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Review

Role of Smad1 in diabetic nephropathy: Molecular mechanisms and implications as a diagnostic marker

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Summary. Diabetic nephropathy (DN) is the leading cause of chronic kidney failure. Moreover, DN is associated with elevated cardiovascular morbidity and mortality. DN is characterized by progressive expansion of the mesangial matrix and thickening of the glomerular basement membrane, resulting in the obliteration of glomerular capillaries. Advanced glycation endproducts (AGEs) produced as the result of hyperglycemia are known to stimulate the production of extracellular matrix (ECM) proteins, resulting in glomerulosclerosis. Exposure of cultured mesangial cells to AGEs results in a receptor-mediated upregulation of mRNA and protein secretion of type IV collagen (Col4), which is a major component of ECM. Here we review recent novel insights into the pathogenesis and diagnosis of DN, with a special emphasis on the emerging concept that diabetic glomerulosclerosis can result from activation of the signaling cascade leading to irreversible ECM overproduction. Finally, we describe signaling pathways involved in the initial change of DN and how these pathways can be manipulated for therapeutic benefit.

Key words: Diabetic nephropathy, Smad1, Type IV collagen, SMA, Biomarker

Introduction

Diabetic nephropathy is the leading cause of end-stage renal disease and a major contributor to morbidity and mortality of diabetic patients throughout the world. It arises due to longstanding diabetes mellitus, and is a prime indication for dialysis in many countries. Nephropathy develops progressively in diabetic patients, and is a major contributory risk factor for death from cardiovascular complications (Parving et al., 2001a). The natural history of diabetic nephropathy is characterized by a prolonged period of clinical silence during which two major changes can be documented (Mauer et al., 1984): functional changes, including increased glomerular filtration rate (GFR) and albuminuria, and structural changes; glomerular basement membrane (GBM) thickening and mesangial expansion. These changes develop into overt proteinuria, tubulointerstitial damage, and then a decline in GFR.

According to the World Health Organization, diabetes affects more than 170 million people worldwide, and this number will rise to 370 million by 2030 (World Health Organization, 2004). Proteinuria was first recognized in diabetes mellitus in the late 18th century. In the 1930s, Kimmelstiel and Wilson described the classic lesions of nodular glomerulosclerosis in diabetes associated with proteinuria and hypertension. By the 1950s, kidney disease was clearly recognized as a common complication of diabetes, with as many as 50% of patients with diabetes of more than 20 years having this complication. About one third of those affected will eventually have progressive deterioration of renal function (Remuzzi et al., 2002). The initial manifestation seems to be the nephromegaly associated with increased RPF and GFR. This is followed by a stage of glomerulopathy in which structural changes can be observed in the glomeruli but without evidence of clinical disease. If untreated, this stage can lead to a state in which diabetic patients develop microalbuminuria, which then progresses to overt diabetic nephropathy with clinically detectable proteinuria. The evolution to overt proteinuria is a critical phase because it accelerates renal damage, inexorably leading to ESRD within a few years. However, the pathophysiologic mechanism underlying the association between albumin excretion

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and CVD is not fully defined. Since it is difficult to prevent the progression of nephropathy in overt nephropathy, treatment in the early phase is recommended.

Persistent proteinuria is the hallmark of DN, a condition that is characterized by a progressive rise in blood pressure, a declining glomerular filtration rate, and a high risk of fatal or nonfatal cardiovascular events. The degree of proteinuria is closely associated with the rates of renal and cardiovascular events (Brenner et al., 2001; Lewis et al., 2001). Albuminuria is one of the most characteristic functional changes in the early phase of DN. At the present time, in type 2 diabetics, microalbuminuria is the earliest clinical sign indicating vascular damage in the glomerulus, which is reflective of vascular disease throughout the body (Keane et al., 1993). Urinary albumin excretion is considered to be a significant predictor of diabetic nephropathy and its control is thought to be important for diabetic treatment, since overt DN has been shown to develop with microalbuminuria. The prevalence of microalbuminuria in type-2 diabetes mellitus is around 37% (Parving et al., 1992; Taneja et al., 2001). In addition, the incidence of microalbuminuria increases with age as well as with increased duration of diabetes mellitus.

