56,57).

Intra-arterial pressures can also be measured directly in conscious mice. These measurements can be accomplished by cannulating arteries with catheters that are tunneled under the skin, exteriorized, and connected to external transducers and monitors via a tether apparatus. Although this approach requires significant surgical and technical proficiency, it is relatively inexpensive and accessible to most laboratories, providing a reasonable method for verifying BP differences that have been detected by indirect methods. However, it is difficult to maintain line patency for recording BP beyond 1 to 2 wk after catheter placement. The recent development of radiotelemetry units for intra-arterial BP measurements in mice has provided a more suitable approach for chronic measurement of intra-arterial pressures in conscious, unrestrained animals (58). These units provide the capacity to obtain direct and continuous measurements of intra-arterial pressure 24 h/d over a period of 2 to 3 mo. Accordingly, they should prove very useful for correlating levels of BP with the extent of kidney injury in experimental models. The major disadvantage of this system is expense. The initial capital outlay for a small-scale telemetry unit is typically twice that of most tail-cuff monitors. Furthermore, individual transmitters cost approximately \$3000 each and must be refurbished periodically for an additional fee.

Albuminuria. Before developing renal failure, humans with overt DN typically develop nephrotic-range albuminuria (albumin excretion rates exceeding 3 g/24 h). Preceding this, albumin excretion rates gradually and progressively increase over the course of years through a phase of microalbuminuria (between 30 and 300 mg/24 h that is negative by urine dipstick), to albuminuria >300 mg/24 h, a phase that is characterized by an overtly positive urine dipstick (19,59,60). *A priori*, it is expected that mice that develop DN should progress through similar phases so that the amount of albuminuria should roughly correlate with the severity of DN.

Albuminuria has been quantified in approximately 100 studies of DN in mice and in the absence of accurate measures of GFR probably remains the best functional surrogate for severity of DN. However, comparisons of the severity of nephropathy in the diverse models of diabetes reported are confounded by use of diverse methods (*e.g.*, quantifying total urine protein, anti-rat antibodies or anti-human antibodies to measure mouse albumin on ELISA), differences in units of reporting (*e.g.*, ng/min, $\mu\text{g}/24\text{ h}$, $\mu\text{g Alb}/\text{mg Cre}$; $\mu\text{g Alb}/\mu\text{Mol creatinine}$, total urine protein $\mu\text{g}/24\text{ h}$, $\mu\text{g Alb}/\text{ml urine}$), and nonstandardized methods of urine collection.

Quantification of proteinuria in mice by dipstick is probably inaccurate (unpublished results). The exact reasons for this are not firmly established; however, the inaccuracy of the dipstick

proteinuria test in mouse urine may be a consequence of sensitivity of this assay to abundant major urinary proteins, normally present in mouse urine (61,62). Albuminuria determined by an immunoassay generally provides a reliable assessment of proteinuria as a result of glomerular injury. Use of antibodies raised against nonmurine albumin may be particularly problematic as mouse albumin amino acid sequence is only 89% identical to rat albumin and 73% identical to human albumin, allowing for differences in the sensitivity of immunoassays. The AMDCC has chosen to use an anti-mouse albumin ELSIA kit and report albuminuria as $\mu\text{g}/24$ h urine collection or μg albumin/mg creatinine. A standardized assay used by consortium members is available at the AMDCC web site (www.amdcc.org).

The values of urine albumin excretion rates in mice that signify glomerular disease have not been firmly established. Furthermore, it is likely that significant variation in albumin excretion rates exist between inbred mouse strains. In the absence of well-defined normal values, a first approximation of abnormal albuminuria would be to extrapolate from human disease and test for values 10-fold greater than nondiabetic controls as reflective of incipient disease. Urine albumin excretion

rates 100- to 1000-fold more than controls should reflect established diabetic renal disease. We have attempted to compare the severity of diabetic albuminuria in various strains and diabetic models studied by limiting our analysis to those studies performed on a 24-h urine collection and using assays specific for mouse albumin (Figure 1). A single study in KK-Ay mice that were fed a high-cholesterol diet suggested that a 1000-fold increase in albuminuria may be achieved in this model; however, whether renal failure ensues in this case remains to be determined (63).

Histopathology. In human DN, renal histopathologic findings include diffuse thickening of the GBM between the endothelium and the podocyte, together with prominent mesangial expansion. This is accompanied by diffuse and sometimes nodular mesangial sclerosis, as well as arteriolar hyalinosis and tubulointerstitial fibrosis (24,25,64). The best histopathologic correlate of renal function in patients with diabetes is fractional volume of the mesangium, an index of mesangial expansion (24,25). Tubulointerstitial volume also correlates well with renal function.

Given the absence of documented renal failure in mouse models of DN, whether specific pathologic criteria are associated with or predictive of renal insufficiency cannot be readily formulated.

