Regulation and consequences of differential gene expression in diabetic kidney disease

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Abstract
DN (diabetic nephropathy) is the leading cause of end-stage renal disease worldwide and develops in 25–40% of patients with Type 1 or Type 2 diabetes mellitus. Elevated blood glucose over long periods together with glomerular hypertension leads to progressive glomerulosclerosis and tubulointerstitial fibrosis in susceptible individuals. Central to the pathology of DN are cytokines and growth factors such as TGF-β (transforming growth factor β) superfamily members, including BMPs (bone morphogenetic protein) and TGF-β1, which play key roles in fibrogenic responses of the kidney, including podocyte loss, mesangial cell hypertrophy, matrix accumulation and tubulointerstitial fibrosis. Many of these responses can be mimicked in in vitro models of cells cultured in high glucose. We have applied differential gene expression technologies to identify novel genes expressed in in vitro and in vivo models of DN and, importantly, in human renal tissue. By mining these datasets and probing the regulation of expression and actions of specific molecules, we have identified novel roles for molecules such as Gremlin, IHG-1 (induced in high glucose-1) and CTGF (connective tissue growth factor) in DN and potential regulators of their bioactions.

Introduction
DN (diabetic nephropathy) is the leading cause of end-stage renal disease and develops in up to 40% of patients with either Type 1 or Type 2 diabetes mellitus [1,2]. The pathological hallmark of DN is glomerulosclerosis, resulting from accumulation of ECM (extracellular matrix) in the glomerular mesangium, triggered by a complex interplay of metabolic and haemodynamic stress (Figure 1). Putative mediators of high-glucose-induced mesangial cell dysfunction include oxidative stress, glomerular hypertension, PKC (protein kinase C) activation, TGF (transforming growth factor) β1, cell sorbitol accumulation and advanced glycation end-products [3]. In DN, the progressive accumulation of ECM in the interstitial space leads to the development of tubulointerstitial fibrosis; the extent of tubular fibrosis is directly related to loss of renal function [2]. Tubulointerstitial fibrosis is proposed to develop through proliferation and activation of resident fibroblasts, recruitment of circulating fibrocytes and by transformation of epithelial cells to a mesenchymal phenotype (EMT (epithelial–mesenchymal transformation)) [4]. TGF-β1 is a pivotal mediator of the pathological changes of kidney disease, resulting in the development of both glomerulosclerosis and tubulointerstitial fibrosis.

Key words: actin-regulatory protein, connective tissue growth factor (CTGF), diabetic kidney disease, diabetic nephropathy, fibrosis, Gremlin.

Abbreviations used: BMP, bone morphogenetic protein; CN, CTGF/hip-12; cycl1/cycl6 and 
CTGF, connective tissue growth factor; DN, diabetic nephropathy; ECM, extracellular matrix, Her1, hairy enhancer of split-1; IHG, induced in high glucose; LX, lipoxin; MAPK, mitogen-activated protein kinase; PFK, phosphofructokinase; PKB, protein kinase B; PKC, protein kinase C; ROS, reactive oxygen species; R-Smad, receptor-regulated Smad; SSH, suppression-subtractive hybridization; TGF, transforming growth factor; UGO, unilateral ureteric obstruction.

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TGF-β1 receptor activation results in phosphorylation of the receptor-regulated Smads (R-Smad) 2 and 3. Phosphorylated R-Smad proteins bind to Smad4 and accumulate in the nucleus, where they activate transcription [5].

Hyperglycaemia is a major stimulus for the development of DN. The mechanisms by which hyperglycaemia perturbs renal cellular function are not fully elucidated, but are thought to be due to both direct effects of high extracellular glucose levels and indirect effects transduced through alterations in glomerular haemodynamics and through the actions of advanced glycation end-products.

In vitro models of renal cells cultured in high glucose have proved to be effective in mimicking cellular responses to hyperglycaemia in vivo. In order to assess the direct effects of high extracellular glucose on mesangial cell function, we used a PCR-based technique, SSH (suppression-subtractive hybridization) [6,7]. SSH identified some 200 genes to be differentially expressed in primary human mesangial cells exposed to high glucose (30 mM glucose compared with 5 mM control) for 7 days, many of which were potential modulators of mesangial cell contractility, turnover and matrix metabolism. Among these genes induced by high extracellular glucose in vitro were genes reported previously to have increased expression in DN (e.g. fibronectin) as well as genes whose induction by high glucose had not been described previously [6,7]. Prominent among the latter were genes encoding actin-regulatory proteins and CTGF (connective tissue growth factor), a modulator of fibroblast matrix production. There were also a number of novel functionally uncharacterized gene transcripts identified as being induced by glucose, including IHG (induced in high glucose)-1 and IHG-2. In this short review, we summarize our findings on...
Figure 1 | The pathophysiology of DN

