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6. DIABETIC NEPHROPATHY

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6.1 Summary

Between 20% and 40% of patients with diabetes ultimately develop diabetic nephropathy, which in the US is the most common cause of endstage renal disease requiring dialysis. Diabetic nephropathy has several distinct phases of development and multiple mechanisms contribute to the development of the disease and its outcomes. This Review provides a summary of the latest published data dealing with these mechanisms; it focuses not only on candidate genes associated with susceptibility to diabetic nephropathy but also on alterations in various cytokines and their interaction with products of advanced glycation and oxidant stress. Additionally, the interactions between fibrotic and hemodynamic cytokines, such as transforming growth factor β1 and angiotensin II, respectively, are discussed in the context of new information concerning nephropathy development. We touch on the expanding clinical data regarding markers of nephropathy, such as microalbuminuria, and put them into context; microalbuminuria reflects cardiovascular and not renal risk. If albuminuria levels continue to increase over time then nephropathy is present. Lastly, we look at advances being made to enable identification of genetically predisposed individuals.

6.2 Introduction

Diabetic nephropathy is the most common cause of end-stage renal disease requiring dialysis in the US (1). The incidence of diabetic nephropathy in this country has increased substantially over the past few years. Advanced diabetic nephropathy is also the leading cause of glomerulosclerosis and end-stage renal disease worldwide (2, 3). Between 20% and 40% of patients with diabetes ultimately develop nephropathy, although the reason why not all patients with diabetes develop this complication is unknown.

The natural history of diabetic nephropathy differs according to the type of diabetes and whether microalbuminuria (defined as > 30 mg but < 300 mg albumin in the urine per day) is present. If untreated, 80% of people who have type 1 diabetes and microalbuminuria will progress to overt nephropathy (i.e. proteinuria characterized by > 300 mg albumin excreted daily), whereas only 20-40% of those with type 2 diabetes over a period of 15 years will progress. As Nielsen *et al*. (4) demonstrated

more than a decade ago, a clear, early predictor of disease progression is increasing systolic blood pressure, even within the prehypertensive range. Among patients who have type 1 diabetes with nephropathy and hypertension, 50% will go on to develop end-stage renal disease within a decade (5). Mortality among dialysis patients with diabetes is 22% higher in the first year following the initiation of dialysis and 15% higher at 5 years than that among dialysis patients without diabetes (6).

Diabetic nephropathy has several distinct phases of development. Functional changes occur in the nephron at the level of the glomerulus, including glomerular hyperfiltration and hyperperfusion, before the onset of any measurable clinical changes. Subsequently, thickening of the glomerular basement membrane, glomerular hypertrophy, and mesangial expansion take place. Seminal studies by Mauer and colleagues (3) and Steinke and colleagues (7) demonstrated that individuals with type 1 diabetes and microalbuminuria in whom these histological alterations were detected were destined to progress to overt nephropathy. Microalbuminuria, however, has a variable course; its progression to macroalbuminuria (> 300 mg per day) is unpredictable and does not always lead to development of nephropathy (7). Moreover, the rate of kidney function decline after the development of nephropathy is highly variable between patients and is influenced by additional factors, including blood pressure and glycemic control.

Multiple mechanisms contribute to the development and outcomes of diabetic nephropathy, such as an interaction between hyperglycemia-induced metabolic and hemodynamic changes and genetic predisposition, which sets the stage for kidney injury (8). Hemodynamic factors are the activation of various vasoactive systems, such as the renin-angiotensin-aldosterone and endothelin systems. In response, secretion of profibrotic cytokines, such as transforming growth factor β1 (TGF-β1), is increased and further hemodynamic changes occur, such as increased systemic and intraglomerular pressure. Metabolic pathway involvement, among other features, leads to nonenzymatic glycosylation, increased protein kinase C (PKC) activity, and abnormal polyol metabolism. Findings from various studies support an association between increased secretion of inflammatory molecules, such as cytokines, growth factors and metalloproteinases, and development of diabetic nephropathy (9). Oxidative stress also seems to play a central part (11). Studies that have used inhibitors of the pathways involved in genesis of diabetic nephropathy have shed light on the pathogenesis of this condition but have not led to expansion of the therapeutic armamentarium to halt the disease process (10). This Review is intended for a clinical audience and we discuss pathological changes to the glomeruli during the development of diabetic nephropathy. Although many factors have been implicated in the pathogenesis of diabetic nephropathy, we have focused on the particular factors outlined above.

