GROWTH FACTORS, CYTOKINES, AND RENAL FIBROSIS DURING THE COURSE OF DIABETIC NEPHROPATHY

J. ROSSERT (1, 2), C. TERRAZ-DURASNEL (1), G. BRIDEAU (1)

SUMMARY - Diabetic nephropathy is characterised by a progressive accumulation of extracellular matrix within the glomerular mesangium and the interstitium. The pathogenesis of this fibrotic process is still poorly understood, but in vitro and in vivo data suggest that TGF-β plays a key role. Local overproduction of TGF-β could be secondary to a synthesis of diacylglycerol, polyols, or glucosamines. It may also be secondary to an accumulation of advanced glycosylation end-products which modify the functions of neighbouring cells. Moreover, clinical as well as experimental data for TGF-β suggest that angiotensin II has a profibrotic effect, and it has been clearly demonstrated that angiotensin-converting enzyme inhibitors have a beneficial effect in patients with insulin-dependent diabetes mellitus. Other molecules such as endothelin-1, lipid peroxidation products, or IGF-1 may also play a role in this fibrotic process. Finally, heavy proteinuria secondary to glomerular lesions enhances the accumulation of extracellular matrix within the interstitium, probably through modifications of tubular cell functions, thereby inducing the release of pro-inflammatory and profibrotic molecules.

RESUMÉ - Facteurs de croissance, cytokines et fibrose rénale au cours de la néphropathie diabétique.


Key-words: diabetic nephropathy, fibrosis, TGF-β.

Mots-clés: néphropathie diabétique, fibrose, TGF-β.
D iabetic nephropathy has long been recognized as a major factor in the morbidity and mortality of patients with diabetes mellitus. It develops in about one-third of patients with insulin-dependent diabetes mellitus and in almost as high a percentage of those with non-insulin-dependent diabetes mellitus. Renal lesions are characterised by early hypertrophy of both glomerular and tubulo-epithelial components of the nephron, a thickening of glomerular basement membrane and tubular basement membrane, and a progressive accumulation of extracellular matrix within the glomerular mesangium and the interstitium (reviewed in [1]). This accumulation of extracellular matrix plays a key role in the progression to renal failure, but its pathogenesis is still poorly understood. In vitro, different molecules produced by resident renal cells and/or inflammatory cells can stimulate the production of extracellular matrix by fibroblastic cells and/or mesangial cells (Table I) (reviewed in [2]). These molecules include cytokines such as IL-1 and IL-4, growth factors such as TGF-β and IGF-1, vasoactive peptides such as angiotensin II and endothelin-1, and other factors such as lipid peroxidation products. The effects of angiotensin II on the synthesis of extracellular matrix appear to be mostly indirect (mediated by other factors such as lipid peroxidation products. The effects of angiotensin II on the synthesis of extracellular matrix are to be mostly indirect (mediated by increased production of TGF-β) since they are abolished by anti-TGF-β antibodies. Similarly, part of the profibrotic effects of endothelin–1 may be mediated by increased production of TGF-β. The potential role of these profibrotic factors in the progression of diabetic nephropathy will be briefly reviewed here.

**TGF-β AND RENAL FIBROSIS IN DIABETIC NEPHROPATHY**

Among all profibrotic molecules, TGF-β is most clearly linked to the development of renal fibrosis during the course of renal diseases, including diabetic nephropathy.

| Table I. Soluble molecules which can stimulate type I collagen production by fibroblastic cells, in vitro [2].

<table>
<thead>
<tr>
<th>Soluble molecules</th>
</tr>
</thead>
<tbody>
<tr>
<td>TGF-β</td>
</tr>
<tr>
<td>IL-1</td>
</tr>
<tr>
<td>IL-4</td>
</tr>
<tr>
<td>IGF-1</td>
</tr>
<tr>
<td>Angiotensin II</td>
</tr>
<tr>
<td>Endothelin-1</td>
</tr>
<tr>
<td>Lipid peroxidation products</td>
</tr>
</tbody>
</table>