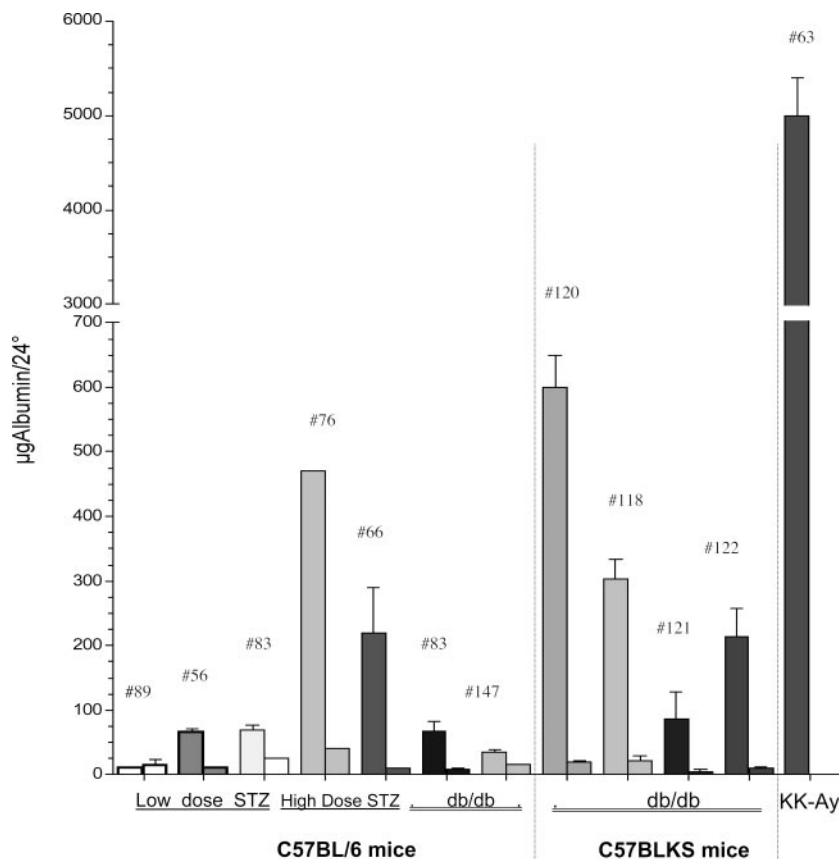


Figure 1. Summary of studies in the literature examining 24-h albumin excretion in mouse models of diabetes (#, reference number). For each study, a pair of bars represent mean \pm SE. On the left, 24-hr albumin excretion in the diabetic cohort; on the right, the nondiabetic cohort. From left to right are albuminuria in C57BL/6 mice treated with multiple low-dose streptozotocin (STZ); C57BL6 high-dose STZ; C57BL6 *db/db* mice, a model of type 2 diabetes; four studies measuring albuminuria in the C57BLKS *db/db* strain show generally higher albuminuria; a single study examining 24-h albuminuria in the KK-Ay strain shows robust albuminuria.

GBM thickening has been reported in several but not all mouse models of DN (65–68). In mice, nodular glomerular sclerosis is generally absent as is arteriolar hyalinosis, two features generally seen only in people with more advanced renal disease (24). The reproducible identification of these two lesions in mouse models of DN remains a benchmark yet to be achieved.

Models of Type 1 Diabetes

High-Dose STZ

STZ-induced type 1 diabetes (Table 1) has been used widely as a model for DN; however, interpreting results in this model may be complicated by nonspecific toxicity of STZ. STZ was

Table 1. Some mouse models of diabetes studied for diabetic nephropathy^a

Mouse Model (Reference No.)	Strains Reported (Reference No.)	Diabetic Type	Advantages	Disadvantages
Streptozotocin (71,81,146, 228–232)	C57BL/6J, C57BLKS, Balb/c, ICR, DBA2, ROP	Type 1	Well established, reproducible timing; may be established in strains both resistant and susceptible to DN	Potential for nonspecific toxicity; strain-dependent dosing necessary; biohazard: potential mutagen
Encephalomyocarditis virus D variant (153,233,234)	DBA, Balb-C	Type 1	May reproduce viral causes of type 1 diabetes in humans; DBA may be prone to DN	Potential for nonspecific renal effects; strain-dependent dosing necessary; biohazard; not widely studied; renal functional effects not characterized
Ins2 Akita (84,88,89)	C57BL/6, C3H	Type 1	Commercially available (JAX); autosomal dominant mutation	Presently only C57BL/6 commercially available; C57BL/6 relatively resistant to nephropathy; hyperglycemia in females is mild
NOD (101,102,235,236)	Inbred line derived from ICR (outbred line)	Type 1	Spontaneous development of β -cell failure may mimic pathophysiology of disease in humans (99); commercially available	Unpredictable timing of development of diabetes; no appropriate control strain; needs insulin therapy to survive long periods; multigenic-cause diabetes precludes easy intercrosses
<i>Db/db</i> (22,134)	C57BL/6, C57BLKS, DBA, FVB (129), CBA (237)	Type 2	Available on multiple strains; commercially available	Infertile; autosomal recessive; mutation in leptin receptor is a very rare cause of obesity and type 2 diabetes in humans
<i>Ob/ob</i> (21)	C57BL/6 (238), FVB/N (238), DBA2 (239,240)	Type 2	Exists on diverse inbred strains; nephropathy uncharacterized; commercially available	Infertility—can be circumvented with leptin; autosomal recessive; nephropathy uncharacterized; mutation of leptin is a very rare cause of obesity and type 2 diabetes in humans
Agouti (Ay)	KK (170,241,242), C57BL (243), C3H (243), FVB (244)	Type 2	KK strain is susceptible to renal injury with significant albuminuria; autosomal dominant; commercially available	Hyperglycemia moderate in males more than in females; onset of diabetes is not well defined; nephropathy may not be robust in strains other than KK
High-fat diet (112)	C57BL/6 is main susceptible strain (245)	Type 2	Onset can be determined by the investigator	Only C57BL/6 reported as susceptible; hyperglycemia not prominent

