

New insights into the molecular pathology and the development of predictive biomarkers in diabetic nephropathy

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ABSTRACT

Diabetic nephropathy (DN) as a cause of end-stage renal disease (ESRD) is increasing worldwide. Moreover, DN is associated with a highly increased incidence of cardiovascular morbidity and mortality. Much research has been conducted in both basic science and clinical therapeutics, which has enhanced the understanding of the pathophysiology of diabetic nephropathy and expanded the potential therapies available. DN is characterized by progressive expansion of the mesangial matrix and thickening of the glomerular basement membrane, resulting in the obliteration of the glomerular capillary lumen, loss of glomerular function and proteinuria. Protein glycation reactions leading to advanced glycation end-products (AGEs) are thought to be the major causes of different diabetic complications. Type IV collagen (Col4) is a major component of extracellular matrix (ECM) and increased Col4 has been linked to the development of glomerulosclerosis in experimental and human diabetic nephropathy. Smad1 transcriptionally regulates Col4 and other glomerulosclerosis-related molecules such as Type I and III collagens and smooth muscle α actin (SMA). Although Smad1 is not expressed in normal glomeruli, bone morphogenetic protein 4 (BMP4) induces and activates Smad1. Conditional transgenic mice for BMP4 exhibits advanced diabetic glomerulosclerosis and marked albuminuria in normal blood glucose condition, suggesting that BMP4 is

an essential factor in the pathophysiological mechanisms in DN. Although microalbuminuria remains the gold standard for early detection of DN, it is not a sufficiently accurate predictor of DN risk. Therefore, it has been deemed necessary to find a novel diagnostic molecular marker specific for the diabetic injuries in the progressive phase of DN, along with the elucidation of the molecular mechanisms in the diabetic glomeruli. This review describes current concepts in the epidemiology, pathophysiology, diagnosis, and treatment of this disorder, with a special emphasis on the molecular mechanisms of diabetic glomerulosclerosis and its accurate diagnosis.

KEYWORDS: diabetic nephropathy, Smad1, type IV collagen, SMA, BMP4, AKL1, AGE.

INTRODUCTION

The prevalence of diabetes around the world has reached epidemic proportions. While diabetes is already estimated to affect more than 8% of the global population (nearly more than 350 million people), this is predictable to grow to over 550 million people by the year 2035 [1]. With worldwide epidemic of diabetes mellitus, diabetic nephropathy which is one of the major causes of microvascular complication has become a serious concern. Diabetic nephropathy is the leading cause of end-stage renal disease and is associated with increased morbidity and mortality of diabetic patients throughout the world [2]. Albuminuria

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was widely considered as the first clinical sign of diabetic kidney disease. However, increasing evidence has shown that a significant number of type 2 diabetes mellitus (DM) patients have a decreased glomerular filtration rate (GFR) without significant albuminuria. A changing concept has been introduced from the classical DN to diabetic chronic kidney disease (DKD), taking into account that histological kidney lesions may vary from the nodular or diffuse glomerulosclerosis to tubulointerstitial and/or vascular lesions. Nonetheless, many studies have demonstrated poor prognosis for DN [3] despite better treatment with many kinds of antihypertensive agents [4, 5]. The natural history of DN is characterized by a prolonged period of clinical silence during which two major changes can be documented [6]: functional changes, including increased GFR and albuminuria, and structural changes; glomerular basement membrane (GBM) thickening and mesangial expansion. These changes develop into overt proteinuria, leading to progressive decline in the GFR.