6.3 Hemodynamic pathways

The early signs of glomerular hyperperfusion and hyperfiltration result from decreased resistance in both the afferent and efferent arterioles of the glomerulus. The afferent arteriole seems to have a greater decrease in resistance than the efferent. Many factors have been reported to be involved in this defective autoregulation, including prostanoids, nitric oxide, vascular endothelial growth factor (VEGF; now formally known as VEGF-A), TGF-β1, and the renin–angiotensin

system, specifically angiotensin II. These early hemodynamic changes facilitate albumin leakage from the glomerular capillaries and overproduction of mesangial cell matrix, as well as thickening of the glomerular basement membrane and injury to podocytes (12). In addition, increased mechanical strain resulting from these hemodynamic changes can induce localized release of certain cytokines and growth factors (13, 14).

The renal hemodynamic changes are mediated partly by the actions of vasoactive hormones, such as angiotensin II and endothelin. Glomerular hypertension and hyperfiltration contribute to the development of diabetic nephropathy because use of renin–angiotensin blockers preserves kidney function and morphology. Blockade of the renin–angiotensin–aldosterone system antagonizes the profibrotic effects of angiotensin II by reducing its stimulation of TGF-β1 (15). Support that such profibrotic effects underlie diabetic nephropathy has also been provided by study of an animal model of diabetic nephropathy (16). Transient blockade of the renin–angiotensin system (for 7 weeks) in prediabetic rats reduced proteinuria and improved glomerular structure. Additionally, the administration of an angiotensin-converting-enzyme inhibitor to patients with type 1 diabetes and nephropathy lowered serum concentrations of TGF-β1 (17). A correlation exists between decreased levels of TGF-β1 in serum and urine and renoprotection, as determined by changes in the glomerular filtration rate over time. We discuss this effect further in the Cytokines section.

6.4 Hyperglycemia and advanced glycosylation end products

Hyperglycemia is a crucial factor in the development of diabetic nephropathy because of its effects on glomerular and mesangial cells, but alone it is not causative. Mesangial cells are crucial for maintenance of glomerular capillary structure and for the modulation of glomerular filtration via smooth-muscle activity. Hyperglycemia is associated with an increase in mesangial cell proliferation and hypertrophy, as well as increased matrix production and basement membrane thickening. *In vitro* studies have demonstrated that hyperglycemia is associated with increased mesangial cell matrix production (18, 19) and mesangial cell apoptosis (20, 21). Mesangial cell expansion seems to be mediated in part by an increase in the mesangial cell glucose concentration, since similar changes in mesangial function can be induced in a normal glucose milieu by overexpression of glucose transporters, such as GLUT1 and GLUT4, thereby increasing glucose entry into the cells (19).

Hyperglycemia might also upregulate VEGF expression in podocytes (14), which could markedly increases vascular permeability (22, 23). Hyperglycemia, however, does not account fully for the risk of diabetic nephropathy, as shown by studies in which kidneys from nondiabetic donors were transplanted into patients with diabetes and nephropathy developed irrespective of the glucose control (24). Hyperglycemia might, therefore, be necessary for but not sufficient to cause renal damage.

Three mechanisms have been postulated that explain how hyperglycemia causes tissue damage: nonenzymatic glycosylation that generates advanced glycosylation end products, activation of PKC, and acceleration of the aldose reductase pathway (25, 26). Oxidative stress seems to be a theme common to all three pathways (27).