Numerous in vivo and in vitro studies have resulted in converging hypotheses that combined insults of hyperglycaemia, inflammation, elevated blood pressure and increased protein overload drive DN. These insults converge at the cellular level by using similar molecular signalling pathways and influencing the expression of cytokine mediators. Central to these processes is the key mediator, TGF-β1. Because of its multifaceted role, inhibiting TGF-β1 has an impact on numerous other processes, many in an adverse way, making it an unsuitable target for therapeutic intervention. Efforts in our group have focused on alternative therapeutic strategies based on modulators of TGF-β1 and downstream mediators of TGF-β1, particularly the BMP antagonist Gremlin, the prototypic CCN family member CTGF and the novel TGF-β1 modulator IHG-1.

actin-regulatory proteins, CTGF, IHG-1, IHG-2 and some novel potential regulators of their bioactions.

Actin-regulatory proteins

Ten of the 70 genes encoding proteins of known function identified in the SSH screen were actin-regulatory proteins. This group of glucose-induced genes function in the regulation of actin filament growth, dissolution and organization, and were of particular interest, as the actin cytoskeleton is a major target for high-glucose-triggered injury in DN and a contributor to the development of diabetic glomerulosclerosis [8].

High glucose induction of actin cytoskeleton regulatory gene expression was reduced by CCCP (carbonyl cyanide m-chlorophenylhydrazone), a potent uncoupler of oxidative phosphorylation that abolishes the electron gradient across the mitochondrial membrane, thus inhibiting ROS (reactive oxygen species) generation [7]. Increased generation of ROS by the mitochondrial electron-transport chain has been proposed to be the causal link between the direct effects of hyperglycaemia and the major biochemical pathways leading to cellular injury in diabetes mellitus (Figure 1). Consistent with this paradigm, antioxidants have been shown to inhibit high-glucose-induced ECM protein synthesis in mesangial cells and prevent both glomerular hypertrophy and ECM accumulation in diabetic animals.

Actin-regulatory proteins in mesangial cells exposed to high glucose were not induced by TGF-β1 or PKC. However, rapid disassembly of the actin cytoskeleton was paralleled by enhanced expression of actin-regulatory genes, suggesting that the induction of the actin-regulatory proteins in high-glucose-treated mesangial cells may represent a homoeostatic drive to correct the morphological disabling effect of loss of cytoskeletal function [7].

These results indicate that induction of genes encoding actin-regulatory proteins is a prominent component of the mesangial cell response to hyperglycaemia in DN. Induction of these genes by glucose is dependent on oxidative stress and independent of PKC and TGF-β1 activity, and represents an adaptive response to actin cytoskeleton disassembly.

Interestingly, further investigations revealed a polymorphism in the promoter of caldesmon (an actin-regulatory protein) to be associated with susceptibility to DN [9].

CTGF

CTGF is a member of a small family of highly homologous proteins termed the CCN family (for cysteine-rich, Cys10/cysteine-rich 61 and Nov) that co-ordinate complex biological
processes during differentiation and tissue repair. In addition to high glucose, TGF-β1 induced the expression of CTGF in primary human mesangial cells, whereas addition of anti-TGF-β1 antibody to mesangial cells cultured under high extracellular glucose conditions partially inhibited the induction of CTGF. The PKC inhibitor GF 109203X similarly inhibited the high glucose induction of CTGF. In the aggregate, these data suggest that high glucose stimulates mesangial CTGF expression by both TGF-β1-dependent and PKC-dependent pathways [6].

The addition of recombinant CTGF to cultured mesangial cells enhanced expression of the ECM proteins collagens I and IV and fibronectin. These proteins typify matrix accumulation seen in DN, demonstrating the potential of CTGF as a stimulus for increased ECM synthesis and mesangial expansion in DN. CTGF expression was also increased more than 2-fold in the glomeruli of STZ (streptozotocin)-treated rats, an in vivo model of Type 1 diabetes, 4 months after the onset of disease, at the time of evident mesangial expansion and proteinuria [6].