Pathophysiological features of DN

Major typical morphological changes are the result of changes in the extracellular matrix (ECM). Thus, basement membranes are thickened and the glomerular mesangial matrix is expanded, due to increased amounts of ECM. Glomerulosclerosis is caused by accumulation of ECM proteins in the mesangial interstitial space, resulting in the narrowing and obliteration of glomerular capillaries [7]. Col4 is a key component of GBM and mesangial ECM, and exists as a triple helix of $\alpha 1$ (IV) and $\alpha 2$ (IV) chains with a noncollagenous globular domain at its carboxyl terminus. During the process of glomerular injuries, mesangial cells overproduce Col4 and secrete type I and type III collagens and osteopontin, which are not normally present in the mesangial matrix [8, 9]. Later, the formation of mesangial nodules represents the characteristic lesions of the Kimmelsteil-Wilson nephropathy with additional extensive tubulointerstitial lesions.

Several factors are involved in the development of DN, including genetic factors, glomerular hyperfiltration [10], oxidative stress, accumulation of advanced glycation end-products (AGEs) [11], acceleration of the polyol pathway, activation of protein kinase C, overexpression of transforming

growth factor- β (TGF- β), followed by increase of extracellular matrices [12]. Hyperglycemia increases the expression of TGF- β in the glomeruli and thereafter TGF- β plays an important role in the AGE response of the glomeruli [13]. Transgenic mice overexpressing TGF- β develop severe glomerulosclerosis [14]. Thus, TGF- β is assumed to be a central mediator of the sclerosing process in DN. However, the effects of therapeutic blockade of TGF- β are still controversial.

AGE/RAGE axis and the extracellular matrix

Prolonged exposure to hyperglycemia is recognized as the principal causal factor of diabetic complications [15]. Its deleterious effects are attributable to the formation of sugar-derived protein adducts and cross-links known as AGEs. These diverse and highly reactive protein adducts have been shown to accumulate in animal and human tissues with aging and at an accelerated rate in diabetes [11]. Importantly, excessive AGEs in tissues or in the circulation are known to stimulate the production of ECM and inhibit its degradation, and to contribute significantly to diabetic complications, including DN [16-18]. Indeed, a number of AGEs, such as carboxymethyl lysine (CML) and pentosidine, have been identified in the kidneys of patients with DM, and their renal ECM accumulation was positively correlated with disease severity [19]. The receptor for advanced glycation endproducts (RAGE) was discovered as a receptor for AGEs such as CML [20]. AGEs, largely *via* RAGE, activate signaling mechanisms that cause cell stress, contribute to cellular dysfunction, and damage target organs, leading to complications. Exposure of cultured mesangial cells to AGEs results in a receptor-mediated upregulation of mRNA and protein secretion of Col4 [21, 22]. Nevertheless, the mechanisms that underlie this regulation remain unknown. *In vitro* and animal experiments have shown that various interventions can inhibit the formation and/or actions of AGEs, in particular the specific AGE inhibitor aminoguanidine [23] and the AGEs' crosslink breaker alagebrium [24], and the B vitamins pyridoxamine [25] and thiamine, and the latter's synthetic derivative, benfotiamine [26]. Beyond the current treatments to treat diabetic complications, such as the optimization of blood pressure and glycaemic control, it is predicted that new

therapies designed to target AGEs may become part of the treatment regimen for diabetic renal disease.

Smad1 is identified as a direct regulatory molecule for Col4

Moving beyond AGE exposure to probe the mechanisms of the downstream signalling pathway of DN, We investigated the role of the transcription factors that regulate the expression of ECM proteins. Although Col4 is the principal component of the GBM, the cellular and molecular mechanisms of the upregulation of Col4 in diabetic conditions remained poorly understood. Bruggeman *et al.* had reported that the 130-bp bidirectional promoter of Col4 contains a large stem-loop structure (CIV) that interacts with several DNA-binding proteins [27] [Fig. 1]. Using an electrophoretic mobility shift assay (EMSA) assay, we demonstrated that an unknown protein binds to the CIV motif only when exposed to AGEs [22]. To identify the protein that binds to the CIV site in the promoter region of the mouse Col4 gene, we used a yeast one-hybrid system to isolate a clone that encodes a specific binding protein that encodes Smad1 [9] [Fig. 2]. Moreover, it was unveiled that glomerular immunoreactivity for Smad1 was correlated with the severity of sclerotic lesions in human diabetic renal glomeruli; the immunoreactive signal was nearly absent in normal glomeruli [9].