^aDN, diabetic nephropathy.

originally identified as an antibiotic (69) composed of a glucosamine-nitrosourea. It was soon recognized to cause diabetes in animals secondary to pancreatic β cell failure (70,71). STZ is presumed to be especially toxic for pancreatic β cells because its glucose moiety is avidly transported into β cells by GLUT2 (72); however, it may be toxic to a variety of other tissues. Given the relative resistance of mice to single doses of STZ (requiring approximately 150 to 200 mg/kg to obtain chronic hyperglycemia), substantial collateral tissue toxicity may occur (73–75). This model of type 1 diabetes typically develops albuminuria (66,76,77), even on the relatively sclerosis-resistant C57BL/6J background (see below); however, the potential for collateral tissue toxicity complicates the interpretation of the cause of this albuminuria. Evidence for robust oxidative stress in STZ-induced diabetes but not other hyperglycemic models of diabetes (e.g., *db/db* and KK mice) has been reported (78). Mice that receive high-dose STZ develop more albuminuria than mice that receive the low-dose STZ regimen (see Figure 1 and text below), despite exhibiting similar levels of hyperglycemia, also consistent with an STZ effect independent of hyperglycemia. Nevertheless, it should be noted that the aforementioned potential for nonspecific renal toxicity has not been proven rigorously and that the high-dose STZ model of diabetes is still widely accepted and commonly used. It also should be noted that reversibility of diabetic lesions after treatment of mice with insulin to normalize plasma glucose would provide another means of excluding nonspecific toxicity as a result of high-dose STZ.

Low-Dose STZ

To mitigate nonspecific cytotoxicity, multiple low-dose STZ injections have been used to induce diabetes, causing repetitive low-grade β cell damage accompanied by lymphocytic infiltration of the pancreatic islets (79,80). This regimen typically calls for daily intraperitoneally injections of 40 to 50 mg/kg STZ for 5 d (71,81). It is notable that inbred strains of mice exhibit substantial differences in susceptibility to both β cell toxicity (82) and nonspecific toxic effects of low-dose STZ (71), so dose adjustments should be made. In general, when administered in strain-appropriate doses, the low-dose STZ regimen produces comparable levels of hyperglycemia as those obtained using the high-dose regimen. The levels of albuminuria (56,83) are generally lower with low-dose STZ than with high-dose STZ, possibly reflecting reduced direct renal toxicity (Figure 1). In addition, we have confirmed that there is no loss of podocyte markers such as WT-1 or apparent decrement in podocyte number 2 wk after completion of the low-dose STZ regimen in C57BL/6J mice (Siu *et al.*, unpublished data), suggesting that this regimen has no toxic effects on podocytes.

Our experience using low-dose STZ in mice has shown that hyperglycemia occurs within 2 wk of the low-dose STZ regimen (for the regimen used, see www.AMDCC.org). Albuminuria develops to a variable degree (depending on strain of mice; see below) within 5 wk. At this early time point, the major histologic feature is glomerular hypertrophy, so it is likely that hemodynamic changes contribute to the development of this early albuminuria. As time progresses, mesangial expansion

and, in some strains, mesangial sclerosis develop. Only a minority of mouse strains (e.g., KK) develop evidence of arteriolar hyalinosis or nodular glomerulosclerosis. These changes occur later (15 to 30 wk of hyperglycemia). At these later time points, the diabetic mice may exhibit significant weight loss, possibly as a result of catabolic effects of insulin deficiency, as well as volume depletion associated with the osmotic diuresis. This may have a significant impact on subsequent survival and more long-term studies. To circumvent this occurrence, intermittent treatment with insulin, sufficient to reverse weight loss but maintain hyperglycemia, may be desirable.

Insulin-2 Akita

To circumvent the potential nonspecific tissue toxicity that occurs in the STZ model of type 1 diabetes, one may use the recently described insulin-2 Akita (*Ins2^{Akita}*) mouse mutant model of type 1 diabetes (84–86). These mice develop pancreatic β cell failure as a result of β cell-selective proteotoxicity resulting from misfolding of insulin2 (87). The mice are commercially available through the Jackson Laboratories. Although originally reported to represent a model of maturity-onset diabetes of the young with insulin resistance, subsequent studies clearly show that this model is insulin responsive. Islets from *Ins2^{Akita}* mice are depleted of β cells, and those remaining β cells release very little mature insulin. This finding together with the finding that mutant mice respond to exogenously administered insulin (84) indicates that *Ins2^{Akita}* mice should serve as a substitute for mice that are rendered insulin dependent diabetic by treatment with alloxan or STZ.