6.5 Glycosylation

Glycosylation of tissue proteins contributes to the development of diabetic nephropathy and other microvascular complications. In chronic hyperglycemia, some of the excess glucose combines with free amino acids on circulating or tissue proteins. This nonenzymatic process affects the glomerular basement membrane and other matrix components in the glomerulus and initially leads to formation of reversible early glycosylation end products and, later, irreversible advanced glycosylation end products. These advanced products can be involved in the pathogenesis of diabetic nephropathy by altering signal transduction via alteration in the level of soluble signals, such as cytokines, hormones and free radicals. Circulating levels of advanced glycosylation end products are raised in people with diabetes, particularly those with renal insufficiency, since they are normally excreted in the urine (28). The net effect is tissue accumulation of advanced glycosylation end products (in part by cross-linking with collagen) that contributes to the associated renal and microvascular complications (29). Moreover, advanced glycosylation end products (AGE) interact with the AGE receptor, and nitric oxide concentrations are reduced in a dose-dependent manner (30).

6.6 Protein kinase C

Other proposed mechanisms by which hyperglycemia promotes the development of diabetic nephropathy include activation of PKC (31). Specifically, activation of this enzyme leads to increased secretion of vasodilatory prostanoids, which contributes to glomerular hyperfiltration. By activation of TGF-β1, PKC might also increase production of extracellular matrix by mesangial cells (32).

The mechanism by which hyperglycemia leads to PKC activation involves *de novo* formation of diacylglycerol and oxidative stress (33). PKC activation induces the activity of mitogen-activated protein kinases (MAPK) in response to extracellular stimuli through dual phosphorylation at conserved threonine and tyrosine residues. The coactivation of PKC and MAPK in the presence of high glucose concentrations indicates that these two families of enzymes are linked (34).

6.7 Aldose reductase pathway

The polyol pathway is implicated in the pathogenesis of diabetic nephropathy. A number of studies have shown a decrease in urinary albumin excretion in animals administered aldose reductase inhibitors (35), but in humans these agents have not been studied widely and the results are inconclusive.

6.8 Prorenin

Initial clinical studies in children and adolescents suggest that increased plasma prorenin activity is a risk factor for the development of diabetic nephropathy (36, 37). The prorenin receptor in the kidney is located in the mesangium and podocytes, and its blockade has a beneficial effect on kidneys in animal models of diabetes. This effect is mediated by intracellular signals that are both dependent on and

independent of the renin–angiotensin system. Prorenin binds to a specific tissue receptor that promotes activation of p44/p42 MAPK (38).

A possible pathogenic role for prorenin in the development of diabetic nephropathy was noted in an experimental model of diabetes-mice with streptozotocin-induced diabetes. Sustained prorenin-receptor blockade abolished MAPK activation and prevented the development of nephropathy despite an unaltered increase in angiotensin II activity (39).

If prorenin is a key player in the pathogenesis of this disease, use of renin inhibitors for hypertension that increase prorenin concentrations should demonstrate a harmful effect. To date, no such adverse effects have been reported.

6.9 Cytokines

Activation of cytokines, profibrotic elements, inflammation, and vascular growth factors such as VEGF might be involved in the matrix accumulation that arises in diabetic nephropathy (40-42). Although some evidence suggests that VEGF increases permeability of the glomerular filtration barrier to proteins (22), levels of this growth factor can be low in patients with diabetic nephropathy. Thus, the role of VEGF in the pathophysiology of nephropathy is unclear.

Hyperglycemia is thought to stimulate VEGF expression and, therefore, act as a mediator of endothelial injury in human diabetes (40, 41). Studies showed initially that in patients with diabetic nephropathy the degree of neovascularization was increased and correlated with expression of VEGF and angiopoetin (43-45). Later findings, however, showed that levels of VEGF messenger RNA were actually decreased in patients with diabetic nephropathy (46). Evidence against the roles of VEGF and angiopoetin demonstrates promotion of vessel leakage and reduction in transendothelial electrical resistance; these two growth factors have key roles in development of retinopathy and contribute to nephropathy development in animal models.