Previous studies have shown that TGF- β plays an important role in the AGE response of the glomeruli, and transgenic mice overexpressing TGF- β develop severe glomerulosclerosis [14]. It is generally known that Smad3 functions as a key intracellular signal transducer for profibrotic TGF- β

responses in various cells. However, the role of the Smad3 pathway in the pathogenesis of DN has yet to be fully understood. Further, the interruption of Smad3 signaling did not improve diabetic nephropathy; i.e., albuminuria was not ameliorated in STZ-diabetic Smad3-knockout mice. Similarly, albuminuria failed to improve in diabetic db/db mice treated with an anti-TGF- β antibody (2G7) [28]. These results suggest that another signaling pathway may function in the development of DN. Members of the TGF- β superfamily bind to two different types of serine/threonine kinase receptors, termed type I and type II receptors [29]. Type II receptors activate type I receptors, which transduce various signals *via* the Smads. Two type I receptors have been described for TGF- β 1, activin receptor-like kinase type 1 (ALK1) and type 5 (ALK5) [30]. Goumans *et al.* [31] described that ALK1 and its effectors [Smad1/5] exert a lateral antagonism of the ALK5 pathway. Accordingly, we examined the expression of ALK1 in mesangial cells under exposure to AGEs. The expression of ALK1 was induced in AGE-treated mesangial cells. In contrast, BSA-treated mesangial cells did not exhibit ALK1 as well as Smad1. Next, it was demonstrated that ALK1, together with Smad1 and Col4, was highly expressed in human DN, corresponding to the progression of diabetic conditions [9]. As both Smad1 and ALK1 are nearly absent in normal mesangial cells and normal glomeruli, ALK1 is thought to act upstream of the excessive production of Col4. These data conclusively show that the ALK1/Smad1 signaling may mediate the development of atherosclerosis, both in diabetic patients and in the aged, by inducing overproduction of ECM [Fig. 3].

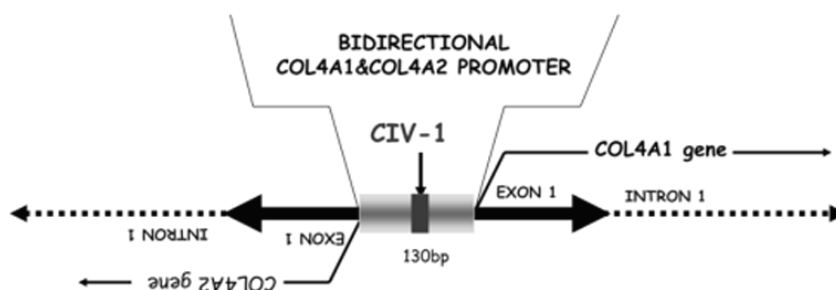


Fig. 1. Promoter of type IV collagen. The 130-bp bidirectional promoter of type IV collagen contains a large stem-loop structure [CIV], which has been shown to interact with several DNA binding proteins.

