

Transcriptome Profiling and the Pathogenesis of Diabetic Complications

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Abstract. Diabetes is an escalating problem worldwide and a major cause of vascular disease, renal failure, and blindness, among other complications. The cellular mediators of high glucose-induced injury include activation of protein kinase C, accumulation of cell sorbitol from increased flux through the aldose reductase pathway, and generation of advanced glycosylation end products and reactive oxygen species, among others. Current strategies for preventing and slowing the progression of the macrovascular and microvascular complications of diabetes include optimization of glycemic control and BP, angiotensin-converting enzyme inhibitors and angiotensin

II blockers, and HMG CoA reductase inhibitors. However, there is an urgent need to develop new therapeutic strategies, as these interventions, although they may slow, rarely halt the progression of diabetic complications. Central to this process is the elucidation of the molecular events that drive this complex disease and that are potential therapeutic targets. This review discusses the promise offered in this regard by global monitoring of cellular or tissue mRNA expression (so-called transcriptomics) and illustrates the potential of this approach by focusing on recent studies on the pathogenesis of diabetic nephropathy.

With the completion of the human genome project, the monitoring of changes in the whole cellular transcriptome is an increasingly attractive method for dissecting the molecular basis of a disease process. Refinement of high-throughput gene analysis techniques has allowed transcriptome profiling of models of disease both *in vitro* and *in vivo* in an unbiased and highly sensitive manner without requiring previous knowledge of the genes involved in a particular disease. Examples of these techniques include differential display PCR, suppression subtraction hybridization (SSH), serial analysis of gene expression (SAGE), and more recently DNA microarray (gene chip) technology. Here we briefly review these techniques and their recent contribution to the study of the pathogenesis of diabetic nephropathy.

Techniques for Transcriptome Profiling

Differential display PCR is a PCR-based technique that allows side-by-side comparison of multiple RNA samples and can facilitate the identification of both suppressed and induced genes. With the use of this technique, increased expression of prolyl 4-hydroxylase α subunit, thrombospondin 1, and a novel glucose-regulated gene encoding a putative zinc finger protein

in mesangial cells in response to high glucose levels has been reported (1).

SSH is a PCR-based technique that allows the creation of subtracted cDNA libraries for the identification of genes differentially expressed in response to an experimental stimulus (2). SSH includes a normalization step that removes bias for the more abundant cellular mRNA and in theory should offer increased sensitivity by comparison with other subtractive techniques. To date, this technique has been successful in identifying an array of diabetes-associated genes, several of which are potential therapeutic targets (3,4). These are discussed later in further detail.

SAGE allows gene expression profiling on a larger scale. SAGE relies on the generation of unique short (10 bp) sequences of cDNA that can be concatomerized, cloned, and sequenced rapidly (5). This strategy provides maximal coverage of the expressed genes for gene identification at the whole genome level while keeping the sequencing analysis at a manageable scale. Although this technique has not been used to probe the changes in gene expression in diabetic nephropathy *per se*, it has permitted analysis of the transcription profile of microdissected normal nephrons and kidney cortical collecting ducts stimulated by aldosterone and vasopressin (6,7).

DNA microarrays (gene chips) have revolutionized our ability to monitor large-scale changes in gene expression in health and disease states. They offer the potential to monitor changes in the entire transcriptome through hybridization of sample mRNA to many thousands of genes immobilized on nylon or glass surfaces. Wada *et al.* (8) used this technique to monitor gene expression in whole kidneys of streptozotocin (STZ)-induced diabetic mice and identified differential expression of

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81 genes (16 upregulated and 65 downregulated), 44 of which were novel genes.

Examples of Potential Disease Drivers Identified by Transcriptome Profiling

SSH has proved particularly successful in identifying genes that are upregulated by high glucose in diabetic nephropathy. Application of this technique to a model of diabetic nephropathy *in vitro*, namely high glucose-treated mesangial cells, identified two major clusters of genes: one that had previously been reported as being involved in experimental and human diabetic nephropathy (*e.g.*, fibronectin, thrombospondin, plasminogen activator inhibitor-1) and a second cohort of genes not previously associated with diabetic nephropathy (DN) (3,4).

Connective Tissue Growth Factor

Among the potential drivers of disease identified by this approach was connective tissue growth factor (CTGF), a cysteine-rich peptide that belongs to the CCN family of growth factors that include members *cef* 10, *nov*, and *wisp*-1. Human CTGF was first identified as a product of human umbilical vein endothelial cells that was both chemotactic and mitogenic for fibroblasts (9). *In vitro*, CTGF has diverse bioactivities depending on cell type (10). Significantly, it induces kidney fibroblast proliferation and extracellular matrix synthesis (11). The finding of CTGF mRNA expression by skin fibroblasts from lesions of patients with scleroderma provided the first evidence of its profibrotic effect *in vivo* (12). Subsequently, immunohistochemical studies demonstrated that CTGF was also expressed in advanced atherosclerotic plaques but was undetectable in the normal artery (13).