The *Ins2^{Akita}* mutation is autosomal dominant. Mice heterozygous for the Akita spontaneous mutation (*Ins2^{Akita}*) are viable and fertile. Mice homozygous for the *Ins2^{Akita}* allele exhibit failure to thrive and die within 1 to 2 mo. Symptoms in heterozygous mutant mice include hyperglycemia, hypoinsulinemia, polydipsia, and polyuria beginning at approximately 3 to 4 wk of age.

At present, this mutation exists on the C57BL/6 and C3H/He strains (88). Studies of *Ins2^{Akita}* mice on the C57BL/6J background show that the hyperglycemia is sexually dimorphic, with the hyperglycemia being substantially worse in male mice than in female mice. As seen with other models of DN in the C57BL/6J strain, albuminuria is not a prominent feature ($10.23 \pm 2.73 \mu\text{g}/24 \text{ h}$ in diabetic mice versus $13.9 \pm 8.8 \mu\text{g}/24 \text{ h}$ in controls) (89). Renal immunopathologic studies show significant deposition of IgA in the glomeruli of Akita mice (88); however, given the association of IgA deposition in glomeruli of up to 20% of humans with diabetes (90) and rat models of diabetes, the significance of these findings is uncertain (91–93). Overall, the severity of DN in the C57BL/6J *Ins2^{Akita}* mouse does not seem to be robust.

Nonobese Diabetic Mouse

The genetic mouse model of type 1 diabetes that has been studied most thoroughly is the nonobese diabetic (NOD) mouse (94–96). These animals develop spontaneous autoimmune destruction of β cells at approximately 5 mo of age, although the precise age of onset of diabetes is somewhat

variable. However, unlike STZ mice, insulin treatment is required to maintain NOD mice for any extended time after the onset of hyperglycemia, indicating more complete insulin deficiency. Moreover, unlike enhanced susceptibility in male mice for STZ diabetes, there is a female predominance in the NOD line with a female:male ratio of approximately 4:1. The characteristics of autoimmune disease contributing to β cell failure in NOD mice have been studied extensively, and the model has a number of similarities with features of human type 1 diabetes (97–99). These include (1) inheritance of specific MHC class II alleles and many non-MHC loci as polygenic susceptibility loci, (2) transmission of the disease by hematopoietic stem cells, (3) the development of an intraislet inflammatory infiltrate (insulinitis) with anti-islet cell antibodies, and (4) a strict dependence of disease on T cells.

Despite the intensive study of the immunopathogenesis of islet cell destruction in the NOD mouse, very little work has been done to study complications of diabetes in this model. In part, this is because of the late and somewhat variable age of onset of diabetes and the requirement for more intensive management, including daily administration of exogenous insulin. In addition, the genetics of the NOD model is complex. The line was initially developed from an outbred ICR mouse line (94–96). Moreover, there are six or seven background loci that must be retained for development of disease. Accordingly, identifying an appropriate nondiabetic control strain has been a problem. Nonobese nondiabetic (NON) mice were derived from nondiabetic progeny in the original crosses that generated the NOD line. However, NON mice contain a diabetes-resistant MHC haplotype (K^b , I-A^{nb1}, I-E^k, D^b) that is distinct from the NOD haplotype (K^d , I-A^{s7}, I-E^{null}, D^b) associated with diabetes susceptibility (100). Although some studies have used the NON animals as controls for the NOD mouse, NON mice seem to develop spontaneous renal disease of uncertain cause (101). Thus, their utility as controls for studies of DN is questionable.

As discussed above, there have been relatively few studies of kidney disease in the NOD line. Nonetheless, those few studies indicate that albuminuria develops in hyperglycemic NOD animals. The levels of albuminuria, assessed by albumin-to-creatinine ratio, are roughly sevenfold higher than in NOD mice before development of hyperglycemia (102). Furthermore, studies of DN using this model have supported roles for TGF- β and advanced glycosylation end products (AGE) in the pathogenesis of mesangial proliferation and sclerosis (103–106). Because of such results along with the pathogenic similarities between NOD mice and humans with type 1 diabetes, this strain is likely to have utility for developing new models of diabetic complications. To circumvent the problems posed by its complicated genetics, AMDCC is working to generate embryonic stem cell lines from the NOD line to facilitate applications of transgenesis and gene targeting to our efforts at model development.

Mouse Models of Type 2 Diabetes

High-Fat Diet

A high-fat diet provides a commonly used approach to induce obesity and insulin resistance in C57BL/6 mice (107–109)

and is particularly useful for the study of accelerated atherosclerosis (104,110,111). The effect of a high-fat diet seems to depend on the strain of mouse studied, with A/J mice being relatively resistant to this effect (107). Although one study suggested that this syndrome was associated with the development of hypertension, a subsequent study failed to confirm this and further found no change in GFR (55,112). To our knowledge, the effects of high fat on albuminuria in these models has not been reported, although one study examined its effect on albuminuria in the KK-Ay mouse and found a dramatic effect (63).

LepR^{db}/LepR^{db}

The *db/db* mutation on the C57BLKS background has been investigated intensively and exhibits many features similar to human DN. Details regarding the identification and nomenclature regarding the *db/db* mouse may be found in a recent review (22). The diabetic gene (*db*) is transmitted as an autosomal recessive trait. The *db* gene encodes for a G-to-T point mutation of the leptin receptor, leading to abnormal splicing and defective signaling of the adipocyte-derived hormone leptin (113,114). This obese and diabetic mutant (*db*) was initially recognized in the C57BLKS/J strain and was subsequently also backcrossed to a pure C57BL/6J background. The C57BLKS/J mouse shares 84% of its alleles with the common C57BL/6 strain and 16% with the DBA/2J strain and was initially maintained by Dr. N. Kaliss (148).