**TGF-β and extracellular matrix production**

TGF-β, a cytokine produced by most cell types, consists of two identical polypeptide chains of 112 disulphide-linked amino acids. In cells, each monomer is translated as a prepropeptide of 390 amino acids and undergoes a series of posttranslational modifications, including cleavage of the signal peptide, glycosylation, phosphorylation, and dimerisation, before being secreted (reviewed in [3]). In extracellular space, pro-TGF-β, which is inactive, is proteolytically trimmed of its N-terminal propeptide to form mature TGF-β. Mature TGF-β associates noncovalently with a dimer of its N-terminal propeptide, called latency-associated protein (LAP), which masks its activity. The LAP component of this latent complex is often disulphide-linked to so-called latent-TGF-β-binding proteins (LTBPs), resulting in the large latent form of TGF-β. These latent proteins increase the stability of TGF-β and target it to matrix and cell surfaces. Recently, a comparison of the phenotype of mice which were null for thrombospondin-1 and TGF-β1 has suggested that thrombospondin-1 is a major activator of TGF-β in vivo [4]. This hypothesis is reinforced by the fact that the phenotype of TGF-β1 null mice can be reproduced in wild type animals by pharmacological blocking of the cleavage of TGF-β1 by thrombospondin-1 [4]. Nevertheless, thrombospondin-1 may not be the only activator of TGF-β in vivo and other agents such as calpain, cathepsin, or plasmin, which can activate TGF-β1 in vitro, could also play a role in vivo. After being released from latent complexes, mature TGF-β signals through heteromeric complexes of type I and type II transmembrane serine/threonine kinase receptors. TGF-β first binds to type II receptor, and then type I receptor recognizes ligand-bound type II receptor to form a heteromeric complex (reviewed in [5]). The formation of this oligomeric complex induces the transphosphorylation of type I receptor by type II receptor kinase. This phosphorylation, which occurs in a cluster of five serine and threonine residues located next to the N terminus of the kinase domain, activates type I receptor kinase. The activated kinase then transiently binds Smad2 and/or Smad3 and phosphorylates these signalling molecules. Smad2 and Smad3 belong to a unique family of intracellular signalling molecules, i.e. the Smad family, which mediate the effects of members of the TGF-β superfamily, such as TGF-βs, BMPs, and activin (reviewed in [6]). After being phosphorylated on serine residues located at their C-terminal end, Smad2 and/or Smad3 form a heteromeric complex with Smad4 and enter the nucleus where they can function as transcriptional activators. In the nucleus, these complexes either bind TGF-β response elements directly or associate with DNA-bound factors and activate transcription through interaction with the co-activator CBP/p300, which bridges between Smad complexes and the basal transcription machinery.
(Fig. 1). It is noteworthy that other proteins of the Smad family, such as Smad6 and Smad7, inhibit the phosphorylation of Smad2 and Smad3 and thus the effects of TGF-β. For example, some effects of IFN-γ are mediated by induction of Smad7, which inhibits the TGF-β signalling pathway [7].

In vitro, TGF-β stimulates the proliferation of fibroblastic cells, enhances the production of extracellular matrix proteins such as type I collagen, and inhibits the degradation of extracellular matrix by decreasing the production of metalloproteases and increasing the synthesis of tissue inhibitors of metalloproteases (TIMPs) (reviewed in [2]). As might be expected from these in vitro actions, TGF-β has been shown to be a major profibrotic cytokine in vivo. In particular, with respect to renal fibrosis, overexpression of TGF-β can induce renal fibrosis, while inhibition of the effects of TGF-β can decrease the accumulation of extracellular matrix in experimental models of glomerulosclerosis. For example, mice which overexpress active TGF-β1 selectively in the kidney develop glomerulosclerosis [8]. Similarly, transgenic mice which harbour a fusion gene consisting of a cDNA encoding mature TGF-β1 under the control of regulatory elements of the albumin gene synthesise high levels of active TGF-β and develop hepatic fibrosis [9]. In addition, the transgenic lines which express the highest levels of TGF-β1 develop a fibrosis of different organs, including kidney [9, 10]. On the contrary, administration of anti-TGF-β1 antibodies or decorin (which is a natural inhibitor of TGF-β) prevented the accumulation of extracellular matrix in an experimental model of glomerulonephritis induced by administration of anti-Thy1.1 antibodies [11, 12].

TGF-β and renal fibrosis in diabetic nephropathy

Data derived from in vivo and in vitro studies suggest that TGF-β plays a key role in the accumulation of extracellular matrix observed during the course of diabetic nephropathy. In vitro, high glucose concentration increases the production of extracellular matrix components such as type I, type III, and type IV collagen and heparan sulphate proteoglycans by mesangial cells. It also increases the production of TGF-β by these cells [13-15] as well as the synthesis of TGF-β type I and type II receptors [16]. Addition of anti-TGF-β antibodies or transfection with antisense oligonucleotide for TGF-β1 decreases the synthesis of extracellular matrix proteins, confirming the autocrine action of TGF-β on extracellular matrix synthesis [14, 15]. Similarly, high ambient glucose stimulates the synthesis of type I collagen by renal cortical fibroblasts and the production of TGF-β by these cells, while anti-TGF-β antibodies decrease the overproduction of collagen [17]. In vivo, analysis of renal biop-
sies from patients with diabetic nephropathy and studies of kidneys from diabetic animals have shown increased expression of TGF-β mRNA and the corresponding protein, which occurs early during the course of the disease [18-20]. Furthermore, in streptozotocin-induced diabetic mice, treatment with anti-TGF-β antibodies decreased renal hypertrophy and the levels of mRNAs encoding extracellular matrix proteins such as type IV collagen and fibronectin [21].