The most reliable diagnostic method is the renal biopsy to diagnose DN, but it is impossible to perform biopsies for all cases. In addition, an extensive study of the glomerular structure in diabetic patients with or without microalbuminuria failed to find a significant difference in the structural changes between the two groups in the absence of raised blood pressure or reduced creatinine clearance (Chavers et al., 1989; Fioretto et al., 1994). These reports do not indicate that albuminuria correlates with glomerulosclerosis in the early phase of DN. The most critical feature of glomerulosclerosis is mesangial expansion, which has been strongly correlated with decline of GFR (Mauer et al., 1984). On the other hand, GBM thickening shows little or no correlation with decline of GFR (Mauer et al., 1984). Therefore, it is important to find a novel diagnostic marker specific for the detection of mesangial expansion in the early phase of DN, along with the elucidation of the precise mechanisms of mesangial expansion. The following review focuses on the molecular mechanisms involved in the initiation and progression of diabetic nephropathy. We will also review biomarkers that can detect or predict the development of glomerulosclerosis.

Pathophysiological features of diabetic nephropathy

Among the various changes seen in DN, glomerular structural changes are very important, because there is widespread agreement that they are generally accompanied by various tubulointerstitial damages and nephron loss leading to ESRD (Kriz and LeHir, 2005). DN is a progressive kidney disease caused by angiopathy of capillaries in the kidney glomeruli and is morphologically characterized by progressive expansion of mesangial matrix and thickening of the GBM. Glomerulosclerosis is caused by accumulation of ECM proteins in the mesangial interstitial space, resulting in the narrowing and obliteration of glomerular capillaries (Steffes et al., 1989). Col4 is a main constituent of GBM and mesangial ECM, and exists as a triple helix of α1 (IV) and α2 (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 (Desmouliere et al., 1993; Abe et al., 2004). Later, the formation of mesangial nodules represents the characteristic lesions of the Kimmelsteil-Wilson nephropathy with additional extensive tubulointerstitial lesions. Therefore, it is crucial to determine risk markers reflecting the initial changes of glomerulosclerosis in the clinically silent period of DN.

Although the exact cause of DN is unknown, various postulated mechanisms include hyperglycemia, AGEs, protein kinase C (PKC), and activation of cytokines. Hyperglycemia increases the expression of transforming growth factor-ß (TGF-ß) in the glomeruli and of matrix proteins specifically stimulated by this cytokine. TGF-ß may contribute to the cellular hypertrophy and enhanced collagen synthesis observed in DN. TGF-ß also plays an important role in the AGE response of the glomeruli (Yang et al., 1994), and transgenic mice overexpressing TGF-ß develop severe glomerulosclerosis (Sanderson et al., 1995). Thus, TGF-ß is assumed be a central mediator of the sclerosing process in DN. In addition to the renal hemodynamic alterations, patients with overt DN (dipstick-positive proteinuria and decreasing GFR) generally develop systemic hypertension. Hypertension is an accelerating factor in all progressive renal diseases, which especially seems the case in DN. The deleterious effects of hypertension are likely directed at the vasculature and microvasculature. Although the pathogenesis of DN is multifactorial, the RAA system plays a particularly important role (Luetscher et al., 1985; Parving et al., 2008). However, direct evidence of transcriptional regulatory mechanisms responsible for ECM overproduction has not been provided yet.

AGE/RAGE axis and the extracellular matrix

Prolonged exposure to hyperglycemia is now recognized as the principal causal factor of diabetic complications (Pirart, 1978; The Diabetes Control and Complications Trial Research Group, 1993). 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 (Brownlee et al., 1988). Prolonged infusion of nondiabetic rats with AGEs has led to the development of similar morphological changes and significant

proteinuria. 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 (Pugliese et al., 1997; Bendayan, 1998; Ling et al., 2001). Indeed, a number of AGEs, such as carboxymethyllysine and pentosidine, have been identified in the kidneys of diabetic patients, and their renal accumulation was positively correlated with disease severity (Sugiyama et al., 1996). AGEs can mediate their effects via specific receptors, such as the receptor for AGEs (RAGE), oligosaccharyl transferase-48 (AGE-R1), 80K-H (AGE-R2), and galectin-3 (AGE-R3) (Vlassara et al., 1995; Li et al., 1996), activating diverse signal transduction cascades and downstream pathways, including generation of reactive oxygen species (ROS). Exposure of cultured mesangial cells to AGEs results in a receptor-mediated upregulation of mRNA and protein secretion of Col4 (Hasslacher et al., 1984; Iehara et al., 1996). However, there is little information regarding the mechanisms that underlie this regulation. In view of the wide occurrence of AGEs and AGEs-derived oxidative stress in diabetes, it would be of great interest to identify and develop AGE inhibitors that can suppress the formation of AGEs (Huijberts et al., 1993). Beyond the current treatments to treat diabetic complications, such as the optimization of blood pressure and glycemic control, it is predicted that new therapies designed to target AGEs, including AGE formation inhibitors and cross-link breakers, as well as targeting ROS using novel highly specific antioxidants, will become part of the treatment regimen for diabetic renal disease. Forbes et al. (2003) demonstrated that the administration of ALT 711, an AGE inhibitor, in diabetic rats readily reduced the glomerulosclerosis index, the tubulointerstitial area, and albuminuria in an experimental model of DN. Unfortunately, however, the development of most AGE inhibitors or breakers has been discontinued due to safety profiles of these compounds (Thornalley, 2003).