In the C57BLKS/J *db/db* mouse, hyperinsulinemia is noted by 10 d of age, and blood glucose levels are slightly elevated at 1 mo of age (7.2 ± 2.3 mM) (115). The *db/db* mouse develops frank hyperglycemia with glucose values of 9.7 ± 1.6 mM by 8 wk of age and 15.7 ± 4.3 mM at 10 wk of age (115). Progressive hyperglycemia is noted with mean levels of glucose of 28.6 ± 13.2 mM at 16 wk of age (115,116). After 5 to 6 mo of age, the body weight and insulin levels begin to fall in association with pancreatic islet cell degeneration (117).

By 2 to 4 mo of age, diabetic mice have a 20 to 30% increase in glomerular size (118). Glomerular hypertrophy at the onset of diabetes may be due to alteration of glomerular hemodynamics as there is evidence of glomerular hyperfiltration in *db/db* mice during the early stages of diabetes (119). After 16 wk of age, there is a very consistent threefold increase in mesangial matrix expansion based on several independent studies (reviewed in reference 22). The range of albuminuria is between 68 and 600 $\mu\text{g}/24$ h in the *db/db* male mouse (22,118,120–122), whereas it is between 4 and 21 $\mu\text{g}/24$ h in the age-matched heterozygous littermate (118,122). The degree of albuminuria does not consistently increase with the duration of diabetes as there are similar levels of albuminuria between 8 and 25 wk (118,122–124). Arteriolar hyalinosis has been described in this model; however, there is virtually no evidence of advanced tubulointerstitial fibrosis.

The progression of diabetes in *db/db* mice on the C57BL/6 background in the diabetic phenotype is less severe than that in C57BLKS/J, and as these mice age, plasma glucose seems to normalize (22,125–129). More recently, some investigators have reported observing that a subset of approximately 50% of

C57BL/6J *db/db* mice develop more persistent hyperglycemia (130,131). In these mice, more robust albuminuria and renal histopathologic diabetic changes have been reported (130,131). Unfortunately, the factors that cause this subgroup of C57BL/6J *db/db* mice to develop persistent hyperglycemia remain to be elucidated. Nevertheless, although the leptin mutation may not produce as robust a model for DN on the C57BL/6 background as it does on the C57BLKS/J background, it does provide a clear advantage for genetic studies because most transgenic and knockout strains are available on this background and can cleanly introgressed onto this strain.

Ob/ob

As opposed to the *db/db* mutants, the *ob/ob* recessive obese mouse carries a mutation in leptin, the ligand for the leptin receptor (132,133). The *Lep^{ob}* mutation exists on DBA2/J and C57BL/6J strains (132,134). Renal structure and function in C57BL/6J *ob/ob* mice is said to be relatively mild (21), but primary studies to support this statement are obscure.

Agouti Mutation

The agouti gene product encodes a 131–amino acid protein that contains a signal sequence. It is produced in the hair follicle, where it acts in a paracrine manner as a high-affinity antagonist of the melanocyte stimulating hormone (MSH) receptor on melanocytes to inhibit α -MSH–induced eumelanin production, causing their yellow coat color (135). Similarly, an agouti-related protein is a potent and selective antagonist of melanocortin receptors 3 and 4 (*Mc3r* and *Mc4r*), receptor subtypes, which are expressed in the hypothalamus and implicated in weight regulation (136). Mice that carry the dominant homozygous lethal mutations in agouti (*A^y*) or viable (*A^{vy}*) exhibit a complex phenotype of obesity and insulin resistance in addition to yellow fur. The unifying molecular feature of all yellow obese Agouti mutations is that they confer ubiquitous and strong expression of the wild-type agouti protein through use of an alternative transcriptional promoter (137,138). Reports of albuminuria in diabetic KK-*Ay* mice suggest that this mutation may be useful for the study of nephropathy (see below and Figure 1) (139,140).

New Zealand Obese Mice

New Zealand obese (NZO) mice have a polygenically inherited form of obesity and type 2 diabetes (141–143). They may be prone to autoimmune disease as the kidneys exhibit light microscopic features of both diabetic and lupus nephropathies: Glomerular proliferation, mesangial deposits, mild basement membrane thickening, and glomerulosclerosis (144). Eosinophilic nodules may be seen in some glomeruli, with occasional hyalinization of the glomerular arterioles and healing arteriolar inflammation (144).

From the preceding discussion, it should be apparent that diverse models of both type 1 and type 2 diabetes are available in mice. Although renal changes have been reported in many of these models, renal failure has not as yet been identified as a consequence of diabetes in any of these models. Whether this is a result of inadequate length of diabetes before study or un-

derlying resistance of specific strains to nephropathy remains to be addressed.