Pathways linking diabetes and TGF-β production

The mechanisms responsible for the increased production of TGF-β during the course of diabetes mellitus are still poorly understood, but recent experimental data suggest that the diacylglycerol-protein kinase C pathway, the glucosamine pathway, and possibly the polyol pathway play important roles.

During glycolysis, glucose is phosphorylated and cleaved to form glyceraldehyde 3-phosphate, which can be transformed into lactate or dihydroxyacetone phosphate and then into glycerol 3-phosphate. Glycerol 3-phosphate is transformed into phosphatidic acid and diacylglycerol (Fig. 2A). Elevated diacylglycerol levels greatly increase the affinity of protein kinase C (PKC) for calcium and phosphatidyl serine, thus allowing prolonged protein kinase C activation (reviewed in [22]). High intracellular levels of glucose thus increase diacylglycerol formation and PKC activation. The links between PKC activation and increased production of TGF-β have been highlighted by the administration of a PKC-β inhibitor to streptozotocin-induced diabetic rats. Administration of LY333531 to diabetic rats, which selectively inhibits the activation of PKC-β by diacylglycerol, prevents glomerular increase in TGF-β1 mRNA [23]. This treatment also prevents any increase in fibronectin mRNA and α1(IV) collagen mRNA [23]. PKC-β seems to be involved not only in the pathophysiology of diabetic nephropathy but also in the pathogenesis of diabetic retinopathy through increased production of VEGF [24, 25]. This could explain the close association between the occurrence of diabetic nephropathy and retinopathy. Another hypothesis is that VEGF plays a direct role in the pathogenesis of diabetic nephropathy, possibly by increasing vascular permeability and thus the mesangial traffic of glucose and profibrotic molecules.

Recent in vitro studies suggest that glucosamine or its metabolites, which are synthesised from glucose through the hexosamine pathway, can increase TGF-β production [26]. In this pathway, glucose is phosphorylated by a hexokinase in glucose-6-phosphate, which is transformed into fructose-6-phosphate and then into glucosamine-6-phosphate. This last reaction is catalysed by glutamine: fructose-6-phosphate aminotransferase (GFAT) (Fig. 2C). In vitro, addition of glucosamine to the culture medium increases the production of hexosamine metabolites and TGF-β by mesangial cells [26]. Conversely, by inhibiting GFAT and thus preventing the production of hexosamine metabolites, it is possible to prevent the increase in

Fig. 2. Metabolic pathways leading to the formation of glucose derivatives which can increase the production of TGF-β by mesangial cells (see text for details). (A): diacylglycerol pathway; (B) polyol pathway; (C) glucosamine pathway. GFAT: glutamine: fructose-6-phosphate aminotransferase.
TGF-β mRNA induced in vitro by high glucose concentrations [26].

The polyol pathway may also play a role in the increased production of TGF-β, and thus of extracellular matrix, by mesangial cells. In this pathway, aldose reductase catalyses the reduction of glucose to sorbitol, which is then transformed into fructose (Fig. 2B). Mesangial cells can metabolise glucose through the polyol pathway [27, 28], and aldose reductase inhibitors prevent glucose-induced activation of PKC and TGF-β production [29, 30]. The link between the polyol pathway and the activation of PKC seems to be indirect, and overproduction of polyols could increase the production of diacylglycerol by modifying the redox state of cells. Despite the in vitro effects of aldose reductase inhibitors on TGF-β production by mesangial cells, the in vivo effects of aldose reductase inhibitors on mesangial matrix accumulation in diabetic animals is still controversial [31, 32].

