Regulation of Transforming Growth Factor β in Diabetic Nephropathy: Implications for Treatment

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Summary: The recognition that the drivers of matrix accumulation is an appropriate therapeutic target for diabetic nephropathy is now accepted by the nephrology and pharmaceutical communities. Interventions focused around transforming growth factor-β (TGF-β) likely will be an important area of clinical investigation in the near future. Understanding the various pathways involved in stimulating TGF-β in the diabetic kidney is of paramount importance in devising strategies to combat the development and progression of diabetic nephropathy. In this review we highlight the major pathways involved in stimulating TGF-β production by increased glucose levels and discuss the therapeutic implications thereof.
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Transforming growth factor-β (TGF-β) has been linked closely to the development and progression of diabetic nephropathy in cell culture, animal models, and the human condition.[1,2] It is likely that there will be several strategies to target TGF-β production and action as a novel means of therapy for diabetic nephropathy within the next 10 years. In this review, we highlight several aspects regarding the regulation of TGF-β in the context of diabetic kidney disease. An aspect that will be highlighted is that there are multiple pathways by which TGF-β may be stimulated in diabetic kidney disease, thus complicating approaches that attempt to block one pathway and not others.

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REGULATION OF TGF-β BY GLUCOSE

Increases in glucose level drive stimulation of TGF-β in cell culture, animal models, and human. We recently showed that a glucose infusion for 2 hours to increase plasma glucose to the 200 to 250 mg/dL range led to stimulation of urinary levels of TGF-β1 in normal human subjects.[3] This study showed that transient increases of blood glucose level, even in healthy individuals, is sufficient to cause production of TGF-β. Because urine levels of TGF-β1 increased and plasma levels were stable, the study suggested that the kidney may indeed be responding to hyperglycemia with increased TGF-β production. The mechanisms involved in this response remain to be proven in human beings, however, several pathways likely are involved based on experiments in cell culture and animal models.

Intracellular Pathways Protein Kinase C

Protein kinase C (PKC), reactive oxygen species (ROS), hydroxyeicosatetraenoic acid (HETE), hexosamines, and the extracellular signal-regulated kinase (ERK)-p38 mitogen-activated protein kinase (MAPK) all have been implicated in mediating glucose-induced stimu-

lation of TGF-β. PKC has been found to be stimulated by glucose increases in mesangial cells and vascular smooth muscle cells.[4-6] The isoform responsible for regulating TGF-β has not been shown conclusively, although there are convincing data that the PKC-β isoform is intimately involved. A PKC-β inhibitor has been shown to block TGF-β stimulation in animal models of diabetic kidney disease and block matrix accumulation.[7,8] These preclinical studies have contributed to recognizing the possible role of PKC-β inhibition in human diabetic kidney disease.[9] The PKC-α isoform likely is not involved in TGF-β stimulation because PKC-α knockout diabetic mice did not show a reduction of renal TGF-β levels as compared with wild-type diabetic mice.[10]

Reactive Oxygen Species

ROS production appears to be critical in the pathophysiology of diabetic vascular complications. High glucose levels induce ROS in mesangial cells[11] and ROS up-regulates TGF-β and extracellular matrix expression.[12] In addition, Nath et al[13] reported that H_2O_2 was able to induce TGF-β messenger RNA (mRNA) expression in rat kidneys and isolated fibroblasts. Scavenging of ROS by α-lipoic acid and manganese superoxide dismutase leads to decreased TGF-β production in diabetic kidney disease and amelioration of diabetic renal pathology and albuminuria.[14,15] Specific pathways that lead to ROS production by high glucose levels in renal cells will need to be determined and may pave the way for directed therapies to focus on ROS-induced TGF-β production. One such pathway is nicotinamide adenine dinucleotide phosphate, reduced form (NADPH) oxidase and the isoform Nox4. Specific inhibition of Nox4 blocks glomerular matrix accumulation in diabetic rats[16] and may be involved in the stimulation of TGF-β. On the other hand, TGF-β itself could cause ROS production via NADH/NADPH oxidase.[17-19] We reported that TGF-β1 induced ROS production in vascular smooth muscle and endothelial cells via NADPH oxidase and that Nox4 is involved in cytoskeletal alterations by TGF-β.[18,19]