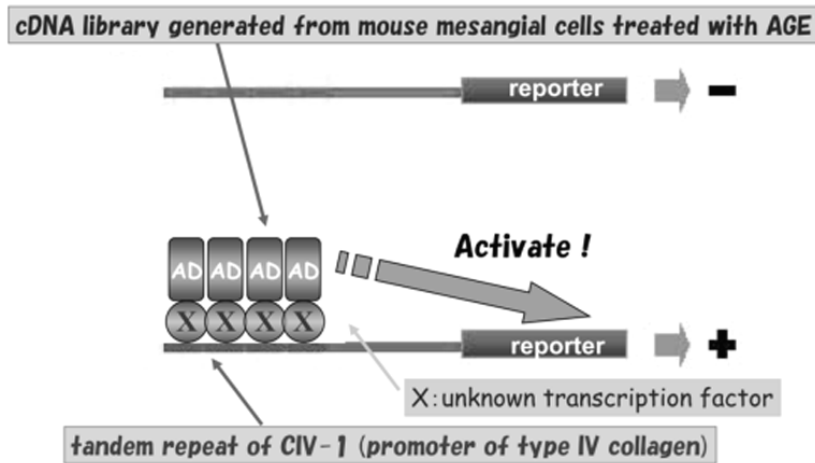


Fig. 2. Cloning of Smad1 by using a yeast one-hybrid assay. The cDNA library from mouse mesangial cells treated with AGE was constructed. Next, reporter plasmids were linked to four tandem copies of the binding sequence [CIV-1], and then transformed into yeast. Clones that contain a fusion protein between GAL4 activating domain and the DNA-binding domain of unknown transcription factor X will strongly activate reporter gene expression through binding to the tandem repeats of DNA sequence X in the reporter gene, allowing the positive selection of rapidly growing clones in a selective media.

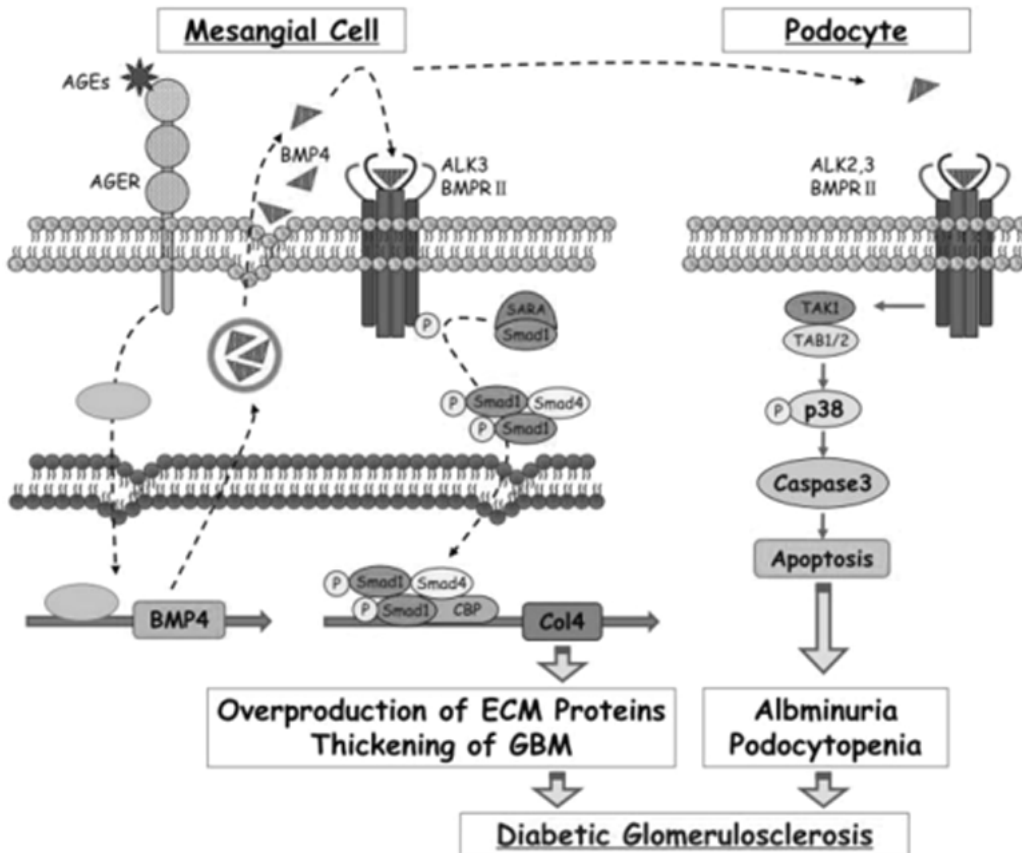


Fig. 3. Schematic illustration of the development of diabetic nephropathy.

