Extracellular Matrix Metabolism in Diabetic Nephropathy

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Abstract. Diabetic nephropathy is characterized by excessive deposition of extracellular matrix proteins in the mesangium and basement membrane of the glomerulus and in the renal tubulointerstitium. This review summarizes the main changes in protein composition of the glomerular mesangium and basement membrane and the evidence that, in the mesangium, these are initiated by changes in glucose metabolism and the formation of advanced glycation end products. Both processes generate reactive oxygen species (ROS). The review includes discussion of how ROS may activate intracellular signaling pathways leading to the activation of redox-sensitive transcription factors. This in turn leads to changes in the expression of genes encoding extracellular matrix proteins and the protease systems responsible for their turnover.

Diabetic Nephropathy: The Magnitude of the Problem

Diabetes mellitus has recently assumed epidemic proportions, partly due to the 2 to 5% increase in the incidence of type 1 diabetes in children, but especially due to the global increase in type 2 diabetes (1). Currently as many as 4% of obese adolescents in the United States may have silent type 2 diabetes (2). Secondary microvascular complications, including nephropathy, develop some years after the onset of diabetes. Genetic background is likely to be important in determining susceptibility to diabetic nephropathy (DN) (3), but exposure of tissues to chronic hyperglycaemia is the main initiating factor (4,5). The prevalence of nephropathy varies according to geographical location, type of diabetes, and the length of time since diagnosis. Microalbuminuria is a sign of early DN, while macroalbuminuria indicates progression (6). While there is a similar prevalence of microalbuminuria in diabetic patients in the United States and Europe (22 and 25%), the prevalence of macroalbuminuria is higher in the United States (27% compared with 12%) (7). In a Japanese study, 44% of type 2 diabetic patients developed DN by 30 yr after diagnosis but only 20% of type 1 patients did so (8). Despite these regional variations, the prevalence of DN is predicted to increase in the decades ahead (9,10). DN is a major cause of end-stage renal disease (11), and new therapeutic approaches are required to limit its development. Evolution of these will depend on understanding the molecular mechanisms driving glomerulosclerosis and interstitial fibrosis in DN. This review surveys current understanding in this area.

Changes in Renal Extracellular Matrices in DN

Similar ultrastructural changes occur in glomeruli in types 1 and 2 diabetes (12,13). The glomerular basement membrane increases in thickness, and the extracellular matrix of the mesangium expands. The basement membrane changes, accompanied by glomerular hyperfiltration, and increased glomerular hydrostatic pressure lead to microalbuminuria. However, the mesangial changes appear to be the main cause of declining renal function in DN (14). As the mesangial matrix expands, it impinges on glomerular capillaries, reducing the surface available for filtration and narrowing or occluding the lumen. Declining glomerular function correlates well with the extent of these changes in both types of diabetes (15,16), although there appears to be more heterogeneity in these structural changes in type 2 (17).

Tubulointerstitial fibrosis occurs in DN, in addition to glomerulosclerosis, but it has been much less well studied. However, decreased creatinine clearance correlates with the interstitial expansion as well as with mesangial expansion (18) and survival rates diminish if interstitial fibrosis is present (19). In this respect, diabetic nephropathy is similar to other renal disorders in which progressive loss of renal function correlates with advancing interstitial fibrosis (20). Interstitial fibrosis in DN may be initiated by the same factors as glomerular fibrosis, as well as being influenced subsequently by factors originating in the glomerulus (21).

Expansion of the mesangial matrix and thickening of the glomerular basement membrane (GBM) in DN could be due to either increased accumulation of proteins that are normally present in these structures or to deposition of proteins that are not present in normal tissue or to both. Table 1 summarizes some of the many reports of matrix proteins that are present in the mesangium and GBM in DN. It is clear that some mesangial proteins such as collagen I and III are only expressed in the late stages of glomerulosclerosis. They are associated with the development of Kimmelstiel-Wilson nodules rather than with the diffuse expansion of the mesangial matrix, which occurs in the early and moderately advanced stages of the disease. Other
proteins such as fibronectin are present in the normal mesangium but increase in the expanding mesangium.

Differences between quantitative electron microscopy-immunogold measurements of the levels of matrix proteins in the mesangium and GBM and data obtained by immunohistochemical and immunofluorescence studies complicates the interpretation of which proteins are responsible for expansion or thickening. Thus an immunogold analysis showed decreased type VI collagen per unit area of the mesangium and decreased total type VI per glomerulus mesangial matrix in “fast track” DN patients with diffuse mesangial expansion compared with controls (30). In contrast, immunohistochemical and immunofluorescence investigations concluded that increased type VI deposition occurs (25,32). Such differences could be due to the immunogold study focusing on an earlier stage of the disease and on type 1 patients, whereas the light microscopy studies were largely concerned with late-stage DN in type 2 diabetic patients. Nevertheless, the quantitative study provokes the question as to precisely which matrix proteins are responsible for diffuse mesangial expansion and whether there may be subtle differences in the molecular changes in type 1 and 2 diabetes. Only further quantitative investigations on well-defined cohorts of patients will provide definitive answers.