Smad1 is identified as a Col4-binding protein

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 remains poorly understood. Bruggeman et al. previously reported that the 130-bp bidirectional promoter of Col4 contains a large stem-loop structure (CIV) that interacts with several DNA-binding proteins (Bruggeman et al., 1992) (Fig. 1). Using a gel mobility shift assay, we demonstrated that an unknown protein binding to the CIV site directly regulates Col4 expression only when exposed to AGEs (Iehara et al., 1996). To identify the protein that binds to the CIV site in the promoter region of the mouse Col4 gene, we constructed a cDNA library from mouse mesangial cells exposed to AGEs. We then used a yeast one-hybrid system to isolate a clone that encodes a specific binding protein from the library and identified the clone as the cDNA that encodes Smad1 (Abe et al., 2004) (Fig. 2). Using chromatin immunoprecipitation (ChIP) and reporter assays, we observed that Smad1 directly and positively regulated the transcription for Col4. Moreover, we examined the expression of Smad1 in mesangial cells exposed to AGEs. The levels of Smad1 mRNA and protein were significantly increased in parallel with the upregulation of Col4 expression. Immunocytochemical analysis revealed that exposure to AGEs induced phosphorylation and nuclear accumulation of Smad1 in mesangial cells. Furthermore, 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 (Abe et al., 2004).

Previous studies have shown that TGF-ß plays an important role in the AGE response of the glomeruli, and transgenic mice overexpressing TGF-ß develop severe glomerulosclerosis (Sanderson et al., 1995). Therapeutic approaches to down-regulate TGF-ß signaling under diabetic conditions provide one strategy for inhibiting the progression of diabetic nephropathy. Thus, TGF-ß is thought to be a central mediator of the sclerosing process in diabetic nephropathy. It is generally known that Smad3 functions as a key intracellular signal transducer for profibrotic TGF-ß responses in various cells. However, the role of the Smad3 pathway in the pathogenesis of DN has only been demonstrated for

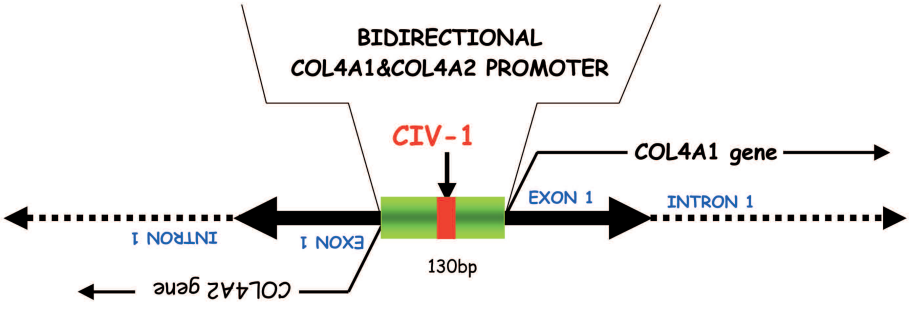

Fig. 1. Promoter of type IV collagen. The 130-bp bidirectional promoter of type IV collagen contains a large stem-loop structure (CIV), which has been shown to interact with several DNA binding proteins.