Smad1 and phenotypic change in diabetic nephropathy

Mesangial cells provide structural support to the glomerular capillary tuft by producing extracellular matrix components that form the mesangial matrix [32]. There is emerging evidence to suggest that the cause of glomerulosclerosis in DN is phenotypic switching of mesangial cells to an activated state. In response to injury, MCs can transdifferentiate into myofibroblasts, a specialized population of mesenchymal cells that synthesize an array of different extracellular matrix proteins (i.e., type I and type III collagens) that are not normally present in the mesangial matrix and markedly up-regulate the expression of smooth muscle-like proteins (i.e., SMA) [8, 9, 33]. It is generally accepted that myofibroblasts represent key players in a variety of pathological conditions involving tissue remodeling in the kidneys. Although myofibroblasts function as a mechanotransducer that may lead to prevention of cell migration and concentrate these cells at the site of injury, the fundamental significance of the switch to myofibroblasts and induction of SMA expression is unclear [34]. Changes in the phenotype of mesangial cells are observed as positive expression of SMA in various glomerular diseases, including DN. In addition, the expression of SMA is associated with mesangial proliferation [35]. Although TGF- β has been reported to induce SMA expression in various cells [36], its precise molecular mechanisms are largely unknown. In a report by Sato *et al.*, it was suggested that the Smad3 pathway is essential for TGF- β -induced epithelial-mesenchymal transition (EMT), and SMA mRNA was detected in both renal tubular epithelial cells and fibroblastic cells adjacent to the renal tubules [37]. Another recent report has shown that TGF- β down-regulates Smad3 in human mesangial cells with a myofibroblastic phenotype [38], but the detailed molecular mechanism is still unclear.

As mentioned above, however, Smad1 transcriptionally upregulates ECM proteins (type IV and type I collagens and osteopontin) in the common process of progressing glomerulosclerosis, thereby playing a key role in the initiation and progression of diabetic nephropathy [9]. Moreover, induction of both Smad1 and SMA expression

coincides with the development of glomerulosclerosis in both type 1 and type 2 diabetic mice or rats [39, 40]. In addition, as a downstream modulator of the TGF- β signaling pathway, it is now clear that Smad1 transduces TGF- β through a type I receptor, ALK1, which is newly induced in MCs in diabetes. In fact, we found that ALK1, together with Smad1 and Col4, was highly expressed in human advanced diabetic nephropathy [9]. Accordingly, it can be considered that ALK1 directly phosphorylates Smad1 and triggers the subsequent mesangial expansion, resulting in diabetic glomerulosclerosis. Thus, Smad1 is thought to be closely involved in the phenotypic change of MCs in diabetes, and Smad1 and/or ALK1 may be a novel therapeutic target of abnormal phenotypes in diabetic nephropathy.

Urinary Smad1 as a biomarker to indicate structural changes in glomeruli in DN

The most reliable diagnostic procedure is renal biopsy, but it is impossible to perform biopsies for all patients with DN. It has been used for decades, is an invasive procedure and, as such, cannot be repeated multiple times; these are all in addition to requiring experienced physicians to perform it. Increased urinary protein excretion may be an early clinical manifestation of diabetic nephropathy [41]. To date, the measurement of albuminuria has been used as a standardized non-invasive test for the diagnosis of early DN [42]. Diabetic kidney disease, however, is not detected by this test in some cases. These reports indicate that albuminuria does not correlate with glomerulosclerosis at all in the early phase of DN. The most critical feature of glomerulosclerosis is mesangial expansion, which has been strongly correlated with decline of GFR [6]. Therefore, it is important to understand the molecular mechanisms underlying the mesangial expansion. Similarly, practical approaches to the diagnosis by novel diagnostic markers specific for the detection of mesangial expansion in the early phase of DN must be established. Recently, clinical urinary proteomic analyses have been widely used to discover biomarkers for disease discovery, diagnosis and monitoring [43]. However, how these biomarkers account for the development of DN has not been clarified yet.