The main components of the normal GBM are type IV collagen, laminin, entactin, and proteoglycans. GBM type IV collagen is comprised predominantly of α3, α4, and α5 chains (39,40). Table 1 summarizes some of the changes that occur during thickening of the GBM in DN. The loss of heparan sulfate proteoglycans with disease progression is associated with proteinuria (44,46), the glycosaminoglycan chains normally forming an anionic charge barrier to protein diffusion across the GBM (49).

<table>
<thead>
<tr>
<th>Protein</th>
<th>Comment</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mesangium</td>
<td></td>
<td></td>
</tr>
<tr>
<td>collagen I</td>
<td>Only detected in late glomerulosclerosis.</td>
<td>22, 23, 24</td>
</tr>
<tr>
<td></td>
<td>May bind decorin and TGF-β.</td>
<td></td>
</tr>
<tr>
<td>collagen III</td>
<td>Only detected in late glomerulosclerosis.</td>
<td>25, 26, 27</td>
</tr>
<tr>
<td>collagen IV</td>
<td>α1(IV), α2(IV) chains expressed in normal mesangium, increased in DN.</td>
<td>28, 29, 30, 31</td>
</tr>
<tr>
<td>collagen V</td>
<td>Minor component in normal mesangium.</td>
<td>25, 32, 33</td>
</tr>
<tr>
<td>collagen VI</td>
<td>Present in normal mesangium. Same distribution as α1(IV) in normal mesangium. Reports of increase in DN not substantiated in type I “fast track” DN, using quantitative immunogold EM.</td>
<td>30, 32–35</td>
</tr>
<tr>
<td>fibronectin</td>
<td>Present in normal mesangium.</td>
<td>32, 36, 37, 38</td>
</tr>
<tr>
<td></td>
<td>Increased in DN.</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Oncofetal, ED-A, and ED-B isoforms expressed in glomerulosclerosis.</td>
<td></td>
</tr>
<tr>
<td>laminin</td>
<td>Minor component in normal mesangium.</td>
<td>25, 32</td>
</tr>
<tr>
<td></td>
<td>Report of increase in diffuse mesangial expansion not confirmed.</td>
<td></td>
</tr>
<tr>
<td>SLR proteoglycans</td>
<td>Includes decorin, biglycan, lumican, fibromodulin; mRNAs for all overexpressed in DN, but proteins barely detected, except in advanced glomerulosclerosis.</td>
<td>23, 24</td>
</tr>
<tr>
<td>GBM</td>
<td></td>
<td></td>
</tr>
<tr>
<td>collagen IV</td>
<td>α3(IV), α4(IV) chains present normally, increased in DN.</td>
<td>28, 31, 39–41</td>
</tr>
<tr>
<td></td>
<td>α1(IV), α2(IV), minor components normally decreased in DN.</td>
<td></td>
</tr>
<tr>
<td>entactin</td>
<td>Present normally.</td>
<td>42</td>
</tr>
<tr>
<td></td>
<td>Increased in DN.</td>
<td></td>
</tr>
<tr>
<td>laminin</td>
<td>Present normally, may be increased in early DN, but generally reported to decrease.</td>
<td>37, 43–45</td>
</tr>
<tr>
<td>heparan sulfate proteoglycan</td>
<td>Present normally, Decreased in DN.</td>
<td>44–48</td>
</tr>
</tbody>
</table>

Table 1. Glomerular matrix proteins in diabetic nephropathy (DN)
mians (24,26,54) and in animal models (51,53). However, glomerular matrix proteins also normally undergo metabolic turnover, being degraded primarily by matrix metalloproteinases (MMP) (55). Any decrease in turnover would promote excessive matrix accumulation, and there is evidence that this too occurs in DN. Glomerular mRNA levels for MMP2 (gelatinase A) and MMP3 (stromelysin) decrease in human DN (54,56), while in STZ-induced diabetes in rats, MMP9 (gelatinase B) mRNA and activity fell and tissue inhibitor of metalloproteinase-1 (TIMP1) mRNA increased (57,58). In this model, MMP3 mRNA levels increased (58). Nevertheless, MMP3 activity and overall glomerular MMP activity decreased (58,59). Activity of MMP is controlled at multiple steps, including their level of synthesis as inactive proenzymes, activation of the proenzymes and, subsequently, inhibition of active enzymes by binding to TIMP (60). Plasmin cleaves and activates MMP proenzymes, while membrane type metalloproteinase-1 (MT1-MMP) is involved specifically in activating MMP2. In vitro evidence indicates that high-glucose conditions reduce plasmin activity by upregulating expression of plasminogen activator inhibitor (PAI-1) in mesangial cells (MC) (61,62) while suppressing expression of MT1-MMP (63). Thus, overall, turnover of mesangial matrix proteins in DN is likely to be compromised by decreased MMP expression, decreased levels of proenzyme activation, and increased expression of the MMP inhibitor, TIMP1.