Further evidence to support a pathogenic role for VEGF in diabetic nephropathy is the observation that VEGF blockade improves albuminuria in an experimental model of the disorder (41, 42). Animal studies that used a neutralizing antibody to VEGF demonstrated the involvement of this growth factor in glomerular hypertrophy and mesangial matrix accumulation (41, 47). High glucose levels, TGF-β1, and angiotensin II stimulate VEGF expression, which leads to the synthesis of endothelial nitric oxide. This action promotes vasodilatation and hyperfiltration, which are the early processes in diabetic nephropathy. VEGF also stimulates the production of the α3 chain of collagen IV, an important component of the glomerular basement membrane. Indirect evidence suggests that increased production of this collagen chain contributes to the thickening of the glomerular basement membrane observed in diabetic nephropathy. In animal studies, administration of an antibody to VEGF decreased urinary albumin excretion compared with that in untreated diabetic controls (22).

Findings from some studies refute a causative role for high VEGF levels in diabetic nephropathy. Instead, results imply that low levels are harmful. Eremina *et al*. (48)

showed in a mouse model that VEGF is produced by podocytes and is necessary for glomerular endothelial cell survival and differentiation as well as for mesangial cell development and differentiation. Gene expression of VEGF is decreased in humans with diabetic nephropathy (49), although whether this effect is due to podocytes loss, leading to reduced production of VEGF, has been questioned. Baelde *et al*. (46) showed that VEGF messenger RNA concentrations were decreased in the glomeruli of patients with diabetic nephropathy and correlated with reduction in the number of podocytes and progression of renal disease.

Hyperglycemia also increases the expression of TGF-β1 in the glomeruli and of matrix proteins specifically stimulated by this cytokine (42). In the glomeruli of rats with streptozotocininduced diabetes, TGF-β1 levels are increased, and use of a neutralizing antibody to TGF-β1 prevents renal changes of diabetic nephropathy in these animals. In addition, connective tissue growth factor and heat shock proteins, which are encoded by TGF-β1-inducible genes, have fibrogenic effects on the kidneys of patients with diabetes. However, diabetes is associated with decreased expression of renal bone morphogenetic protein 7, which in turn seems to counter the profibrogenic actions of TGF-β1 (17). Evidence clearly shows that TGF-β1 contributes to the cellular hypertrophy and increased synthesis of collagen, both of which occur in diabetic nephropathy (17, 42, 50, 51). Further evidence for these actions is provided by studies in which the combination of an antibody to TGF-β1 plus an angiotensin-converting-enzyme inhibitor normalized levels of protein in the urine of rats with diabetic nephropathy; proteinuria was only partly resolved with the use of an angiotensin-converting-enzyme inhibitor alone (52). Glomerulosclerosis and tubulointerstitial injury were also improved by the combined therapy.

The administration of hepatocyte growth factor, which specifically blocks the profibrotic actions of TGF-β1, ameliorates diabetic nephropathy in mice (53).

Inflammatory cytokines also contribute to the development and progression of diabetic nephropathy, specifically interleukin 1 (IL-1), IL-6 and IL-18 and tumor necrosis factor. Concentrations of all these cytokines were increased in models of diabetic nephropathy and seemed to affect the disease via multiple mechanisms. In addition, raised levels of several of these cytokines in serum and urine correlate with progression of nephropathy, indicated by increased urinary albumin excretion (54).

Each cytokines has several different effects. IL-1 alters the expression of chemotactic factors and adhesion molecules, alters intraglomerular hemodynamics (by affecting mesangial cell prostaglandin synthesis), might increase vascular endothelial cell permeability, and increases hyaluron production by renal tubular epithelial cells (which in turn could increase glomerular cellularity) (55). IL-6 has a strong association with the development of glomerular basement membrane thickening as well as possible relationships with increased endothelial permeability and mesangial cell proliferation. IL-18 induces the production of other inflammatory cytokines, such as IL-1, interferon γ and tumor necrosis factor, and might be associated with endothelial cell apoptosis. Tumor necrosis factor has the widest variety of biological activities and effects that contribute to development of diabetic nephropathy - too many to describe here. Importantly, though, it causes direct renal injury as a cytotoxin, as well as affecting apoptosis, glomerular hemodynamics, endothelial

permeability, and cell-cell adhesion. It also seems to play an important part in the early hypertrophy and hyperfunction of diabetic nephropathy (54, 56, 57).