Advanced glycation end-products (AGES) constitute another candidate for bridging between hyperglycaemia and increased synthesis of TGF-β. These products result from non-enzymatic glycation of proteins, which successively yield to the formation of Schiff base intermediates, Amadori products, and AGES. In patients with diabetes mellitus, AGES accumulate in glomeruli during the course of diabetic nephropathy [33]. Furthermore, administration of AGE-modified albumin to rats for 5 months induced glomerular hypertrophy, thickening of glomerular basement membrane, and accumulation of mesangial matrix [34]. Similarly, administration of AGE-modified albumin for 4 weeks in mice increased TGF-β mRNA in glomerular cells as well as mRNA levels for type IV collagen, laminin, and heparan sulphate proteoglycans [35]. In vitro, AGES added to cultures of mesangial cells increased the levels of mRNA for type IV collagen, laminin, and heparan sulphate proteoglycans and the production of type IV collagen [36]. These profibrotic effects were specific to AGES since they were abolished by addition of anti-AGE antibodies to cultures of mesangial cells and by administration of aminoguanidine to rats injected with AGES [36, 34]. Nevertheless, these effects were probably indirect (mediated by increased PDGF production) since in vitro anti-PDGF antibodies abrogated glomerular response to AGES [36]. It is noteworthy that aminoguanidine, which antagonizes the formation of AGES, prevented diabetic nephropathy partially in streptozotocin-diabetic rats [37, 38]. It delayed albuminuria, mesangial expansion, and thickening of glomerular basement membrane.

In addition to AGES, Amadori products could play a role in the pathogenesis of diabetic nephropathy. In vitro, Amadori products increased type IV collagen mRNA levels in mesangial cells [39]. In vivo, administration of a monoclonal antibody which specifically reacts with Amadori-modified glycated albumin to db/db mice slowed the development of glomerulosclerosis [40, 41]. This effect of Amadori products could also be mediated by increased production of TGF-β and increased expression of TGF-β receptors [42].

Glomerular hypertrophy and increased glomerular pressure, which are observed early in the course of diabetic nephropathy, may stretch mesangial cells and thus play a role in the pathogenesis of glomerulosclerosis. In vitro, cyclic stretching of mesangial cells increased the production of both TGF-β and extracellular matrix proteins such as collagen (type I and type IV), laminin, and fibronectin [43, 44]. The increased production of extracellular matrix proteins was largely inhibited by anti-TGF-β antibodies, which suggests that stretch increased production of TGF-β. In turn, TGF-β increased extracellular matrix production in an autocrine pathway [44]. The increased production of TGF-β appeared to be mediated by tyrosine kinase-dependent mechanisms since it was abolished by tyrosine kinase inhibitors [45]. Activation of tyrosine kinases could be secondary to integrin-induced cytoskeletal changes and/or ion flow through mechanosensitive cation channels [46]. In vitro, the effect of stretch on extracellular matrix and TGF-β production seems to be largely enhanced by high ambient glucose, which suggests that, in vivo, hyperglycaemia and glomerular hypertension or hypertrophy have synergistic effects [44].

### Vasoactive Peptides and Renal Fibrosis in Diabetic Nephropathy

Besides TGF-β, vasoactive factors such as angiotensin II and endothelin-1 may play a role in the stimulation of extracellular matrix production during the course of diabetic nephropathy.

In vitro, culture of mesangial or fibroblastic cells in the presence of angiotensin II increased the production of extracellular matrix protein, although this effect appeared to be mainly indirect (mediated by increased production of TGF-β) since it was largely reduced by addition of anti-TGF-β antibodies to the culture medium [47, 48]. In vivo, the role of angiotensin II in the pathogenesis of renal fibrosis has been mainly suggested by the beneficial effect of angiotensin-converting enzyme (ACE) inhibitors on the progression of chronic renal failure (reviewed in [49]). Different studies have shown that ACE inhibitors in patients with type I diabetes and overt diabetic nephropathy or in diabetic animals decrease the progression of diabetic nephropathy and reduce proteinuria by mechanisms which can be attributed only partly to an effective control of systemic hypertension (reviewed in [50, 51]). In a recent study, ACE inhibitors have also been shown to inhibit extracellular matrix accumulation within the renal interstitium in patients with
type 2 diabetes [52]. Conversely, overproduction of angiotensin II in diabetic rats accelerated the development of renal glomerulosclerosis [53]. Nevertheless, it is noteworthy that the effects of diabetes on the expression of the different components of the renin-angiotensin system are still unclear (reviewed in [54]). Different studies have found low plasma renin activities in patients with diabetic nephropathy, but renal renin content may be elevated in diabetic rats [55], and there may be increased renal expression of elements of the renin-angiotensin system in diabetic patients [56]. In addition, high ambient glucose seems to increase the expression of angiotensin II type 1 receptors [57]. Furthermore, angiotensin II activates PKC, which appears to be an important mediator of the profibrotic effects of hyperglycaemia (cf. supra), and can thus enhance the deleterious effects of hyperglycaemia.