Inbred Mice: Strain Dependence of DN

Studies have identified certain strains (*e.g.*, 129, ROP, NON, KK/HIJ) as more prone to glomerulosclerosis than other more commonly studied strains (*e.g.*, C57BL/6, FVB/NJ) that seem relatively resistant to renal disease. It is noteworthy that studies of DN have been performed in fewer than 5% of the available mouse strains. The following section overviews the literature regarding the sensitivity of the kidney to diabetes in the major strains studied so far.

C57BL6

Although C57BL/6 is the most widely used inbred strain, studies of kidney disease in this strain suggest that it is relatively resistant to renal injury (145,146) (other than a possible subgroup, mentioned below, regarding a cohort of this strain that may be more susceptible to DN in the *db/db* model). The C57BL/6J strain provided the DNA source for the first high-quality draft sequence of the mouse genome. This strain is used in a wide variety of research areas, including cardiovascular biology, developmental biology, diabetes, and obesity. Overall, C57BL/6 mice breed well, are long lived, and have a low susceptibility to tumors. Other characteristics include a high susceptibility to diet-induced obesity, type 2 diabetes, and atherosclerosis. C57BL/6J mice that are fed a high-fat diet develop obesity, hyperglycemia, and hyperinsulinemia (55,112); however, this does not seem to have an impact on renal function (112). In contrast to their resistance to glomerulosclerosis, C57BL/6J mice provide a reasonably robust strain for studying of atherosclerosis when fed an atherogenic diet (111). In the experience of the AMDCC and others, the leptin receptor (*db/db*) mutation in this background often results in relatively mild and transient hyperglycemia and modest albuminuria (22,147) when compared with the presence of this mutation in the C57BLKS strain (see below).

C57BLKS (Formerly C57BL/KsJ)

The C57BLKS strain was originally derived from C57BL/6J mice maintained by Dr. N. Kaliss (148) and may be somewhat more susceptible to renal disease than the parental C57BL/6. Genomic analysis shows that 84% of the alleles in C57BLKS are shared with C57BL/6 and 16% are shared with DBA/2J, consistent with genetic contamination of the C57BL/6J progenitors early in the strain's history (148). Studies indicate that on the C57BLKS background, type 2 diabetic *db/db* mice have lesions consistent with DN (thickened basement membrane, mesangial expansion), whereas they are resistant to DN on a C57BL/6 background (22,149–151). Although these models have not been completely characterized, this difference suggests two possibilities: Either the worse severity of hyperglycemia that develops in C57BLKS causes this (111) (a combination of peripheral insulin resistance and insulin deficiency develops in C57BLKS^{*Lepr^{db}*}, whereas C57BL/6^{*Lepr^{db}*} have only peripheral insulin resistance), or C57BLKS expresses modifier genes that predispose to DN. These mice seem to be relatively prone to

atherosclerosis (111) and also develop myocardial disease (152). In support of the former possibility is the general experience including that of the AMDCC that fasting blood glucose is sustained at much higher levels in *db/db* mice on this background than in *db/db* mice on a C57BL/6J background.

DBA/2J

In contrast to C57BL/6, the DBA/2J strain seems to be prone to diabetic albuminuria (153,154), but this remains to be rigorously tested. DBA/2J are relatively resistant to the development of atherosclerosis on a semisynthetic high-fat diet (155) and are hyporesponsive to diets that contain high levels of fat and cholesterol (156). Spontaneous calcified heart lesions progress with age, and dystrophic cardiac calcification may be related to disturbed calcium metabolism (157,158).

129/SvJ

Multiple substrains of 129 exist, and care must be taken to determine the precise genetic composition of the specific 129 substrain in use for appropriate interpretation of data. This strain of mice has been found to be more susceptible to elevated BP, nephrosclerosis (both glomerular and tubulointerstitial), and albuminuria in the setting of DOCA-salt hypertension than the C57BL/6 strain (159). In addition, 129/Sv mice develop more significant glomerulosclerosis, proteinuria, increases in BP, and apparent renal failure than do C57BL/6 mice after 5/6 nephrectomy (145). This susceptibility to renal injury and hypertension makes 129/Sv a potentially attractive strain for DN studies. No reports to date have compared renal or BP effects of diabetes in animals on this background with C57BL/6J mice or other strains. Unfortunately, the 129/J strains of mice with the *db/db* mutation are resistant to hyperglycemia (40).

ROP

The ROP strain of mouse originated from a heterogeneous stock being heterozygous for three mutant alleles: *Ra/+* (ragged), *Os/+* (osteosyndactylism), and *Pt/+* (pintail). Studies of *Os/+* ROP mice show that they possess approximately 50% fewer nephrons in the *Os/+* mice than in the *+/+* mice (160). In general, wild-type mice (*Os^{+/+}*, *Ra^{+/+}*, and *Pt^{+/+}*) of ROP background seem to be more prone to glomerulosclerosis than C57BL/6 mice after a reduction in renal mass (161–163). Mice on the ROP background, in the absence of *Os*, *Ra*, or *Pt* mutant alleles, may also exhibit increased propensity for glomerulosclerosis after combined nephron mass reduction and diabetes (146).