6.10 Lipid mediators

Small lipids derived from arachidonic acid have been implicated in the pathogenesis of diabetic nephropathy. Cyclo-oxygenase 2 breaks down arachidonic acid into several different prostanoids. In a rat model of streptozotocin-induced diabetes, levels of inflammatory prostanoids, such as prostaglandins E_2 and I_2, were raised (58). Furthermore, increased expression of cyclooxygenase 2 has been reported in animal studies of diabetes and in the macula densa of kidneys from humans with diabetes (59). In diabetic rats, inhibition of cyclo-oxygenase 2 is associated with decreased glomerular hyperfiltration (60). A more detailed characterization of how the production of prostanoids affects the pathogenesis of diabetic nephropathy is needed.

Arachidonic acid can also be oxidized by lipoxygenases (61). Evidence is accumulating that some of the products derived from the actions of lipoxygenases contribute to diabetic nephropathy. Specifically, levels of lipoxygenases 12 and 15 are increased in diabetic animals. In addition, high glucose levels increase expression of lipoxygenases 12 and 15 in cultured mesangial cells. To conclude, this pathway has a key mediatory role in the critical processes of mesangial cell hypertrophy and extracellular matrix accumulation mediated by TGF-β1 and angiotensin II (61).

6.11 Oxidative stress

Generally, metabolic activity within the nephron produces a large amount of reactive oxygen species that are counterbalanced by a large number of antioxidant enzymes and free radical scavenging systems. Reactive oxygen species mediate many negative biological effects, including peroxidation of cell membrane lipids, oxidation of proteins, renal vasoconstriction and damage to DNA. Unfortunately, hyperglycemia tips the balance towards production of reactive oxygen species, most of which seem to be generated in the mitochondria (62). The metabolism of glucose through harmful alternate pathways, such as via PKC activation and advanced glycation end-product formation, in the setting of hyperglycemia also seems partly dependent on reactive oxygen species (62-64).

Hyperglycemia specifically induces oxidative stress, even before diabetes becomes clinically apparent. Concentrations of markers of DNA damage induced by reactive oxygen species are higher in patients with more-severe nephropathy (i.e. proteinuria versus microalbuminuria). Furthermore, histological analysis of human kidney biopsy specimens has detected products of glyco-oxidation (combined products of glycation and protein oxidation) and lipoxidation in the mesangial matrix and glomeruli, whereas these lesions are much less common in specimens from individuals without diabetes (64, 65).

6.12 Nephrin

Podocytes (specialized visceral epithelial cells) are important for the maintenance of the dynamic functional barrier (66). Nephrin, a protein found in these cells, is crucial for maintaining the integrity of the intact filtration barrier. The renal expression of nephrin might be impaired in diabetic nephropathy.Patients with diabetic nephropathy have markedly reduced renal nephrin expression and fewer electron-dense slit diaphragms compared with patients without diabetes and minimal nephropathic changes or controls (67). Furthermore, nephrin excretion is raised 17-30% in patients with diabetes (with and without albuminuria) compared with that in individuals without diabetes. Thus, nephrin excretion could be an early finding of podocyte injury, even before the onset of albuminuria (13, 14). Treatment with blockers of the renin-angiotensin-aldosterone system might help protect nephrin expression. In a study of patients with type 2 diabetes, treatment with an angiotensin-converting-enzyme inhibitor for 2 years maintained nephrin expression at control levels compared with that in untreated patients with diabetes (23). By contrast, the expression of two other important podocyte and slit diaphragm proteins, podocin and CD2AP, was similar in the three groups. Comparable decreases in renal nephrin expression were reported in other studies of diabetic nephropathy (68, 69).