**Mesangial Cell Responses to Hyperglycemic Conditions In Vitro**

Many in vitro investigations have exposed MC to high concentrations of glucose, or to albumin-Amadori adducts, or to albumin-advanced glycation end products (AGE) to mimic the conditions they experience in vivo. The formation of Amadori-protein adducts and AGE-proteins is initiated in vivo by the non-enzymatic interaction of protein amino-groups with glucose (64) or with metabolites of glucose such as methylglyoxal (65). Increased levels of glycated proteins occur in the plasma, renal, and other tissues in diabetes (66–68). MC respond to culture medium containing high glucose concentration or glycated-albumin by upregulating the expression of many of the matrix proteins that accumulate in the mesangium in DN, and by changes in the expression of MMP and proteinase inhibitors. Some examples are shown in Table 2. It is apparent that both induce similar effects, even though those of glucose depend on its metabolism, following uptake into the cell by facilitative glucose transporters (75), while glycated-proteins interact with cell surface receptors (76,77).

As well as being exposed to circulating glycated-proteins, mesangial and other cells are likely to be affected by the function of intracellular proteins being compromised by AGE formation and by cellular interactions with insoluble glycated matrix proteins. The latter lead to some of the same effects in vitro as are induced by high glucose; for example, increased expression of MMP2 and TIMP1 and decreased expression of MT1-MMP (78). Glycation of type IV collagen and laminin affects their structure and assembly into polymers (79–81). Formation of advanced glycation end products of collagen IV or glomerular basement membrane also markedly inhibits their susceptibility to cleavage by MMP3 and MMP9 (82), impeding their turnover.

Other glomerular cells also respond to high-glucose conditions or to glycated-albumin in vitro. For example, glomerular visceral epithelial cells exposed to high glucose have been reported to upregulate expression of transforming growth factor-β (TGF-β) and fibronectin (83) and TGF-β type II receptor (84), while glycated-albumin stimulates the production of collagen IV and fibronectin in glomerular endothelial cells (85).

The response of MC to hyperglycemia is clearly complex, and differential gene screening techniques have proved useful for understanding more fully the changes in gene expression that occur after exposure to high glucose. mRNA differential display indicated that a large number of genes undergo changes in expression after exposure of human MC to 30 mM D-glucose (86), and suppression subtraction hybridization (SSH) subsequently identified 70 known genes that were induced under these conditions, a further 26 showing similarity to 70 known genes. For example, increased expression of MMP2 and TIMP1 and decreased expression of MT1-MMP (78). Glycation of type IV collagen and laminin affects their structure and assembly into polymers (79–81). Formation of advanced glycation end products of collagen IV or glomerular basement membrane also markedly inhibits their susceptibility to cleavage by MMP3 and MMP9 (82), impeding their turnover.

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Differential screening of MC exposed to high glucose revealed increased expression of genes that, potentially, play a

| Table 2. Mesangial cell protein expression in vitro in response to hyperglycemic stimuli* |
|-----------------------------------------------|-----------------------------|--------------------------|--------------------------|
| **Stimulus**                                 | **Proteins**                | **Expression**           | **References**           |
| High glucose (11 to 30 mM)                   | Collagens I, III, IV, Fibronectin, laminin, decorin, versican, perlecan | Increased                | 69–71                    |
|                                              | PAI-1                       | Increased                | 61                       |
|                                              | TIMP1                       | t-PA activity decreased  | 61                       |
|                                              | MMP2                        | Increased                | 61                       |
|                                              | MMP7, MMP9, MT1-MMP         | Increased, but activation reduced | 61, 63                  |
| Glycated albumin                            | Collagen IV, fibronectin    | Increased                | 72–74                    |
|                                              | MMP9                        | Decreased                | 74                       |