FVB

FVB/NJ was derived from outbred Swiss mice at NIH inbred for the *fv1b* allele, which confers sensitivity to the Friend leukemia virus B strain. Because of the prominent pronuclei in their fertilized eggs and the large litter size, FVB/NJ are commonly used for transgenic injection. In this context, FVB/N mice transgenic for a calmodulin minigene regulated by the rat insulin II promoter have been shown to develop type 1 diabetes (164,165). Initial studies characterizing the severity of DN in this line (designated OVE26) suggest that whereas they develop

glomerular capillary basement membrane thickening, they do not develop significant albuminuria (68,166). The *LepR^{db}* mutation has also been backcrossed onto the FVB/NJ strain, and the kidneys from these mice exhibit mesangial sclerosis, although albuminuria and GFR have not been reported (134).

NON Mice

The NON strain is closely related to NOD mice and was developed as a “control” for NOD mice. The name was derived from “nonobese nondiabetic”; however, NON/LtJ mice are not “normal” as they exhibit tendencies toward autoimmune diseases. NON/LtJ mice harbor genes that predispose to type 2 diabetes, as evidenced by early, impaired glucose tolerance in males and females, and by the development of moderate maturity-onset obesity in the presence of low plasma insulin levels.

The renal phenotype in these mice is not well characterized but is reported to develop spontaneous glomerular lesions that resemble nodular glomerulosclerosis, although they are not overtly diabetic (101). The susceptibility of NON mice to DN using STZ or other models of diabetes has not been reported.

KK Mice

Although exhibiting only mild insulin resistance, the KK mouse seems to be predisposed to development of renal lesions very reminiscent of DN (167,168). These mice were originally established from inbreeding a Japanese mouse by Kondo (169). The severity of hyperglycemia and insulin resistance is exacerbated by introduction of the agouti (*Ay*) allele into the KK background (170). Of note is that both KK and KK-*Ay* mice develop nodular glomerulosclerosis and mesangial proliferation (171), which are improved by glycemic control (167,172,173). Albuminuria progressively increases with age; however, reports of renal insufficiency or failure have not been forthcoming.

Monogenic Mutations and Transgenic Mice

Despite the preceding caveats regarding the difficulties in phenotyping renal function in mice, the use of transgenic mouse models has provided a better understanding of the factors that exacerbate DN. The following review is not meant to be comprehensive but only to provide an overview of some of the more recent developments in the field. The reader is referred to other recent articles for additional perspectives on this topic (174).

Apolipoprotein E

Although control of hyperglycemia can decrease the development of DN, there is evidence suggesting that a genetic susceptibility to development of DN is related to abnormalities in lipid metabolism. Hypertriglyceridemia is among the main phenotypic features that distinguish people who are prone to nephropathy (175).

Apolipoprotein E (ApoE) is a protein of 34 kD that circulates on plasma lipoproteins at a concentration of 3 to 5 mg/dl (176,177). In the human population, the ApoE gene, located on chromosome 19q13.2, has three common alleles—2, 3, and

4—coding for the three main isoforms of the ApoE protein: E2, E3, and E4 (178). ApoE isoforms differ in their ability to bind to LDL receptors, with E4 having the greatest binding capacity and E2 having defective binding and decreased triglyceride clearance (179). Many but not all studies in patients with either type 1 or type 2 diabetes have implicated the presence of the E2 allele as a risk factor for development and/or progression of DN (180–190). The underlying mechanism(s) that might be involved in the development of DN in patients with the ApoE2 polymorphism have not yet been determined. It is possible that the associated hypertriglyceridemia may predispose to renal injury. It has also been suggested that ApoE may play a role in tissue growth and/or repair after injury separate from its effects on maintenance of lipoprotein homeostasis (191).

In mice with targeted disruption of the ApoE gene locus, no tissues or cells synthesize or secrete ApoE, so ApoE is absent from the plasma. As a consequence, the clearance of remnant lipoproteins is blocked, and serum cholesterol and triglycerides increase to levels of approximately 450 and 250 mg/dl, respectively, similar to levels in human type III hyperlipoproteinemia as a result of ApoE deficiency. ApoE deficiency causes increased susceptibility to atherosclerosis and represents a murine model of spontaneous (non-diet-induced) atherosclerosis (192). Of note, fasting glucose and insulin levels are not elevated in ApoE^{-/-} mice on a normal diet compared with C57BL/6J mice. It is interesting that a recent study in low-dose STZ-treated C57BL6 mice showed that deletion of the ApoE allele was associated with more severe nephropathy as assessed by histopathology and albuminuria (which increased approximately fivefold, albeit still <100 µg/24 h) (193).

Endothelial Nitric Oxide Synthase

Endothelial dysfunction is present in diabetes and is associated with impaired vascular nitric oxide (NO) synthesis. A number of polymorphisms in the endothelial NO synthase gene (*eNOS*), located on human chromosome 7, have been linked to vasculopathies (194–198). Recent studies have reported an association between *eNOS* polymorphisms that lead to decreased *eNOS* expression and development of advanced nephropathy in patients with type 1 (199–201) and type 2 diabetes (202,203). Other studies have found an association of these polymorphisms with ESRD (204,205). However, not all studies have detected any potential association of these *eNOS* polymorphisms with DN (206–210).

Although direct examination of development of DN has not yet been undertaken in mice with gene deletion of *eNOS*, it is noteworthy that studies of the phenotype of mice with combined *eNOS* and ApoE deficiency indicate accelerated atherosclerosis, hypertension, and progressive renal dysfunction characterized by smaller kidney weights and glomerular lipid deposits (211–214).