interstitial fibrosis in a model of obstructive nephropathy (Sato et al., 2003). 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-ß antibody (2G7) (Ziyadeh et al., 2000). These results suggest the existence of another signaling pathway involved in the development of DN. Members of the TGF-ß superfamily bind to two different types of serine/threonine kinase receptors, termed type I and type II receptors (ten Dijke et al., 1996). Type II receptors activate type I receptors, which transduce various signals via the Smads. TGF-ß is able to activate two distinct TGF-ß type I receptors and signal transduction pathways: the activin-like kinase 5 (ALK5)/Smad2/3 pathway and ALK1/Smad1/5-regulated pathway (Chen and Massagué, 1999; Oh et al., 2000). 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. We also demonstrated that ALK1, together with Smad1 and Col4, was highly expressed in human DN, corresponding to the progression of diabetic conditions (Abe et al., 2004). 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. ALK1 is one of the type I receptor members for TGF-ß family proteins and has been linked to an inherited multisystemic vascular disorder, hereditary hemorrhagic telangectasia 2 (HHT2) (Johnson et al., 1996). Furthermore, ALK1 is highly expressed in vascular endothelial cells (Attisano et al., 1993; Massagué and Wotton, 2000), and may be essential for vascular maturation and stabilization (Urness et al., 2000; Larsson et al., 2001). Accordingly, we speculate 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). These results should lead to not only a better understanding of the mechanisms responsible for the initiation and progression of diabetic conditions, but also the development of novel therapeutic strategies for the treatment of diabetic vascular complications.

Activation of Smad1 in the angiotensin II signaling pathway

The renin-angiotensin system (RAS) has key regulatory functions for blood pressure and fluid homeostasis. The benefit of blocking the RAS in patients with diabetes who are at risk of ESRD is now well established (Lewis et al., 1993, 2001; Brenner et al., 2001; Parving et al., 2001b; Gæde et al., 2003). Epidemiologic data indicate that the presence of albuminuria can predict increased cardiovascular morbidity and mortality independent of other cardiovascular risk factors (Keane and Eknoyan, 1993). Gerstein et al. analyzed information from the Heart Outcomes Prevention Evaluation (HOPE) study and noted that microalbuminuria is a powerful predictor for major cardiovascular events and all-cause mortality in patients with and without diabetes (Gerstein et al., 2001). In practice, angiotensin-converting-enzyme (ACE) inhibitors and Ang II type 1 receptor blockers (ARB) are widely used in diabetic patients and have been proved clinically effective in slowing the decline in renal function (Ruggenenti et al., 1998; Brenner et al., 2001; Lewis et al., 2001). Ang II is known to play a pivotal role in the development of DN. Activation of RAS contributes to oxidative stress, which might also potentially increase AGEs (Tikellis et al., 2006). Thus, Ang II has many actions that might cause or contribute to DN. Collectively, renal RAS is activated in DN and is

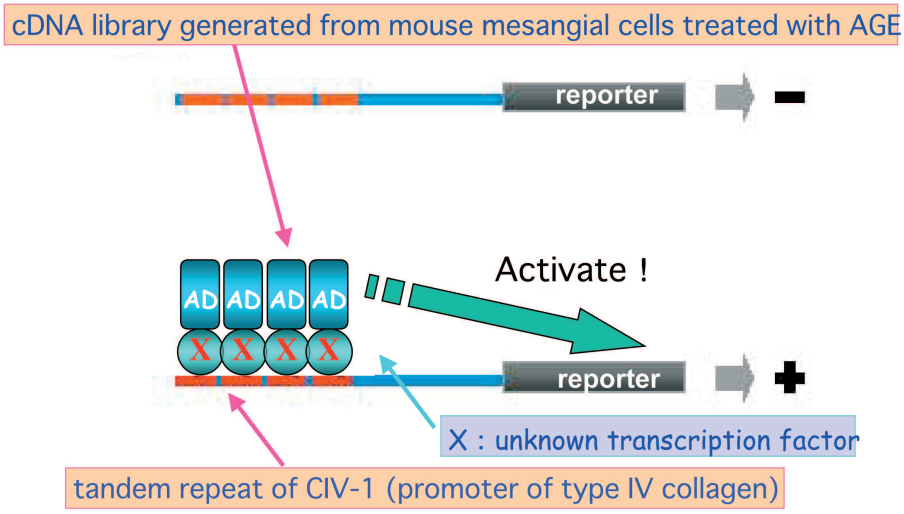

Fig. 2. Cloning of Smad1 by using a yeast one-hybrid assay. The cDNA library from mouse mesangial cells treated with AGE was constructed. Next, reporter plasmids were linked to four tandem copies of the binding sequence (CIV-1), and then transformed into yeast. Clones that contain a fusion protein between 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.

involved in the pathogenesis of the disease. The necessity for aggressive blood pressure control is undisputed in the medical community. Therefore, preventing the development of microalbuminuria is thought to be a key treatment goal for renoprotection (UK Prospective Diabetes Study (UKPDS) Group, 1998) and, possibly, for cardioprotection (Ritz, 2003). At present, however, blockade of RAS is not considered to halt the development of diabetic nephropathy. Furthermore, the mechanisms governing the overproduction of ECM in the activation of RAS leading to glomerulosclerosis in mesangial cells have not been determined.