*PAI-1, plasminogen activator inhibitor; TIMP1, tissue inhibitor of metalloproteinase-1; MMP, matrix metalloproteinase.
role in the development of glomerulosclerosis, including connective tissue growth factor (CTGF) (87,89). Its role in promoting the synthesis of mesangial matrix proteins is discussed below. However, expression screening also indicated that mRNA for both subunits of the fibronectin receptor (α5β1 integrin) is increased in high-glucose conditions (N.A. Wahab and R.M. Mason, unpublished work). Further experiments demonstrated that TGF-β, acting at least in part through a CTGF-dependent pathway, increases the number of α5β1 receptors on the cell surface and their occupation by ligand (90). This promotes increased deposition of insoluble fibronectin matrix around the cells, a process that can be inhibited by anti-β1-neutralizing antibody (90). This mechanism may represent one mechanism for the development of fibrosis in the mesangium in DN. It is also conceivable that an increased number of mesangial cell surface α5β1 integrin sites ligated by fibronectin may modulate cell behavior by an outside-in signaling mechanism. MC cultured in three-dimensional collagen gels show fibronectin-dependent activation of phosphatidylinositol-4-phosphate 5-kinase by outside-in signaling (91). Fibronectin signaling stimulates protein kinase C (PKC) translocation to the cell membrane in CHO cells (92) and induces hypertrophy of cardiac myocytes, accompanied by increased transcription of the brain natriuretic peptide gene (93).

Changes in Intermediary Metabolism in Response to Hyperglycemia

A high plasma glucose concentration leads to elevated intracellular glucose levels in cells such as MC in which uptake is not regulated by insulin. The increased uptake in MC is probably achieved by increased expression of GLUT1 in response to high glucose. GLUT1 is a high affinity, low capacity facilitative glucose transporter which is near saturated at normal physiologic concentrations of glucose (75). Glucose is normally metabolized through glycolysis and the tricarboxylic acid cycle, generating CO2 and the reduced coenzymes NADH and FADH2. The latter reoxidize by donating electrons to the respiratory chain (electron transport chain) in the inner mitochondrial membrane, where they reduce molecular oxygen to form water. Passage of electrons through the chain results in a proton gradient across the membrane which drives oxidative phosphorylation, producing ATP. High intracellular glucose levels generate increased production of several glucose metabolites which are normally present in only low concentration and the glucose and three of these products stimulate activity of four other pathways, and actions downstream of them. Thus (1) diacylglycerol (DAG), derived from the glycolytic intermediate dihydroxyacetone phosphate (DHAP), activates several isoforms of PKC; (2) dicarboxyl metabolites such as glyoxal and methylglyoxal, derived from glucose and the glycolytic intermediates DHAP and glyceraldehyde-3-phosphate (G3P), initiate AGE formation; (3) fructose-6-phosphate, a glycolytic intermediate, when present in raised concentration, enters and increases flux through the hexosamine biosynthetic pathway, generating UDP-N-acetylglucosamine. This promotes glycosylation of Sp1 and possibly of other transcription factors, modulating their activity; (4) glucose itself, which when present in raised concentration enters the aldose reductase pathway and is converted to sorbitol. This utilizes the reduced coenzyme NADPH. The resulting NADP is converted back to NADPH using the cell’s antioxidant, reduced glutathione, depleting the latter and increasing cell susceptibility to reactive oxygen species (ROS). The details of activation of these four pathways in high glucose have been reviewed extensively recently (94,95). Increased production of NADH and FADH2 and donation of electrons from them to the respiratory chain increases the proton gradient across the inner mitochondrial membrane, which inhibits electron transport at complex III of the chain. Electrons carried by coenzyme Q, which normally pass to complex III, then generate intracellular ROS by reducing O2 to superoxide ion O2⁻ (94). This also inhibits activity of the glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase (96), potentially raising the level of metabolic intermediates between glucose and G3P and their entry into the four pathways. The formation of AGE also generates extracellular ROS due to the autooxidation of glucose (97). This may lead to the activation of latent TGFβ (L-TGFβ) (98). Extracellular AGE also bind to the cell surface receptor, RAGE, which is expressed in various renal cell types (76,77). This initiates intracellular ROS production (99).

Attenuation of DN in Animal Models of Diabetes

Support for the involvement of ROS and AGE in inducing DN in vivo comes from studies on the effect of various inhibitors on animal models. Thus, overexpression of Cu²⁺/Zn²⁺ superoxide dismutase in mice protects them from glomerular injury after induction of diabetes with STZ (100). Inhibitors of non-enzymatic glycation of proteins attenuate glomerular and interstitial damage in db/db mice (101–106) and in diabetic transgenic mice overexpressing RAGE (a receptor for AGE), which otherwise develop advanced glomerulosclerosis (107). Likewise, inhibition of PKCβ attenuates mesangial expansion in db/db mice (52) and glomerular dysfunction in the STZ rat (108). Sorbinil, an inhibitor of the aldose reductase pathway, reduced GBM width in STZ rats on low-protein diets but not in those on higher-protein diets (109). However, definitive evidence of the efficacy of antioxidants, or inhibitors of AGE formation, PKC activation, or of the aldose reductase pathways, in attenuating the development of DN in humans is still awaited (95).