CTGF induced not only ECM protein expression in human mesangial cells, but also cell migration and cytoskeletal rearrangement [7]. These functional responses were associated with recruitment of Src and phosphorylation of p42/44 MAPK (mitogen-activated protein kinase) and PKB (protein kinase B). Inhibition of CTGF-induced p42/44 MAPK or PI3K (phosphoinositide 3-kinase)/PKB pathway resulted in reduced levels of fibronectin expression. Anti-β3 integrin antibodies also inhibited the activation of both the p42/44 MAPK and PKB by CTGF, also resulting in reduced fibronectin expression.

CTGF induced mesangial cell migration also by activation of β3 integrin, an activation that was also sensitive to the inhibition of the p42/44 MAPK and PI3K pathways. Adhesion of the mesangial cells to type I collagen was promoted following stimulation by CTGF and increased expression of α1 integrin.

Actin cytoskeletal disassembly was observed to occur in a transient fashion over a 24 h period following treatment with CTGF. An associated loss of focal adhesions was observed in mesangial cells, as was evident by the loss of punctate vinculin. However, these processes were independent of both p42/44 MAPK and PI3K pathway activation [10].

These data indicate that CTGF is a mediator of TGF-β1-driven matrix production within the diabetic kidney. In addition, CTGF induces ECM production by the induction of signalling processes via β3 integrin-specific activation of p42/44 MAPK and PI3K pathways. CTGF also regulates actin cytoskeleton disassembly in a β3 integrin/MAPK/PI3K-independent manner, indicating that CTGF is a complex pleiotropic factor with the potential to amplify primary pathophysiological responses [11,12].

**Gremlin**

IHG-2 was identified by cloning *in silico* to be Gremlin, a member of the DAN (differential screening-selected gene ab-

errant in neuroblastoma) family of BMP (bone morphogenetic protein) antagonists [13,14]. Gremlin has been reported to influence diverse processes in growth, differentiation and development [13,15]. Gremlin expression was increased by both cyclic mechanical strain *in vitro*, a model of the glomerular hypertension associated with DN, and in the kidneys isolated from rats with STZ-induced diabetes *in vivo* [14].

Gremlin expression in kidney biopsy specimens from patients with DN was increased when compared with normal kidney or biopsy specimens from patients with minimal change disease, a non-scarring renal disorder [16]. Abundant Gremlin expression was observed in the tubular compartment in advanced human DN. Although Gremlin expression was detected in occasional glomeruli, the majority of expression was observed in areas of tubulointerstitial fibrosis, where it co-localized with TGF-β1 expression. In addition, Gremlin mRNA levels correlated directly with elevated serum creatinine levels and tubulointerstitial fibrosis score in patients with DN. These data suggest a role for Gremlin in the pathogenesis of tubulointerstitial fibrosis [16].

Given the strong correlation with disease development and Gremlin expression, we used bioinformatic analysis to investigate whether Gremlin gene sequence and structure could be used to identify other genes implicated in DN. Two genes were identified using this approach: Jagged1 (the Notch ligand) and its downstream effector, Hes1 (hairy enhancer of split-1) [17]. Both of these genes had promoter structure and predicted microRNA-binding elements similar to those found in the Gremlin gene. TGF-β1 increased Gremlin, Jagged1 and Hes1 expression in human kidney epithelial cells *in vitro*, and all three genes also showed increased expression in biopsies from DN patients. In addition, increased expression of Gremlin, Jagged1 and Hes1 co-localized in areas of tubulointerstitial fibrosis. Furthermore, Notch pathway gene clustering showed that samples from DN patients grouped together, distinct from both control living donors and patients with minimal change disease [17].

**IHG-1**

IHG-1 is a novel evolutionary conserved gene transcript expressed ubiquitously in human tissue [18]. IHG-1 expression in microdissected tubule-rich kidney biopsy specimens from patients with DN was increased almost 10-fold when compared with control subjects. *In situ* hybridization and Southwestern immunohistochemistry reveal increased tubular IHG-1 levels co-localized with TGF-β1, and activated Smad3 in DN [18]. These data suggest an association between expression of this gene and tubulointerstitial change in DN.

We decided to investigate the expression of IHG-1 in UUO (unilateral ureteric obstruction), an acute model of renal fibrosis, in order to investigate further the association between IHG-1 expression and the development of fibrogenesis in the tubulointerstitium. As fibrosis in this model does not occur secondarily to a pre-existing systemic disorder, it allowed us to examine whether increased expression of IHG-1 was a feature of tubulointerstitial fibrosis. IHG-1 mRNA
levels were significantly increased (almost 3-fold) in UUO at day 3 before the development of fibrosis and were still significantly increased at day 10 post-obstruction when the tubulointerstitial lesion was well advanced. IHG-1 protein levels were increased both at 3 and 10 days [18].