Lipoxygenases

Lipoxygenases (LOs) are a family of enzymes that insert molecular oxygen into polysaturated fatty acids. They are classified as 5-, 8-, 12-, and 15-LO. 12-LO activation can produce 12(S)-HETE[20] and has been shown to stimulate TGF-β in human macrophages.[21] High glucose levels have been shown to stimulate 12-/15-LO expression, and 12(S)-HETE (12-LO product) induces cellular hypertrophy and fibronectin expression in rat mesangial cells.[22] Recently, the Natarajan group[23] clarified the cross-talk between the TGF-β and 12-/15-LO pathway in mesangial cells. Direct addition of 12(S)-HETE to rat mesangial cells stimulated the murine TGF-β1 promoter, TGF-β1 mRNA, and protein expression, along with p-Smad 2/3 activation. Reciprocally, TGF-β treatment of rat mesangial cells increased 12-/15-LO mRNA expression and 12(S)-HETE production significantly. In addition, mesangial cells from 12-/15-LO knock-out mice expressed less TGF-β, and mesangial cells overexpressing 12-/15-LO produced more TGF-β. The investigators[23] suggested that 12-/15-LO and TGF-β could cross-talk and activate each other during the initiation and progression of diabetic kidney disease.

Hexosamines

Hexosamines, such as glucosamine-6-phosphate, can be formed under the control of the rate-limiting enzyme glutamine:fructose-6-phosphate amidotransferase (GFAT). Immunostaining of GFAT showed increased staining in diabetic kidneys, primarily in glomerular mesangial and epithelial cells.[24] Inhibition of GFAT by chemical inhibitors can block TGF-β expression in mesangial cells treated with high glucose levels.[25] The PKC and p38 pathways appear to be involved in mediating hexosamine-induced TGF-β production in human mesangial cells.[26]

ERK and p38 MAPK

ERK and p38 MAPK are activated in mesangial cells exposed to high glucose levels and in rat glomeruli of early type 1 diabetes.[27,28] In a type 2 diabetic model, ERK activity was reported to be activated significantly in renal cortex of

db/db mice as compared with nondiabetic mice.[29] ERK activation may well be the result of upstream PKC activation because PKC inhibition also inhibits ERK activation.[30] There is a growing body of evidence supporting a role for p38 in diabetic kidney disease[31] and its regulation of TGF-β.[32] The mechanism of high glucose–induced p38 MAPK in monocytes and mesangial cells may be mediated via ROS[33,34] and possibly independent of PKC. High glucose levels have been shown to stimulate p38 in mesangial cells, podocytes, endothelial cells,[35] and glomeruli in diabetic kidney disease.[36] Inhibition of p38 may mediate renal TGF-β production in several models of kidney disease[37] and in proximal tubular cells.[32]

TRANSCRIPTION FACTORS INVOLVED IN GLUCOSE-INDUCED TGF-β STIMULATION

The major transcription factors involved in mediating glucose-induced TGF-β1 promoter activity has been postulated to include the activator protein 1 (AP-1) complex and the family of upstream stimulatory factors (USF). Activation of PKC is well known to stimulate the *c-fos* and *c-jun* proto-oncogenes that form complexes for the AP1 binding site of the human and murine TGF-β1 promoter.[38,39] Mutagenesis of either 1 of the 2 or both AP-1 binding sites abolished the high glucose and phorbol myristate acetate (PMA) effect in the human promoter.[38] Furthermore, addition of the AP-1 inhibitor curcumin blocked the glucose response.[38] Interestingly, curcumin treatment of diabetic rats ameliorates kidney disease, although no measurements of TGF-β1 were performed.[40,41] However, to date there are no in vivo studies to clearly implicate AP-1 in glucose-mediated renal TGF-β production.

Our group initially identified the CACGTG element or E-box as an important site for glucose regulation in the murine TGF-β1 promoter.[42] High glucose levels increased the binding of mesangial cell nuclear proteins to this E-box element. Additional studies by our laboratory and others now clearly have determined that USF1 and USF2 are involved in binding the murine TGF-β1 promoter[43] and the human TGF-β1 promoter.[44] High glucose levels induced stimulation of the human TGF-β promoter via the E-box and USF activity is regulated by the hexosamine pathway.[44] In murine mesangial cells, chromatin immunoprecipitation assay revealed in vivo binding of USF1, but not USF2, to a glucose-responsive region of the TGF-β1 promoter. Furthermore, glucose fluctuation with high carbohydrate feeding led to the stimulation of renal TGF-β1 mRNA levels in wild-type and USF2 knockout mice, but not in the USF1 knockout mice.[43] Therefore, there are convincing data that both USF1 and USF2 are involved in regulating TGF-β1 production by high glucose levels in mesangial cells and, at least in the murine system, the available evidence favors a dominant role for USF1. Future studies in various diabetic models are required to identify modulators of USF1 and USF2 to better understand the role of this family of transcription factors in the development of diabetic nephropathy.