To date, there are no in vivo studies on the effect of blocking the hexosamine biosynthetic pathway on glomerular function in diabetic models. However, inhibition of glutamine:fructose-6-phosphate-amidotransferase (GFAT), the rate-limiting enzyme of the pathway, inhibits high-glucose−induced TGF-β production (110) and NF-κB-dependent promoter activation (111), two key events in the response to high glucose (see below). Overexpression of GFAT activates the PAI-1 promoter (112), another key event.

Growth Factors and Hormones Affecting Matrix Protein Expression in DN

In vitro and in vivo studies have shown that several different growth factors are upregulated in DN, and the signaling path-
ways these activate ultimately regulate transcription factors affecting glomerular ECM accumulation. The principle factors and hormones involved are angiotensin II (AngII), TGF-β, CTGF, platelet derived growth factor (PDGF), growth hormone (GH), and insulin-like growth factors (IGF).

**Angiotensin II.** Activation of the renal renin-angiotensin system (RAS) and its involvement in the pathogenesis of DN is indicated by several studies that showed that both angiotensin-converting enzyme (ACE) inhibitors and angiotensin-receptor-I antagonists attenuate DN (11,113–114). The entire RAS is present in the kidney (115). In vitro studies show increased angiotensin expression in mesangial and tubular cells in response to glucose (116,117). This response was shown to be mediated, at least in part, by ROS and subsequent activation of p38 MAPK in tubular cells (118). AngII, the active octapeptide derived from angiotensinogen, also stimulates ROS generation in vascular smooth muscle cells (119) and MC (120). The ROS signaling pathway activates NF-κB, which mediates further angiotensinogen expression, as ROS generation could be part of a positive feedback loop (121), perpetuating activity of the RAS in DN. Increased AngII is also generated in hypertension, a disorder that frequently accompanies diabetes and accelerates progression of DN (122). Several intrarenal hemodynamic changes, including glomerular capillary hypertension, occur in early experimental diabetes. This and the subsequent development of albuminuria and glomerular structural changes is attenuated by ACE inhibition (123), demonstrating the importance of hemodynamic factors in the pathogenesis of DN. It is noteworthy that intraglomerular hypertension can occur in diabetes even in the absence of systemic hypertension (124). Although AngII is associated with both systemic and renal hemodynamic effects, it also has direct nonhemodynamic effects on glomerular cells, particularly MC. AngII has been shown to increase ECM accumulation by MC, primarily via stimulation of TGF-β expression (53,115,125–127). The mechanism by which AngII transactivates TGF-β was found to be similar to that for hyperglycemia. Both transactivate the growth factor gene through two AP-1 regulatory elements (128–130). This activation appears to be PKC- and p38 MAPK-dependent (129,130).

**Transforming Growth Factor-β.** Numerous studies indicate that hyperglycemia induces an increase in TGF-β expression on both the mRNA and protein levels in experimental and human diabetes (51,127,130–132) as well as in cultured MC (71,133,134). TGF-β appears to be involved in both the early and later stages of DN. In STZ-diabetic rats, renal TGF-β expression increased markedly as early as 24 h after the onset of hyperglycemia (135). A similar increase was also reported in the non-obese diabetic (NOD) mouse (136,137) and in the diabetic BB rat (136). Sustained elevated expression of TGF-β occurs in STZ-diabetic rats with diabetes of 24-wk duration (138) and in the kidney of 16-wk-old diabetic db/db mice (139). All TGF-β isoforms, TGF-β1, β2, and β3, and the TGF-β type II receptor were reported to be elevated in experimental diabetes (139–141). Increased renal production of TGF-β in patients with diabetes and DN is also well documented (127,142,143). In addition, many in vitro studies have shown that TGF-β expression is increased in various renal cells cultured under high-glucose conditions or with AGE (71,133,143–145).

It is now clear that TGF-β is the key cytokine mediating the production of different ECM proteins in MC, epithelial cells, renal interstitial cells, and fibroblasts (71,133,144,146–151). TGF-β also influences matrix-degrading enzymes by inhibiting the synthesis of collagenses and stimulating the production of TIMP and PAI-1 (149,150,152–154). TGF-β can also influence local matrix deposition by upregulating different ECM receptors (90,150).