In renal systems, CTGF was initially reported in renal mesangial cells exposed to high extracellular glucose *in vitro* (14). Evidence for involvement of CTGF in renal fibrosis *in vivo* was first intimated by Ito *et al.* (15), who subsequently reported significant upregulation of CTGF expression, as determined by *in situ* hybridization, in a variety of tubulointerstitial and inflammatory disorders including diabetic nephropathy.

Using SSH, our group confirmed that ambient high glucose was a potent stimulus for CTGF expression in human mesangial cells *in vitro* and demonstrated its expression in glomeruli from rats with STZ-induced diabetic nephropathy (3). In these studies, recombinant CTGF stimulated production of collagens I and IV by mesangial cells (3). Riser *et al.* (16) also found high glucose as a stimulus for CTGF expression, at both the mRNA and protein levels, in rat mesangial cells. In addition, these investigators identified cyclic mechanical strain (which simulates the glomerular hypertension that exists in the diabetic kidney *in vivo*) as another inducer of CTGF mRNA expression but not protein levels *in vitro* (16).

There is compelling evidence that TGF- β is a stimulus for mesangial matrix accumulation in diabetic nephropathy (17). Against this background, TGF- β triggers CTGF release by fibroblasts and chondrocytes (18,19) and the CTGF promoter contains a TGF- β response element (20). We demonstrated that exogenous TGF- β_1 directly upregulated CTGF mRNA

expression in mesangial cells, an effect that was similar to that induced by high glucose (3). Furthermore, the high glucose-triggered effect was inhibited by a neutralizing anti-TGF- β_1 antibody. It is interesting that this inhibitory effect of TGF- β_1 antibody was only partial, suggesting that there may be a TGF- β -independent component to this response (3). Riser *et al.* (16) also demonstrated that TGF- β upregulated CTGF expression in mesangial cells but that this effect was completely inhibited by the addition of an antibody that neutralizes TGF- β_1 , -2, and -3 activity. High glucose activates protein kinase C (PKC) in a variety of cell types, including mesangial cells, and PKC has been proposed as a therapeutic target in this setting (21). It is interesting that in our study, inhibition of the PKC pathway did not affect TGF- β -mediated upregulation of CTGF, but it did attenuate high glucose-induced CTGF expression (3).

The mechanisms through which CTGF modulates mesangial cell function are still being defined. Recently, Crean *et al.* (22) reported that CTGF stimulation of mesangial cells initiated early recruitment of Src and activation of PI3K and p42/44 MAPK pathways by signaling through β_3 integrins. Furthermore, they also demonstrated transient actin cytoskeleton disassembly in mesangial cells after the addition of CTGF. This finding was of interest in view of previous work that had suggested that cytoskeletal alteration is a contributor to the decreased afferent arteriolar tone found in glomerulosclerosis (23). The cytoskeleton disassembly process was accompanied by dissolution of focal adhesions as evidenced by the disappearance of punctate vinculin staining. However, this result, unlike the fibronectin-inducing effect of CTGF, was mediated in a β_3 integrin/MAPK/PI3K-independent manner (22).

CTGF has also been reported to have a direct role in modulation of the mesangial cell cycle (24). This is consistent with the fact that established diabetic nephropathy is associated with mesangial cell hypertrophy rather than with cell proliferation (25). CTGF stimulates mesangial cells to enter the cell cycle and arrests progression at the G1 phase (24). This was achieved through the induction of the negative regulators of the cell cycle CDKI p15^{INK4}, p21^{Cip1}, and p27^{Kip1}, which subsequently bound to and inactivated cyclin D/CDK4/6 and cyclin E/CDK2 kinase complexes (24). Cells arrested in the G1 phase showed increased size and exhibited enhanced RNA and protein synthesis, resulting in cellular hypertrophy (24). Furthermore, the investigators provided evidence that TGF- β had a positive regulatory effect on expression of all three CDKI and that this effect was mediated through CTGF (24).