Several reports indicate that RAS is accelerated in the early phase of DN (Kagami et al., 1994) and that intrarenal Ang II expression is already augmented before overt DN (Anderson, 1997). In vitro studies have shown that Ang II stimulates mesangial cell matrix biosynthesis via the AT1 receptor, and this is mediated by TGF-ß (Kagami et al., 1994; Wolf and Zidayeh, 1997). In rat mesangial cells, glucose-induced TGF-ß secretion is abrogated by ARBs (Singh et al., 1999). The renoprotective effect of RAS blockade could result from abrogation of some of these mechanisms. However, the precise underlying intracellular molecular mechanisms regulating the TGFß-mediated effects of Ang II in ECM overproduction have not been fully elucidated. Therefore, we examined whether Ang II can modulate Smad1-mediated signaling involved in mesangial matrix expansion in diabetic nephropathy *in vivo* and *in vitro* (Mima et al., 2006), and showed a direct link between Src and Smad1 activation, and the subsequent increase of Col4 synthesis in mesangial cells (Fig. 3). Furthermore, administration of ARB (olmesartan) or Src inhibitors attenuated diabetic mesangial matrix expansion, independent of its effects on blood pressure and glucose metabolism (Mima et al., 2006). These results have clarified the intracellular molecular mechanism involved in the influence of Ang II on the development of DN.

Smad1 and phenotypic change in diabetic nephropathy

Mesangial cells provide structural support to the glomerulus by producing extracellular matrix components that form the mesangial matrix (Schlondorff, 1987). Emerging evidence suggests 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) (Johnson et al., 1991; Desmouliere et al., 1993; Abe et al., 2004). Myofibroblasts are key participants 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 (Ronnov-Jessen and Petersen, 1996).

Mesangial cells with phenotypic change of positive SMA have been observed in various glomerular diseases, including DN. The expression of SMA is associated with mesangial proliferation (Groma et al., 1997). Hyperglycemia has been implicated as an important factor in the development of phenotypic changes, as many such changes have been induced in vitro by exposing mesangial cells to elevated glucose levels (Ayo et al., 1991; Ziyadeh et al., 1994). High glucose exerts its deleterious effects by numerous pathways; in particular, the multifunctional cytokine TGF-ß has been implicated as a principal mediator of DN (MacKay et al., 1989; Border et al., 1990; Sharma and Zidayeh, 1995). Diabetic kidney disease is thought to be associated with increased expression of TGF-ß in glomerular and tubular epithelial cells. TGF-ß has been previously reported to induce SMA expression in various cells (Orlandi et al., 1994). The role of the Smad3 pathway in the pathogenesis of interstitial fibrosis was demonstrated in a model of obstructive

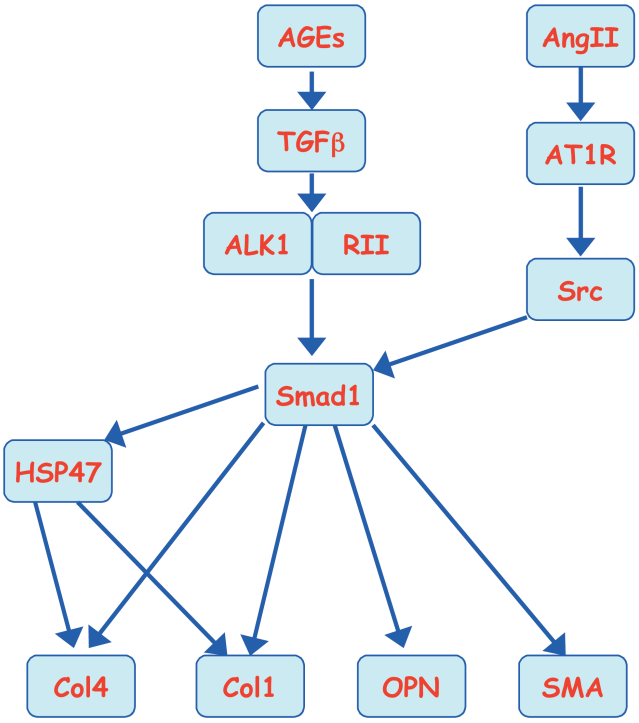

Fig. 3. Schematic drawing of the upstream molecular signaling pathways of Smad1 expression that could lead to glomerulosclerosis in diabetic nephropathy. AT1R; type 1 angiotensin II receptor, OPN; osteopontin, RII; TGF-ß receptor type II.

nephropathy. In the same report, it was suggested that the Smad3 pathway is essential for TGF-ß-induced epithelial-mesenchymal transition (EMT), and SMA mRNA was detected in both renal tubular epithelial cells and fibroblastic cells adjacent to the renal tubules (Sato et al., 2003). Another recent report has shown that TGF-ß down-regulates Smad3 in human mesangial cells with a myofibroblastic phenotype (Poncelet et al., 2007).