Although the main signaling pathway of TGF-β is the Smad pathway (155) and is activated in the STZ-diabetic mouse model (156), TGF-β also activates other pathways such as the MAPK (ERK, SAPK/JNK, and p38 MAPK) (157,158). TGF-β induces PKA activation in MC by a mechanism involving the degradation of the inhibitory peptide of PKA (PKI) (159).

**Connective Tissue Growth Factor.** CTGF is another profibrotic cytokine and has also been shown to be involved in both the early and later stages of DN. Its expression is increased in experimental diabetic glomerulosclerosis (160,161). Elevated CTGF levels in glomeruli of NOD mice appear to correlate with the duration of diabetes (89). Elevated CTGF expression was also detected in human DN (89,162,163). In vitro studies have shown that CTGF is induced in renal cells by both high glucose and AGE (87,89,160), as well as ROS (164), which they generate. The induction of CTGF in MC by hyperglycemia seems to be partly TGF-β–dependent and partly PKC-dependent (165,166).

CTGF is one of the TGF-β-inducible immediate early genes (167) and is induced in cultured MC by TGF-β (168–170). TGF-β induces CTGF gene expression via Smad binding elements (SBE) and a unique TGF-β response element in the CTGF promoter (170–172). The induction of CTGF by TGF-β seems to be PKC- and MAPK-dependent (170).

Emerging evidence from in vitro studies of renal cells, including MC, indicates that CTGF is a crucial mediator for TGF-β-stimulated matrix protein expression. CTGF has been shown to mediate TGF-β–induced increases in fibronectin (89,168,173), collagen type I (174–176), and the fibronectin receptor (α5β1 integrin) (90).

Other ECM proteins that have been reported to be induced by CTGF in different renal cells include collagens I, III, and IV, tenascin, and thrombospondin-1 (TSP-1) (87,177,178). Induction of FN by CTGF in MC appears to be mediated by the activation of MAPK and PKB pathways (179). Recent work in our laboratory has shown that MC exposed to CTGF down-regulate expression of Smad7 (Wahab and Mason, unpublished work). Smad7 is an inhibitor of the Smad signaling pathway (155); we therefore propose that CTGF promotes increased TGF-β signaling through this pathway due to decreased availability of Smad7. We hypothesize that this accounts, at least in part, for CTGF’s profibrotic activity.

**Platelet-Derived Growth Factor.** Glomerular mRNA levels for PDGF-B chain are enhanced fivefold in experimental diabetes (106). PDGF has been reported to mediate both high glucose– and AGE-dependent induction of type III collagen in
cultured rat MC. However, its effect seems to be through augmenting the production of TGF-β (180,181). Anti-PDGF antibodies also attenuated the increased production of type IV collagen in MC exposed to AGE, again suggesting that PDGF acts as an intermediate factor (182).

**Growth Hormone and Insulin-Like Growth Factors.** Classically, GH secreted from the pituitary induces the synthesis of IGF in various tissues through activation of the GH receptor (GHR). Two lines of evidence implicate GH in the pathogenesis of glomerular fibrosis in experimental diabetes. One is the renoprotective effect of long-acting somatostatin analogs and GHR antagonists (183). The other is the failure of STZ-treated mice, which are either transgenic for a mutated analogs and GHR antagonists (183). The other is the failure of STZ-treated mice, which are either transgenic for a mutated

GH, which is an antagonist of native GH, or in which the GHR/binding protein gene has been knocked out, to develop glomerulosclerosis, independent of IGF. Plasma GH levels are raised in type I diabetic patients with poor glycemic control, while the concentration of IGF-I is low and that of IGF binding protein 1 (IGFBP1), a modulator of its activity, is raised (e.g., 188). Intraportal insulin deficiency decreases hepatic IGF-I synthesis and increases production of IGFBP in type I diabetes, while hypersecretion of GH occurs as a result of lowered negative feedback of IGF-1 on secretion (189).

How GH induces glomerulosclerosis directly is not known, but mRNA for GHR has been detected in MC and they respond to the hormone in vitro by upregulating iNOS transcripts and nitric oxide production (190). However, iNOS knockout mice show more advanced mesangial expansion, increased GBM staining for collagen IV, and tubulointerstitial fibrosis, after induction of diabetes with STZ than do controls, suggesting a protective effect for NO in vivo with respect to matrix deposition (191). On binding its cellular receptor, GH activates the Jak/Stat, MAPK, and PI3K signaling pathways (192), so it may potentially regulate the transcription of a number of genes.