AGE

Generation of AGE, nonenzymatically glycosylated protein derivatives resulting from prolonged hyperglycemic exposure, is a cardinal feature of the diabetic milieu, and early and advanced steps of the complex process of reactions that lead to

glycation of proteins are now well understood (215). Human diabetic kidney (and other tissues) are characterized by glomerular accumulation of AGE, in particular carboxymethyl lysine-modified adducts of proteins (216). AGE may perturb cell function through receptor-independent mechanisms and through receptor-mediated signaling pathways. Several cell surface receptors/binding proteins have been shown to bind AGE, including the macrophage scavenger receptor type II, OST-48, 80K-H, galectin-3, CD36, and receptor for AGE (RAGE) (reviewed in reference 216). Whereas some of these interactions may function to remove/scavenge AGE, AGE binding to RAGE, a signal transduction receptor of the Ig superfamily, mediates diverse cellular responses. For example, Oldfield *et al.* (217) demonstrated that AGE mediate tubular epithelial to myofibroblast transdifferentiation through RAGE *in vitro* and that this effect is dependent on the pro-sclerotic cytokine TGF-β. Thus, an AGE-dependent pathway may play a role in the development of tubulointerstitial fibrosis in the diabetic kidney. RAGE may activate diverse signaling mediators in endothelial cells and macrophages, including p21^{ras}, ERK1/2 MAP kinases, Nf-κB, and NADPH oxidase species (216,218). However, in the diabetic kidney in humans and mice, RAGE is expressed principally by glomerular visceral epithelial cells (podocytes) (219,220). It is interesting that neutralizing soluble RAGE ameliorated glomerular damage in the hyperglycemic *db/db* mouse (220).

Recently, Yamamoto *et al.* (221) reported an interesting new transgenic model that provides further evidence of a role of RAGE in acceleration of diabetes-induced glomerular lesions. In this model, the human RAGE gene is overexpressed specifically in endothelial cells under control of an endothelial cell-specific mouse *flk-1* promoter (*flk-1*-RAGE) on an inbred CD-1 genetic background. Persistent hyperglycemia was induced in *Flk1*-RAGE mice by interbreeding with diabetic insulin-*eNOS* transgenic mice, and *flk-1*-RAGE/*ins-eNOS* double-transgenic mice exhibited increased HgbA_{1c} and serum AGE levels similar to *ins-eNOS* diabetic controls. Kidney enlargement and glomerular lesions, including albuminuria, mesangial expansion, glomerular hypertrophy, and glomerulosclerosis, were detectable at younger age (4 mo) in *flk-1*-RAGE/*ins-eNOS* mice, compared with diabetic *ins-eNOS* mice (6 mo). Thus, aberrant endothelial expression of RAGE accelerated a glomerular phenotype in diabetic mice that mimics features of diabetic glomerulopathy in humans, including albuminuria, mesangial expansion, and glomerular hypertrophy. However, cardinal histopathologic features of progressive DN in humans, including arteriolar hyalinosis and tubulointerstitial fibrosis, and progressive renal insufficiency (see the Working Definition of Diabetic Nephropathy section), have not been described in this model to date. In collaboration with H. Yamamoto, in-depth phenotype examination and validation using AMDCC standards is under way.

GLUT1

A role for the GLUT1 glucose transporter in the pathogenesis of DN has been suggested over the past decade (222,223). This transporter has been identified as a major glucose transporter of the glomerulus (224), and high glucose exposure or diabetes

increases its expression in cultured mesangial cells and whole glomeruli (225,226). Presumably, increased plasma membrane expression of GLUT1 in susceptible cells in the kidney would lead to increased glucose metabolic flux and glucotoxicity perhaps as a result of enhanced generation of reactive oxygen species. In cultured mesangial cells, GLUT1 overexpression leads to enhanced protein kinase C activation and fibronectin accumulation, whereas suppression of GLUT1 levels reduces protein kinase C activation and fibronectin accumulation (225,226). Similar results in transgenic mice have been presented in preliminary form. Normoglycemic GLUT1 transgenic mice develop increased mesangial expansion and albuminuria, whereas mice with an antisense GLUT1 transgene that reduces glomerular GLUT1 expression are protected from these changes despite the presence of hyperglycemia (225,227). Thus, differences in GLUT1 expression could explain part of the variable susceptibility to DN in different strains of mice. Such a possibility is now being explored.

Conclusions

Mice provide an experimental model of unparalleled flexibility for studying mammalian diseases, including DN. Inbred strains of mice exhibit substantial differences in the renal effects of diabetes. Much remains to be established regarding the course of DN in this species and defining those strains and/or mutants that are most susceptible to renal injury from diabetes. It will be especially important to determine whether renal function declines in any of these mouse models of DN. Technical refinements in the measurement of plasma creatinine, GFR, and uniform reporting of albuminuria and histopathology should facilitate progress in this field and allow better comparisons of results obtained by different laboratories. Together with the unique genetic reagents available in mice, the identification of a mouse model of DN that closely mirrors human disease will significantly enhance our understanding of DN and accelerate our progress toward a treatment for this disease.

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