In contrast, 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 (Abe et al., 2004). Moreover, induction of Smad1 and SMA expression coincides with the development of glomerulosclerosis in both type 1 and type 2 diabetic mice or rats (Matsubara et al., 2006; Mima et al., 2006). As a downstream modulator of the TGF, signaling pathway, it is now clear that Smad1 transduces TGF, through a type I receptor, ALK1, which is newly induced in MCs in diabetes. We found that ALK1, together with Smad1 and Col4, was highly expressed in human advanced diabetic nephropathy (Abe et al., 2004). Accordingly, it can be considered that ALK1 directly phosphorylates Smad1and brings on 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. In addition, because this phenotypic alteration, which is characterized by SMA gene induction, is a common phenomenon in the process of sclerotic or fibrotic changes in many organs, such as the liver (Ramadori et al., 1990), lung (Zhang et al., 1994), and skin (Sundberg et al., 1996), Smad1 is thought to also be involved in tissue remodeling in other organs.

Smad1 and diabetic glomerulosclerosis-related genes

Glomerulosclerosis is the common pathological feature in most immunological and non-immunological renal diseases, and is tightly related to the progression of renal failure. In morphological studies, glomerulosclerosis is characterized by the depletion of glomerular cells and the accumulation of extracellular matrix, including type I, III and IV collagens, fibronectin, laminin and proteoglycans (Makino et al., 1993). In addition, it has been well accepted that expression of heat-shock protein 47 (HSP47), which is a collagen-binding molecular chaperon (Nagata, 1998), and osteopontin (OPN) (Xie et al., 2001) are key factors in the progression of renal injuries and sclerosis. Although numerous attempts have been made to elucidate the precise molecular pathogenesis of glomerulosclerosis, no key molecule has been identified. We first demonstrated that Smad1 directly regulated Col4 expression in diabetic nephropathy. In addition, Col1, HSP47, SMA, and OPN have also been shown to be regulated by Smad1 signaling in MCs (Fig. 3) (Abe et al., 2004). Taken together, these results indicate that the phenotypic switch to osteoblast/chondroblast-like cells may result in an irreversible process, and thereby implies that activation of Smad1 signaling may introduce irreversible changes in the diabetic kidney. It has been well established that Smad1 transduces bone morphogenetic protein (BMP) signals (Massagué and Wotton, 2000) and is critically important in the development of the kidneys (Vrljicak et al., 2004).

In humans with diabetes, expansion of the mesangial area and subsequent loss of the capillary-filtering surface by squashing of the glomerular capillaries correlates closely with a declining glomerular filtration rate (Fig. 4). Therefore, once ECM expansion begins, unless it is

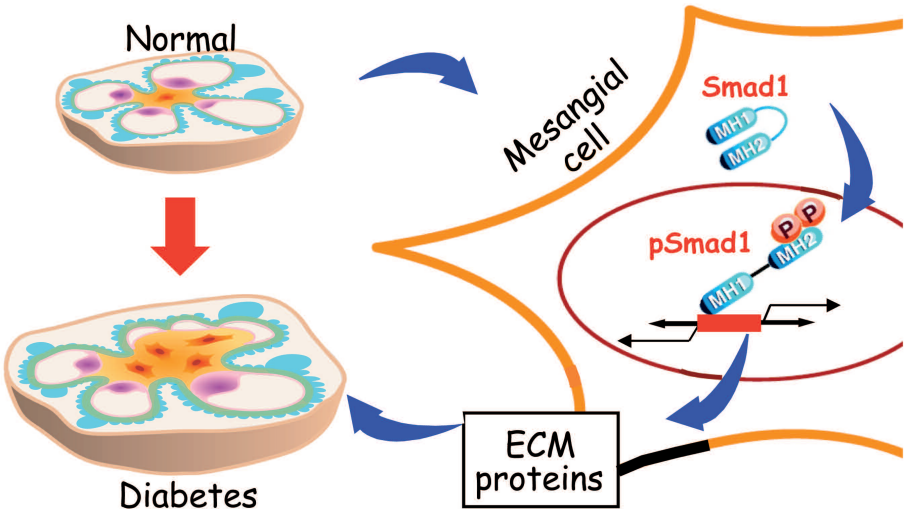

Fig. 4. Schematic Illustration of the development of diabetic nephropathy.