IGF-I may be expressed in the kidney independently of GH. MC cultured on glycated-albumin increase IGF-I production (193), and the growth factor directly stimulates synthesis of laminin, fibronectin, proteoglycan, and type IV collagen in these cells (194–196). IGF-I and IGF-BP species have also been reported to increase in the kidney in the early stages of experimental DN (197–200), while the expression level of IGF-IR rises in a later phase (201). IGF-I and IGF-II signal through two specific receptors, IGF-IR and IGFII/mannose-6-phosphate receptor (IGF-II/mannose-6-PR) (202,203). IGF-I activates the phosphatidylinositol-3-kinase (PI3K) and ERK1/2 MAPK pathways (196,204). It has also been shown to stimulate NF-κB (205). In the presence of high glucose, IGF increased IGF-I-stimulated insulin receptor substrate-1/2 phosphorylation and AP-1 transcriptional activity, while decreasing IGFBP-2 expression (204). Thus, despite lower circulating levels of IGF-I in diabetes, locally produced growth factor may influence MC in vivo. MC from diabetic NOD mice secrete increased amounts of IGF-I, and this probably contributes to the increased ECM accumulation, largely through an IGF-I-mediated reduction in MMP2 activity (206).

**ROS and the Activation of Pathways Stimulating Matrix Accumulation**

Although further in vitro and in vivo confirmatory studies are required, current data strongly suggest that intracellular ROS, from either AGE-RAGE interaction or glucose metabolism (94,207), have a direct role in overproduction of ECM proteins and that this can be counteracted by antioxidants (208). It has been shown that ROS activate the PKC, MAPK, and JAK-STAT pathways (209–212), which lead to the activation of redox-sensitive transcription factors including NF-κB, AP-1 (Fos and Jun proteins), STAT, and Egr-1 (99,213–215). These enhance the transactivation of genes coding for cytokines such as TGF-β and CTGF that upregulate ECM protein expression (164,216–217).

**Other Factors Modulating the Activity of TGF-β**

Thombospondin-1. TSP-1 is one of five isoforms of thrombospondin and is synthesized and secreted by a variety of renal cells, including MC (86,218). Exposure to high glucose upregulates TSP-1 expression in MC (86), and increased levels of glycoprotein occur in the glomeruli in diabetic animals (219) and in DN in man (220). In addition to interacting with many other matrix proteins (221,222), TSP-1 also modulates their level of synthesis (223). This appears to be primarily through its ability to activate latent TGF-β (224). In vitro studies indicate that TSP-1–dependent TGF-β1 activation is important in the mesangial cell response to high glucose (225,226). Interestingly, TSP-1 null mice have a similar phenotype to TGF-β null mice (227), suggesting an important role for the TSP-1 activation mechanism in vivo.

Decorin. The expression of decorin (DCN), a small proteoglycan containing a single dermatan/chondroitin sulfate chain, is markedly upregulated in MC exposed to high glucose (71,228) and in the glomerulus in DN, although protein accumulation only occurs in the late stage of the disease (24). Decorin binds to collagen type I (229) and to other extracellular proteins, including activated TGF-β, which it sequesters in the matrix (230), possibly as a ternary complex with fibrillar collagen in advancing DN (24). Decorin-TGF-β complexes are also excreted in the urine in late DN (24). Administration of DCN inhibits TGF-β–mediated ECM expression in experimental nephritis (230). We recently showed that DCN inhibits TGF-β–mediated upregulation of PAI-1 in MC through a mechanism that involves Ca2+-dependent phosphorylation of Smad2 at a key regulatory site, thus modulating the TGF-β Smad signaling pathway (231). Despite all these studies the role of decorin in DN remains unresolved.

**Hyperglycemia and the Regulation of Gene Expression**

Any hypothesis explaining the overall coordinated program of changes in gene expression in hyperglycemia that lead to glomerulosclerosis must take into account the activation mechanism of the genes involved. An E-box has been implicated as
a carbohydrate response element in TGF-β and several other glucose-regulated promoters (232,233). However, the concept of a single glucose response element being implicated in the overall changes in gene expression becomes less attractive when considering the very large number of genes upregulated by hyperglycemia. Table 3 summarizes promoter region analyses for a number of these human genes and includes known hyperglycemia-associated stimuli, the response elements, transcription factors, and signaling pathways activating them.

We propose that ROS are the overall activators of these signaling pathways (Figure 1). Thus matrix-associated latent transcription factors, and signaling pathways activating them. hyperglycemia-associated stimuli, the response elements, and transcription factors, and signaling pathways activating them. The rapid activation of cellular “redox-sensitive” transcription factors such as NF-κB, AP-1 (fos and jun proteins), Erg-1, and Stat1. These, together with activated Smads (phosphorylated Smad2 and 3 complexed with Smad4), coordinate the transcription of a number of genes, including angiotensinogen, TSP-1, and CTGF. AngII derived from angiotensinogen stimulates further generation of ROS and expression of TGF-β. Secreted CTGF works in concert with TGF-β activated by TSP-1 and ROS to transactivate subsequent waves of genes, including those encoding structural proteins whose accumulation leads to glomerulosclerosis in DN (Figure 1).