Endothelin-1, a potent vasoconstrictor, is also profibrotic in vivo. For example, transgenic mice, which overexpressed endothelin-1 in different organs including kidney, had normal blood pressure but developed glomerulosclerosis and renal interstitial fibrosis [58]. Similarly, transgenic rats, which overexpressed endothelin-2 in glomeruli, developed glomerulosclerosis, whereas they had normal blood pressure [59]. On the contrary, an endothelin receptor antagonist in rats partially protected against hepatic fibrosis induced by administration of carbon tetrachloride or bile duct ligation [60]. This antagonist also protected against arteriosclerosis induced by administration of L-NAME through mechanisms independent of its effects on blood pressure [61]. In patients with diabetes mellitus, the profibrotic role of endothelin-1 is suggested by two lines of evidence. Firstly, glomerular expression of endothelin-1 was increased in streptozotocin-induced diabetic rats [62]. Secondly, an endothelin receptor A antagonist decreased glomerular mRNA levels for type I, type III, and type IV collagen, and for laminins in streptozotocin-diabetic rats—effects which were not mediated by modifications of blood pressure [63]. In the latter study, the endothelin receptor A antagonist also decreased mRNA levels for TGF-β, suggesting that some of the effects of endothelin-1 are mediated by TGF-β [63].

OTHER PATHWAYS LINKING RENAL FIBROSIS AND DIABETES MELLITUS

Reactive oxygen species

Hyperglycaemia leads to increased production of reactive oxygen species and thus of lipid peroxidation products in isolated glomeruli [64], while lipid peroxidation products can increase the production of collagen by fibroblastic cells (reviewed in [2]). Furthermore, an increased amount of lipid peroxidation products was detected in kidney biopsies from diabetic patients [65]. Thus, part of the profibrotic effects of hyperglycaemia may be mediated through increased production of lipid peroxidation products. One study showed in vivo that taurine, a potent antioxidant, improves streptozotocin-induced diabetic nephropathy [66].

Insulin growth factor 1 (IGF-1)

Both IGF-1 and IGF-1 receptor levels are increased in glomeruli of diabetic rats [67, 68], and in vitro data suggest that this growth factor may be profibrotic. IGF-1 increases extracellular matrix production by fibroblastic cells (reviewed in [2]) and decreases the activity of MMP-2, a matrix metalloprotease [69]. Furthermore, by increasing glomerular volume, IGF-1 can stretch mesangial cells and thus increase TGF-β production (cf. supra) [70]. Nevertheless, transgenic mice which overexpressed IGF-1 had enlarged glomeruli but did not develop glomerulosclerosis, which suggests that IGF-1 may enhance fibrotic reaction in vivo, but not induce it [71].

Proteinuria

During the course of glomerular diseases, heavy proteinuria can induce renal interstitial fibrosis (Fig. 3) (reviewed in [72]). In vitro data and studies of rats with experimentally-induced heavy proteinuria suggest that proteinuria can modify the functions of proximal tubular cells and induce the production of pro-inflammatory molecules as well as profibrotic cytokines. Among these molecules which can be released in the interstitium, MCP-1, osteopontin, TGF-β, endothelin-1, and free oxygen radicals could play important roles (reviewed in [73]). The course of diabetic nephropathy is characterised by early onset of proteinuria, and it is quite likely that proteinuria plays a role in the occurrence of interstitial fibrosis and the progression to renal failure. Furthermore, as with non-diabetic glomerulopathies, part of the protective effects of ACE inhibitors are probably mediated by a decrease in proteinuria levels [72]. It is noteworthy that VEGF, which is involved in the pathogenesis of diabetic retinopathy, may also play a role in the pathophysiology of proteinuria by increasing glomerular permeability [24].

In conclusion, in vitro and in vivo data suggest that TGF-β is a major profibrotic factor, and that its production can be increased through many pathways during the course of diabetic nephropathy (Fig. 4). Nevertheless, due to the pleiotropic effects of this growth factor, it is not clear whether systemic inhibition of the effects of TGF-β could be beneficial in patients with diabetic nephropathy. To protect diabetic patients against renal fibrosis, another attractive possibility would be to prevent the accumulation of glucose.
within renal cells by decreasing the activity of glucose transporters such as Glut-1, or to inhibit its metabolism through pathways such as the diacylglycerol, glucosamine or polyol pathway. It could also be useful to prevent the accumulation of AGEs by administration of molecules such as aminoguanidine or phenacylthiazolium bromide. A more futuristic, but probably more potent, possibility would be to switch off genes encoding extracellular matrix proteins in mesangial cells and/or fibroblastic cells. In this respect, it would be very important to delineate the molecular mechanisms responsible for differentiation of mesenchymal cells into fibroblastic cells, or for the activation of genes such as type I collagen genes.

REFERENCES