otherwise prevented or controlled, renal damage will progress irreversibly. In addition, it has been proposed that glomerular hemodynamic changes or glomerular growth response may promote the development of glomerulosclerosis (Mackenzie et al., 1996). However, the current treatments for DN, including optimal metabolic and blood pressure control, proteinuria reduction, and RAS inhibition, have only slowed the rate and extent of decline in renal functions. Therefore, novel therapies based on molecular pathophysiology are needed to improve the outcome for DN patients.

Urinary Smad1 as a biomarker to indicate structural changes in diabetic nephropathy

Although the presence of diabetic glomerulosclerosis can also be inferred from the clinical presentation, an invasive procedure, the kidney biopsy, is required to make a definitive diagnosis. Therefore, the non-invasive diagnosis of kidney diseases is a challenge in clinical nephrology. To date, the measurement of albuminuria has been used as a standardized non-invasive test for the diagnosis of early DN (Caramori et al., 2000). Diabetic kidney disease, however, is not detected by this test in some cases. Biopsy studies have shown that albuminuric patients with type 2 diabetes without retinopathy frequently suffer from nondiabetic kidney disease (Parving et al., 1992; Fioretto et al., 1996; Cordonnier et al., 1999), but the prevalence is not known.

Recently, by using a variety of proteomics techniques, several protein biomarkers have been identified as non-invasive indicators of kidney injury in diabetes. Jain et al. used 2DE and MALDI-TOF-MS to identify urinary protein markers for accurate prediction of nephropathy in type 2 diabetic patients. Along with albumin, four proteins were identified, including zinc α-2 glycoprotein and α-1 acid glycoprotein, in the urine of diabetic patients with microalbuminuria (Jain et al., 2005). Rao et al. applied 2D-DIGE and LC/ESI-MS/MS to identify urinary protein biomarkers of DN in urine from type 2 diabetic patients. They reported that seven proteins, including vitamin D-binding protein and hemopexin, were progressively upregulated with increasing albuminuria, and four proteins, including retinol-binding protein and transthyretin, were progressively downregulated (Rao et al., 2007). These proteins can be used as markers for specific and accurate clinical evaluation of DN. However, it has not been clarified how these proteins obtained from proteomics using mass spectrometry contribute to the development of DN.

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, it is necessary to establish a non-invasive marker reflecting both predictable and therapeutic effects. The optimal approach to diagnosis stems directly from a consideration of the pathology and pathophysiology of the disease. In this context, it has been shown that Smad1 is absent in the renal glomeruli of normal adult mice (Huang et al., 2000). We recently showed that AGEs induce the expression of Smad1 in adult mouse glomeruli. Therefore, Smad1 may be the earliest indicator of renal dysfunction. We 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 6 weeks diabetes in db/db mice (Matsubara et al., 2006; Mima et al., 2008). 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 (Fig. 5). In addition, to mimic the human situation, some animals were treated with the ARB olmesartan, which is known to block DN development. Olmesartan treatment significantly ameliorated glomerulosclerosis and dramatically decreased urinary Smad1. These findings indicate that urinary Smad1 could be a novel predictor