TGF-β ACTIVATION: LATENCY-ASSOCIATED PROTEIN, THROMBOSPONDIN, AND DECORIN

TGF-β usually is secreted in large latent complexes without biological activity. It consists of 3 components: a disulphide-bonded homodimer of mature TGF-β, noncovalently bound to the latency-associated protein (LAP; homodimers of the N terminal fragment of precursor TGF-β), and a covalently attached molecule of latent TGF-β binding protein.[45-47] LAP and TGF-β compose the small latent complex. In this latent complex, TGF-β cannot bind to its surface receptors. Thus, the dissociation of TGF-β from LAP is a critical regulatory mechanism.[45] The administration of LAP reduces glucose-induced fibronectin production in mesangial cells,[48] however, no studies to date have comprehensively examined the regulation of latency associated peptide with active TGF-β in the setting of diabetic kidney disease.

Thrombospondin 1 (TSP1) is a homotrimeric multifunctional glycoprotein expressed by a variety of cell types such as platelets, vascular smooth muscle cells, and mesangial cells. It frequently is expressed at sites of inflammation and wound healing,[49] is overexpressed in diabetic vessels[50] and diabetic kidneys,[51] and is secreted by mesangial cells.[52] Murphy-Ullrich

and Hook[53] first reported that TGF-β could bind to TSP1 under physiologic conditions. More detailed studies identified 2 important sites in the TSP1 molecule that were responsible for this complex interaction. One is the WxxW (WSHW, WSPW, or WGPW) motif from the type I repeats of the TSP1 that binds active TGF-β, and the other site is the (K)RFK-sequence that binds the N-terminal LSKL-sequence of the LAP.[54,55] Additional studies indicate that high glucose level–induced activation of TGF-β is largely dependent on TSP1. There are convincing data that high glucose levels stimulate TSP1 gene transcription via the USF2 transcription factor[56] and TSP1 then binds to the LAP, thus dissociating active TGF-β. Interruption of this step may have therapeutic implications.

Decorin is a multifunctional extracellular proteoglycan,[57] and its core protein neutralizes TGF-β and antagonizes its prosclerotic effect.[58,59] Exogenous decorin suppressed TGF-β mRNA expression of the kidney.[58] Mogyorosi and Ziyadeh[60] reported a rapid and sustained increase of decorin mRNA expression in an animal model of type 1 diabetes. Furthermore, decorin expression was enhanced by high glucose levels in mesangial and proximal tubular cells.[60] The stimulation of decorin by high glucose levels has been shown by several studies[61,62] and appears to be via a CAMP response element binding protein (CREB)-dependent pathway.[63] It was notable that decorin expression in mesangial and proximal tubular cells was suppressed by exogenous TGF-β treatment, both in high and normal glucose level conditions.[60] Because both decorin and TGF-β were up-regulated in the diabetic state, Mogyorosi and Ziyadeh[60] speculated that TGF-β and decorin act in a negative feedback loop with each other.

In a human study, Schaefer et al[64] revealed that small proteoglycans including decorin mRNA were up-regulated in all stages of diabetic nephropathy, both in the tubulointerstitium and in glomeruli. They pointed out that the glomerular expression and protein accumulation of decorin were not prominent compared with mRNA expression. They suggested that this phenomenon could be explained by assuming that decorin was secreted into the mesangial matrix and then cleared via the vasculature or the urinary tract, in part as complexes with TGF-β. In advanced diabetic nephropathy, decorin deposition was found in fibrotic areas and was colocalized with deposits of type I collagen.[64] Decorin may modulate the progression of TGF-β–mediated renal fibrosis through the formation of complexes of decorin type I collagen and TGF-β. Decorin deficiency could result in an imbalance of decorin and TGF-β counterregulation and lead to excess TGF-β activity and progressive matrix accumulation. Further studies are needed to clarify the relative role of decorin in mediating TGF-β activity and progression in diabetic nephropathy.