Earlier diagnosis may lead to better long-term outcomes for patients with DN. Changes in GBM structures occur very early in DN, even before microalbuminuria. To identify reliable biomarkers for the early changes of DN, it is absolutely imperative to uncover the molecular mechanisms involved in the initiation of DN. Thus, the optimal approach to diagnosis stems directly from a consideration of the pathology and pathophysiology of the disease. In this context, our has shown that AGEs induce the expression of Smad1 in the glomeruli and that Smad1 may be the earliest indicator of renal dysfunction. We first examined whether the presence of urinary Smad1 in an early phase of diabetes can predict later development of glomerulosclerosis in diabetic nephropathy, and how ARB might be able to modulate structural changes and urinary markers. Smad1 and albumin in the urine were examined 4 weeks after injection of streptozotocin in rats or in 6-week old db/db mice [39, 40]. There was a very good correlation between urinary Smad1 levels and the development of mesangial expansion, whereas the correlation between albuminuria and mesangial expansion was not statistically significant. Clinical studies are underway to investigate the concept that urinary Smad1 could be an early predictor in diabetic patients.

Critical role of BMP4 for the initiation and progression of DN

It is generally known that Smad1 is directly phosphorylated by TGF- β type I receptors as well as bone morphogenetic proteins (BMPs) through type I and II BMP receptors [44]. Although BMPs are well known to be required for the normal development of various tissues and organs, including the kidneys [45], the role of BMPs in adults or in diseases is unclear. For this reason, we generated inducible Bmp4 transgenic mouse lines by using the tamoxifen-regulated Cre-loxP system [46]. Tamoxifen-inducible Bmp4 transgenic mice revealed extensive expansion of the mesangial matrix, compared with noninducible glomeruli. These transgenic mice also showed significant induction of glomerular expressions of Smad1, pSmad1, Col4, and Col1 compared with noninducible mice. Furthermore, the Bmp4 tgm exhibited marked thickening of the GBM as well as mesangial

expansion in electron microscopic analyses, both of which are characteristic of human DN. Albuminuria was dramatically increased in inducible Bmp4 tgm compared with noninducible mice. Collectively, inducible Bmp4 tgm were able to mimic diabetic changes in glomeruli by exhibiting pathological features remarkably resembling human DN in a nondiabetic condition. Therefore, activation of BMP4-Smad1 signaling pathway plays a critical role in the pathogenesis in DN. Furthermore, to confirm the role of BMP4, we demonstrated that diabetic Smad1-Tg and wild-type mice treated with a BMP4-neutralizing antibody exhibit decreased Smad1 phosphorylation and ~40% less mesangial expansion than those treated with control IgG [47].

Future perspectives

Patients with diabetic kidney disease have exceptionally high rates of cardiovascular morbidity and mortality. It is also clear that the current therapeutic approach of glycemic control can slow [48], but cannot completely prevent the development or progression of DN in most patients. Despite extensive investigations, DN has remained an unresolved problem. Here, we propose the molecular mechanism of the development of DN, focusing on the earliest structural changes in the expansion of the mesangium due to accumulation of ECM proteins. We unveiled the critical role of BMP4-Smad1 signaling axis in the initiation and progression of DN. Very recently, we reported that phosphorylation of the linker domain in Smad1 protein suppressed the progression of DN *in vivo* [49]. Furthermore, the pharmacological agents that inhibit the activity of Smad1 signaling may halt the progression of diabetic glomerulosclerosis. However, further clinical studies are needed to illuminate their therapeutic potential in treating diabetic patients with nephropathy. Concerted clinical and basic research efforts will be needed to understand DKD pathogenesis and to identify novel drug targets.

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CONFLICT OF INTEREST STATEMENT

The authors declare that they have no conflicts of interest with regard to the contents of this article.

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