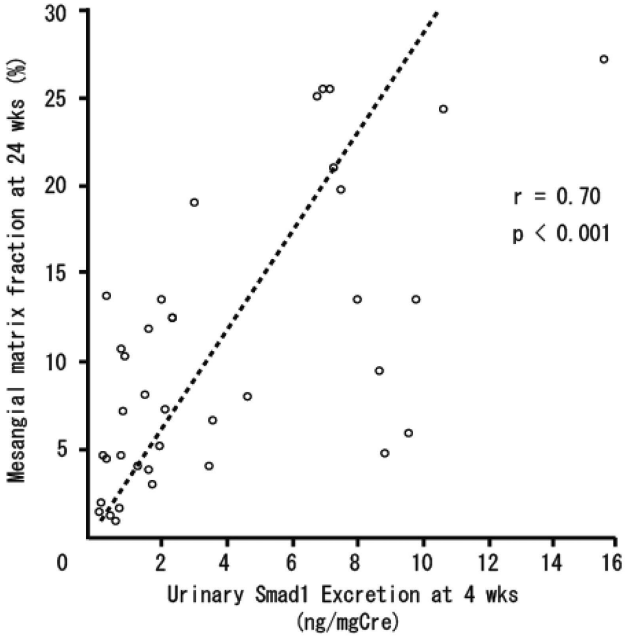

Fig. 5. The correlation between urinary excretion of Smad1 and mesangial matrix fraction. The correlation between urinary excretion of Smad1 and mesangial matrix fraction in diabetic rats. Quantification of urinary concentration of Smad1 was described elsewhere (Matsubara et al., 2006).

for later onset of morphological changes and can be used to monitor the effect of ARB in DN (Mima et al., 2008). Clinical studies are underway to investigate the concept that urinary Smad1 could be an early predictor in diabetic patients.

Blocking of Smad1 attenuates overproduction of ECM proteins

Because strategies to prevent and control DN would be expected to result in a reduction in patient morbidity and mortality, as well as significant cost savings, it is hoped that the studies presented here will lay the foundation for more effective therapies that will halt the development of DN. Even though powerful instruments are currently available to lower blood pressure substantially and achieve specific pharmacological blockade of RAS, we are still confronted with patients whose proteinuria and loss of renal function is not controlled satisfactorily by this approach. Some workers have said that insufficient diabetic control played an important role in the pathogenesis of diabetic microangiopathy, as well as the aging factor and the duration of diabetes (Joslin et al., 1959). In most patients, DN still progresses inexorably to ESRD. Patients with advanced DN not only have a high incidence of cardiovascular disease (CVD), but CV morbidity and mortality is the leading cause of death in these subjects. Novel therapies are urgently required to improve outcomes for patients with this disease. Therapy is ideally based on controlling the pathologic effects of mesangial matrix expansion and proteinuria.

Previous studies have shown that TGF-ß is a key mediator of ECM accumulation in experimental and human kidney disease, which leads to progressive glomerular scarring and renal failure (Sanderson et al., 1995). Therapeutic approaches to downregulate TGF-ß signaling under diabetic conditions constitute one strategy to inhibit the progression of diabetic nephropathy. For example, the use of endogenous proteoglycan decorin (a natural inhibitor of TGF-ß) (Isaka et al., 1996) or neutralizing anti-TGF-ß (Chen et al., 2003) has been shown to prevent the development of diabetic glomerulosclerosis. However, prolonged inhibition of TGF-ß may lead to unwanted adverse effects, because TGF-ß has anti-proliferative effects in some cancers, and there is a report that Smad3-deficient animals developed metastatic colon tumors (Zhu et al., 1998). Since Smad1 directly regulates the expression of extracellular matrix proteins in DN, antisense or siRNA targeting Smad1 could be used as novel chemo-therapeutics against diabetic complications. Selective inhibition by antisense (AS) for the Smad1-signaling pathway under the condition of exposure to AGEs resulted in significant attenuation of Col4, while control oligos had no effect on the expression of these genes. Similarly, the mRNA levels of both Col1 and OPN were significantly decreased (Fig. 4). These data indicate that Smad1 plays a critical role in the control of Col4 expression.

We also observed that chronic exposure to AGEs induced a sustained increase in Smad1 gene activation and expression and led to glomerulosclerosis, suggesting that Smad1 is a critical modulator in diabetic conditions. Therefore, direct inhibitors for Smad1 and suppression of Smad1 phosphorylation (for example, ALK1 inhibitors) will lead to novel therapeutic approaches and should be useful in combination with the current therapies.

Future perspectives

The development of diabetic kidney disease in diabetic patients is a huge clinical problem associated with increased morbidity and mortality, along with impaired quality of life. It is also clear that the current therapeutic approach of glycemic control can slow (The Diabetes Control and Complications Trial Research Group, 1993; UK Prospective Diabetes Study (UKPDS) Group, 1998), but cannot completely prevent the development or progression of DN in most patients. Established diabetics are more likely to develop serious cardiovascular and cerebrovascular disease than are new diabetics and are at increased risk from stroke and myocardial infarction caused by vascular occlusion. Despite extensive investigations, DN has remained an unresolved problem. Here, we provide 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 Smad1 in the initiation and progression of DN. This undoubtedly represents an initial breakthrough into a very promising non-invasive marker for early glomerular injury. 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.

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