It is becoming clear that the coordinated expression of TGF-β and CTGF is crucial for the induction of ECM proteins and thus for the development of DN. The expression of some ECM proteins, such as fibronectin, is CTGF-dependent, and its promoter region does not contain any Smad binding elements (SBE) (89). It is also noteworthy that previous experiments have shown that TGF-β induces fibronectin expression via a MAPK-dependent pathway and not via a Smad-dependent pathway (250), although a recent report indicates that a Smad-dependent pathway may operate in mice (251). In contrast, the transcription of a number of other matrix proteins, for example collagen I, PAI-1, and TIMP-1, is TGF-β-dependent, and their promoter regions contain SBE (Table 3). However, it appears that increased signaling by TGF-β is also markedly influenced by CTGF, as we have found that the latter rapidly inhibits the expression level of Smad7. This inhibitory Smad mediates an

<table>
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<td>Collagen I</td>
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<td>Collagen V</td>
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<td>AP-1*, CRE*, SBE*, GCE*, SBE*</td>
<td>NIE</td>
<td>NIE</td>
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<tr>
<td>Collagen VI</td>
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<td>AP-1*, CRE*, SBE*, GCE*, SBE*</td>
<td>NIE</td>
<td>NIE</td>
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<td>Decorin</td>
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<td>CRE-like, SBE*</td>
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* Promoter-reporter studies using different promoter regions (see references) and our in silico analysis (*) indicate the presence of common regulatory elements known to be redox-sensitive, as well as of the Smad-binding element (SBE). NIE, not identified experimentally; CTGF, connective tissue growth factor; Ang, angiotensin; TSP, thrombospondin; FN, fibronectin; HG, high glucose; AGE, advanced glycation end products; ROS, reactive oxygen species.
in intracellular negative feedback that limits TGF-β signaling (Figure 1), apparently by blocking the association of R-Smads (Smad2 and 3) with the TGF-β receptor complex and, thereby, their phosphorylation (252). Overexpression of Smad7 has been shown to block TGF-β–dependent induction of collagen I in MC (253). Thus CTGF may mediate the induction of ECM protein expression both directly and indirectly by potentiating the TGF-β/Smad signaling pathway. A signaling receptor for CTGF has not yet been characterized, but rapid phosphorylation of MAPK after exposure to the growth factor indicates that one must be present in responsive cells (179,254).

The promoters of MMP genes also show remarkable conservation of regulatory elements (255), including AP-1, ETS, and the TGF-β inhibitory element, TIE. The downregulation of MMP3 (256), collagenase, MMP9, and MMP7 (257) is thought to be mediated by TIE. There is also evidence for modulation of MMP7 mRNA stability through ATTTA motifs in the 3′-untranslated region (258). With respect to this we have shown that high-glucose conditions switch off synthesis of a protein, HGRG-14, in MC (259). This is achieved by production in high glucose of an HGRG-14 mRNA with a long 3′ UTR containing several destabilizing ATTTA motifs, which has a short half-life and is not translated. In low glucose, an mRNA with a short 3′ UTR and no ATTTA motifs is produced that has a longer half life and is translated (259).

**Concluding Comments**

The response of glomerular cells, especially MC, to hyperglycemia, is driven primarily by the generation of ROS and then TGF-β and CTGF. This upregulates the transcription of many matrix genes and represses that of MMP, events which in vivo lead to glomerulosclerosis (Figure 1). It is likely that the response of other renal cells to hyperglycemia has many features in common with this pathway, but this has been less well studied to date. There are clearly many points at which therapeutic approaches could be tried to provide renoprotection in diabetes. These include ACE inhibitors and AT-1 receptor antagonists, which are already in use clinically, and novel agents to oppose the actions of ROS, TSP-1, TGF-β, and CTGF. It is likely that targeting multiple points in altered metabolism in the diabetic kidney will be more successful in attenuating the development of DN, due to its complexity, rather than a single approach.

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*Figure 1. Model to explain the mechanisms by which hyperglycemia promotes ECM protein accumulation by mesangial cells. Hyperglycemic conditions generate reactive oxygen species (ROS), which activate a cascade of events. Downstream of these, TGF-β and connective tissue growth factor (CTGF) work in a coordinated manner to promote increased expression of ECM proteins. See text for details.*
Acknowledgments
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