Similarly to DN, activation of the TGF-β1 pathway in UUO is a pivotal event leading to development of fibrosis. As IHG-1 expression was increased in kidney tubules in advanced DN and in the rat model of tubulointerstitial fibrosis, we examined the impact of IHG-1 overexpression on TGF-β1 signal transduction. Increased activity of TGF-β1 in the tubulointerstitium results in increased expression and accumulation of ECM proteins, resulting in compartment-specific pathological matrix remodelling and scarring. IHG-1 amplified TGF-β1-induced transcriptional activation by enhancing levels of phospho-Smad3. Overexpression of IHG-1 in proximal tubular epithelial cells increased CTGF and fibronectin expression following TGF-β1 stimulation. Furthermore, inhibition of endogenous IHG-1 expression suppressed transcriptional responses to TGF-β1, underscoring the importance of IHG-1 as an intrinsic regulator of TGF-β1. These data indicate that IHG-1 facilitates increases and prolonged phosphorylation of Smad3, enhancing transcriptional responses to TGF-β1 along a fibrotic pathway [18].

Regardless of the initiating causes of renal disease, Smad signalling is believed to be the final common pathway for fibrogenesis. Increased IHG-1 levels are likely to contribute to the TGF-β1-induced profibrotic changes in tubular cells which prime for tubulointerstitial fibrosis.

**LXs (lipoxins)**

There is a growing appreciation that activation of the innate immune system may play an important role in the development of DN [19]. In this context, we have investigated the potential of LXA4 to protect renal cells from various profibrotic stimuli. LXs are endogenously produced eicosanoids with anti-inflammatory and pro-resolution activities [20,21]. More recently, we have demonstrated that LXA4 can modulate growth factor receptor activation in primary human mesangial cells [22,23] and can protect renal epithelia from mesenchymal transformation and bronchial fibroblast activation in response to multiple stimuli, including TGF-β1 [24,25]. In this context, we propose the potential of LXA4 and/or its stable synthetic analogues [26] as potential fibrosuppressants in multiple conditions such as DN.

**Summary**

In summary, we have used SSH to identify 200 mesangial cell genes whose expression is enhanced or suppressed in response to high extracellular glucose. A cohort of actin-regulatory proteins dominated the up-regulated genes and appeared to be induced through signalling pathways triggered by oxidative stress. Disruption of the actin cytoskeleton by cytochalasin D mimicked the effects of high glucose, suggesting that this response may represent an attempt at cytoskeletal repair. CTGF also identified in the screen is strongly associated with development of diabetic renal disease and functions as a mediator of TGF-β1-driven matrix production within the diabetic kidney. In addition, CTGF induces ECM production by the induction of signaling processes via β3 integrin-specific activation of p42/44 MAPK and PI3K pathways. CTGF also regulates actin cytoskeleton disassembly in mesangial cells in a β3 integrin/MAPK/PI3K-independent manner, indicating that CTGF is a multifactorial growth factor with the ability to enhance primary pathophysiological responses in the diabetic kidney.

Further investigation of two novel glucose-induced genes identified IHG-2 to be Gremlin, a BMP antagonist with an essential role in kidney development. Increased Gremlin expression was associated with the development of tubulointerstitial fibrosis in DN. Increased expression of the novel gene IHG-1 also was associated with the development of tubulointerstitial fibrosis. IHG-1 was found to amplify the activity of TGF-β1, a key mediator of diabetic renal disease. IHG-1 enhanced TGF-β1 transcriptional activity through prolongation of Smad3 phosphorylation. By investigating the regulation of expression and actions of specific molecules, we have identified novel roles for mediators such as Gremlin, IHG-1 and CTGF and potential regulations of their bioactions.

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**References**


Murphy, M., McMahon, R., Lappen, D.W. and Brady, H.R. (2002) Gremlins: is this what renal fibrogenesis has come to? Exp. Nephrol. 10, 241-244

McMahon, R., Murphy, M., Clarkson, M., Taal, M., Mackenzie, H.S., Godson, C., Martin, F. and Brady, H.R. (2000) HGF-2, a mesangial cell gene induced by high glucose, is human gremlin: regulation by extracellular glucose concentration, cyclic mechanical strain, and transforming growth factor-β1. J. Biol. Chem. 275, 9901-9904


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