6.13 Genetic susceptibility

Genotype seems to be an important determinant of both incidence and severity of diabetic nephropathy (9, 31, 70). The increase in risk cannot be explained by the duration of diabetes or hypertension, or the degree of glycemic control. Environmental and genetic factors must, therefore, have roles in the pathogenesis of diabetic nephropathy. In patients with type 1 or type 2 diabetes, the likelihood of developing diabetic nephropathy is markedly increased in those who have a sibling or parent with diabetic nephropathy (71, 72). One study evaluated Pima Indian families in whom two successive generations had type 2 diabetes (72). The likelihood of the offspring developing overt proteinuria was 14% if neither parent had proteinuria, 23% if one parent had proteinuria and 46% if both parents had proteinuria.

Advances in molecular genetics have led to the development of a system for genotyping single-nucleotide polymorphisms and have enabled exploration of loci involved in diabetic nephropathy in genome-wide association studies. In the search for susceptibility genes for microvascular complications of diabetes in Pima Indians, four loci on chromosomes 3, 7, 9 and 20 were identified (73). Additional loci are identified as diabetic nephropathy susceptibility genes areas on chromosomes 7q21.3, 10p15.3, 14q23.1 and 18q22.3 (74, 75).

Association studies of candidate genes have been the most common approach to identify genes involved in susceptibility to diabetic nephropathy. The greatest risks seem to be associated with genes encoding angiotensinconverting enzyme, angiotensin II receptor, cytokines, proteins involved in glucose or lipid metabolism, and extracellular matrix proteins.

The angiotensin-converting-enzyme gene (ACE) polymorphism has been explored in several studies. The insertion-deletion polymorphism is responsible for the difference

between individuals in plasma levels of angiotensin-converting enzyme. In patients with type 2 diabetes, the DD polymorphism of the ACE gene has been associated with an increased risk of developing diabetic nephropathy, severe proteinuria, progressive renal failure, and of mortality during dialysis (76-78). In addition, an analysis of more than 1,000 white patients with type 1 diabetes showed a strong correlation between genetic variation in the ACE gene and the development of nephropathy (79). Other studies have, however, produced conflicting data. A critical review of 19 studies that examined a possible link between this gene and diabetic nephropathy failed to confirm an association among white people with either type 1 or type 2 diabetes, although a possible association in Asians could not be excluded (78). Ongoing studies are taking a multigene approach, since the likelihood of diabetic nephropathy being a single-gene disease is low.

6.14 Conclusions

While progression to diabetic nephropathy cannot yet be prevented, the pathogenesis is better characterized than a decade ago. The hemodynamic changes of glomerular hyperperfusion and hyperfiltration become evident before the earliest measurable clinical signs of nephropathy but do not predict the demise of kidney function. Various structural changes, including podocyte foot process effacement, decrease in podocyte number, thickening of the glomerular basement membrane and mesangial expansion, all occur with the early changes. These features, when assessed independently, cannot, however, predict disease progression. Hyperglycemia plays a central part in a cascade of damaging effects mediated by cytokines and growth factors that produces oxidative stress, abnormal glycosylation, lipid peroxidation, and the production of further inflammatory elements. With the anticipated completion of current studies that are evaluating the genetics of nephropathy in the next 2-4 years, understanding of how to integrate genetic and environmental susceptibility factors into risk assessment should be improved. This knowledge should give clinicians the ability to predict earlier who will develop nephropathy and, therefore, improve prevention of this devastating disease.

Key points

- Microalbuminuria is not a predictor of nephropathy development in individuals with diabetes
- Multiple mechanisms are operative in diabetes that are related to injury to the kidney and, in susceptible individuals, contribute to nephropathy development
- Defects in nephrin and podocin are central to the development of macroalbuminuria and associated with nephropathy progression
- Abnormally high concentrations of lipids contribute to β-cell injury and development of albuminuria

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