In summary, CTGF is expressed by mesangial cells in response to various stimuli, including TGF- β , high ambient glucose levels, and cyclic mechanical strain. It displays matrix-enhancing effects with the induction of fibronectin and collagen I and IV in mesangial cells *in vitro* via activation of PI3K and p42/44 MAPK pathways. It also plays a role in mediating mesangial cell hypertrophy and cytoskeletal disassembly, processes previously implicated in glomerulosclerosis. Whether CTGF is a therapeutic target in DN remains to be answered. CTGF has antiproliferative effects for some cancer cell lines *in vitro* (26) and is also expressed in atheromatous plaques, where

it is postulated to have a role in fibrous cap formation (13). Thus, intervention studies targeting CTGF will have to address not just its potential antifibrotic actions but also its potentially protective effects in other disease states.

Gremlin

The reappearance of developmental genes in the context of acquired disease in adulthood represents an emerging paradigm in the study of disease pathogenesis and suggests that the fibrosis of chronic disease may be an attempt at tissue repair, albeit a frequently frustrated and ineffective one (27). In this regard, SSH identified the human homologue of a rat developmental gene, *drm/gremlin*, as a high glucose–triggered gene in cultured human mesangial cells (28). Gremlin is a 184–amino acid protein that, together with DAN and cerberus, is a member of the cysteine knot superfamily of proteins (29). Vertebrate limb outgrowth and patterning depend on reciprocal interactions between sonic hedgehog (SHH) signaling from the posterior mesenchyme and fibroblast growth factor (FGF) signaling from a specialized ectodermal structure, the apical ectodermal ridge (30). Gremlin is an important modulator of the FGF/SHH feedback loop, by antagonizing bone morphogenetic protein repression of FGF signaling (31). In our studies, mesangial cell gremlin mRNA levels were positively regulated by high extracellular glucose, cyclic mechanical strain, and TGF- β_1 (28). In addition, gremlin mRNA levels were elevated in the renal cortex of rats with STZ-induced diabetic nephropathy *in vivo* (28). Preliminary studies suggest that gremlin overexpression may modulate both mesangial cell growth and transdifferentiation of cultured tubular epithelial cells to a more fibroblast-like phenotype (32).

Actin Regulatory Binding Proteins

The actin cytoskeleton architecture, in conjunction with the stress fibers of filamentous actin and myosin II, plays an important role in the mechanical strength and elasticity of the glomerulus. Previous studies have identified high glucose as having a deleterious effect on the contractility of mesangial cells *in vitro*, with disassembly of F-actin and loss of stress fibers as one of the mechanisms identified (33–35). This cytoskeletal alteration has also been postulated to contribute to the mesangial and vascular smooth muscle cell hyporesponsiveness to vasopressors seen in models of DN both *in vivo* and *in vitro* (36,37). More recently, Cortes *et al.* (23) reported cytoskeletal disorganization involving F-actin containing stress fibers in the mesangial cells in experimental diabetes *in vivo* and suggested that the resultant impairment in contractility may lead to the well-documented perturbation in glomerular perfusion and filtration that is seen in DN.

It is interesting that one of the largest functional gene clusters identified by SSH in our study of high glucose–stimulated mesangial cells included genes encoding actin cytoskeleton regulatory proteins such as caldesmon, ADP-ribosylation factor GTPase-activating protein (ASAP-1), ARP-3, profilin, 14-3-3 ζ and cyclase-associated protein (4). The upregulation of these proteins may have a significant role in the context of the cytoskeletal disarray that characterizes DN. For example, over-

expression of ASAP-1 interferes with cytoskeletal remodeling events and focal adhesion turnover in other systems (38). Cyclase-associated protein may halt filament growth through binding to the barbed end of the actin filament and thus prevent further incorporation of G-actin into F-actin, effectively (39). Myosin regulatory light chain (MRLC) is a component of myosin filaments and plays an important role via its interaction with myosin light chain kinase in the regulation of cell contractile activity (40). Profilin and ARP-3 encode proteins involved in key regulatory pathways of actin cytoskeletal turnover (41).

In our study, the changes in the expression of mRNA encoding actin cytoskeletal regulatory proteins induced by high glucose was largely independent of TGF- β , in contrast to the regulation of CTGF and gremlin expression (3,28). It is interesting that carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), which inhibits the generation of reactive oxygen species, attenuated the expression of these actin cytoskeletal regulatory genes in the presence of high ambient glucose, providing further evidence that oxidative stress may play a key role in cytoskeletal disruption in this setting (4).

Conclusion

In conclusion, improvements in high-throughput techniques that permit transcriptome profiling have already made significant contributions to our understanding of the pathogenesis of DN and identified putative mediators of diabetic complications such as CTGF, gremlin, and actin cytoskeleton regulatory proteins. These techniques, when coupled with our wider understanding of the molecular pathways invoked by disease, should continue to open exciting new avenues for exploration and fuel the quest for the ultimate therapy in DN.

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