ROLE OF MACROPHAGE IN TGF-β PRODUCTION

Most studies have focused on mesangial and proximal tubular cells as the cells responsible for TGF-β production in diabetic kidney disease[32,65-67]; however, there are a variety of other cell types that may contribute as much or more to renal TGF-β production in diabetic kidney disease. Macrophages are a rich source of TGF-β and it is now clear that macrophage infiltration is a characteristic feature of diabetic nephropathy.[68,69]

The role of macrophages in diabetic kidney disease remains to be clarified. Depletion of leukocytes by irradiation in the diabetic rat leads to reduced α3 (IV) collagen mRNA expression in the glomeruli.[70] Moreover, diabetic intracellular-adhesion molecule (ICAM)-1 knockout mice showed reduced macrophage infiltration and decreased TGF-β and type IV collagen expression coincident with reduced mesangial matrix expansion and reduced albuminuria.[68] It is likely that there is an important cell–cell interaction between macrophages and mesangial cells because it has been reported that the culture supernatant of macrophages stimulates mesangial cells to produce fibronectin.[71] Together with these findings, one could speculate that infiltrated glomerular macrophages and mesangial cells conspire to both secrete TGF-β and promote a positive feedback loop. A potential protective role also may apply to macrophages in early diabetic kidney disease. Glomerular mesangial cell production of hyaluronan leads to the attraction of macrophages in early diabetes in the rat.[72] Macrophages appear to be

responsible for the removal of glomerular hyaluronan, and macrophages then may depart. If macrophages remain it is likely that they may contribute to pathology. Because it is likely true for all infiltrating cell types, the context in which the cell is present is critical in understanding its role. A complex dual role for macrophages also has been suggested in the development of atherosclerosis[73] and in models of glomerulonephritis.[74,75]

TGF-β AND PODOCYTES

There is a growing body of evidence that podocytes occupy a significant role in the pathogenesis of diabetic nephropathy, diabetic glomerular proteinuria, and matrix accumulation.[76-78] The role of the podocytes as a cell type producing TGF-β in diabetic nephropathy has not been clarified. Human diabetic nephropathy kidney sections have been shown to have increased glomerular mRNA and protein for TGF-β1 in podocytes and mesangial cells.[51] It remains to be established whether the high-glucose level condition stimulates TGF-β expression in podocytes. In human podocytes, high glucose levels induced increased TGF-β expression in both protein and mRNA levels.[79] However, De La Cruz et al[80] reported that high glucose levels did not stimulate the production of TGF-β1 in cultured mouse podocytes, although high glucose levels did stimulate the expression of TGF-β type II receptor. In addition, Chen et al[81,82] showed that angiotensin (Ang)II could induce podocyte dysfunction via TGF-β and vascular endothelial growth factor activation, and these factors could lead to glomerular basement membrane (GBM) thickness and albuminuria in diabetic nephropathy. Of note, it is intriguing that anti–TGF-β approaches are associated with a reduction of albuminuria in some experimental studies of diabetic nephropathy,[83] but not in others.[84,85]

IMPLICATIONS FOR ANTI–TGF-β APPROACHES

Based on the convincing body of work implicating stimulation of TGF-β in the diabetic milieu and its critical role in progressive nephropathy,[86] a new theoretical basis for therapy has emerged.[2,87] Emerging from the hemodynamic and metabolic control paradigms, the next decade or so may be characterized by an antifibrotic and cell-based therapeutic approach. The multiple pathways by which TGF-β may be stimulated in the diabetic condition leads to the consideration of a multipronged strategy to improve metabolic control, decrease hemodynamic stress, and reduce local AngII effects. However, with the understanding of ROS and glucose-induced intracellular signaling pathways to stimulate TGF-β1, it is clear that even suboptimal glycemic control would lead to ongoing production of TGF-β in diabetes. Accumulating evidence suggests that blood glucose level fluctuations are correlated with the complications of diabetes.[88] Future studies that determine the metabolic pathways activated by repeated glycemic fluctuations are likely to be important in understanding the basis for renal TGF-β production and serve as targets for therapeutic interventions. Furthermore, there will be heterogeneity in the pathways involved among various ethnic groups and sexes. Translational studies in human beings will facilitate future personalized approaches for interventions to block TGF-β production in the kidney.

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