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TGF-β1-induced-EMT and therapeutic intervention in Diabetic Nephropathy

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Short title: TGF-β, EMT and DN

Key words: TGF-β, EMT, DN

Word count: 4845

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Abstract:

 Background/Aims: Epithelial–to-mesenchymal cell transformation (EMT) is the trans-differentiation of tubular epithelial cells into myofibroblasts, an event underlying progressive chronic kidney disease in diabetes, resulting in fibrosis. Mainly reported in proximal regions of the kidney, EMT is now recognized as a key contributor to the loss of renal function throughout the nephron in diabetic nephropathy (DN). Concomitant up-regulation of TGF-β in diabetes makes this pro-fibrotic cytokine an obvious candidate in the development of these fibrotic complications. This article reviews recent findings clarifying our understanding of the role of TGF-β and associated sub-cellular proteins in EMT. **Methods:** To understand the pathology of EMT and the role of TGF-β, we reviewed the literature using Pubmed for English Language articles that contained key words related to EMT, TGF-β and DN. **Results:** EMT and phenotypic plasticity of epithelial cells throughout the nephron involves cytoskeletal reorganization and *de novo* acquisition of classic mesenchymal markers. Concurrent down-regulation of epithelial adhesion molecules results in a loss of function and decreased cell coupling, contributing to a loss of epithelial integrity. TGF-β1 is pivotal in mediating these phenotypic changes. **Conclusion:** TGF-β induced EMT is a key contributor to fibrotic scar formation as seen in DN and novel routes for future therapeutic intervention are discussed.

Introduction:

Diabetic nephropathy (DN) is the single commonest cause of entry into the renal replacement therapy programme and the leading cause of end-stage renal failure in diabetes mellitus [1]. DN refers to a set of structural and functional changes which arise in response to chronic glycaemic assault [2]. Structural abnormalities include; hypertrophy, glomerular basement membrane thickening, tubular atrophy and interstitial fibrosis [3]. These changes contribute to increased glomerular filtration rate, proteinuria, systemic hypertension and loss of renal function [3]. Histologically, DN is characterised by an accumulation of extracellular matrix (ECM) proteins in both the glomerular mesangium and tubular interstitum culminating in a decline in excretory function and excessive renal scarring [3][4]. Renal fibrosis manifests itself as glomeruosclerosis, tubulinterstitial fibrosis, infiltration of inflammatory mediators and the activation of alpha-smooth muscle actin (α-SMA)-positive myofibroblasts. Tubular epithelial to mesenchymal transition (EMT), or the transdifferentiation of tubular epithelial cells into myofibroblasts, mediates excessive deposition of the ECM. This ability to switch phenotype stems from a unique plasticity of epithelial cells and is implicated in the generation of interstital fibroblasts in the diseased kidney. EMT of proximal tubule cells (PTC) has been clearly documented in DN and overwhelming evidence implicates Transforming Growth Factor-beta 1 (TGF-β1) as the predominant agent mediating these fibrotic changes. This review discusses our current knowledge of TGF-β1 induced EMT in DN in both the PTC and collecting duct of the kidney. Finally, we discuss potential therapeutic strategies to inhibit renal fibrogenesis in diabetes.

Epithelial-to-mesenchymal-transition in the kidney:

Developmentally, renal tubules are derived from the metanephric mesenchyme through a process termed mesenchymal-to-epithelial transdifferentiation (MET). This cellular differentiation is not static and cells retain the ability to revert back to their original mesenchymal phenotype through EMT. Commonly associated with epithelia of embryonic origin, this plasticity is critical in early stages of development. In adults, EMT is associated with tissue injury and repair; a process instigated as the demand for fibroblasts and wound healing increases [5].

In EMT, the loss of epitheial characteristics coincides with the acquisition of proteins associated with a mesenchymal phenoptye. These morphological and phenotypic changes occur at four different stages: (i) the loss of epithelial cell adhesion molecules such as E-cadherin and zonula occludens protein ZO-1 are replaced by the (ii) mesenchymal markers α-SMA and the intermediate filament protein vimentin. The loss of cell adhesion is accompanied by (iii) cytoskeletal remodelling and morphological changes resulting in tubular basement membrane disruption. (iv). Consequently these cells possess the ability to migrate from the tubular basement membrane (TBM) into the interstium. This migratory capacity leads to increased deposition of the ECM and renders EMT key in the pathology of tubulointerstitial fibrosis. Normally, tubular epithelial cells form a highly coupled epithelial sheet held together by the adhesion molecule Epithelial (E)-cadherin. Loss of E-cadherin expression occurs in the early stages of EMT and results in the dissociation of cells within the epithelial sheet [6]. This represents the beginning of a series of events culminating in transition from an epithelial-to-mesenchymal phenotype. Changes in E-cadherin are rapidly accompanied by an up-regulation of mesenchymal markers. Re-organisation of the actin cytoskeleton into stress fibres containing *de novo* expressed α-SMA is accompanied by an

exchange of cytokeratin for vimentin filaments and the expression of fibroblast-specific-protein (FSP1), a Ca^{2+}-binding protein involved in motility, invasion, and tubulin polymerization. These morphological and phenotypic changes support matrix remodelling and the migration across the TBM into the interstitial environment where these cells further exacerbate fibrosis [7].

The migratory phenotype, used to define EMT remains controversial, especially in the context of fibrosis [8]. Loss of an epithelial phenotype can be clearly identified by a loss in the expression of specific epithelial proteins, in particular E-cadherin. Acquisition of a mesenchymal phenotype is more difficult to attribute [9] and may explain why EMT is often overlooked in studies of renal disease. Markers classically used to define EMT include vimentin and α-SMA [10]. However, vimentin is not specific for fibroblastoid cells [11], whilst α-SMA, the most commonly used marker in EMT exhibits heterogeneity of expression [12]. Clarification of EMT in fibrosis is dependent on the identification of a complex interplay of both phenotypic and morphological changes.

The pathology of EMT:

A role for EMT in the progression of chronic kidney disease (CKD) was first demonstrated by Iwano *et al* in a model of unilateral ureteral obstruction (UUO) where 36% of the matrix producing cells resident within the tubulointerstitial space were found to be of epithelial origin and derived from renal tubular epithelium through EMT [13]. The underlying pathology of EMT has since been observed in human renal biopsies from diseased kidney, a feature confirmed by the presence of myofibroblasts in which the proportion of cells undergoing EMT was found to correlate to both the level of serum creatinine and the degree of interstitial fibrosis [14][15]. In models of renal disease, EMT occurs in response to

hypoxia, reactive oxygen species, advanced glycation end products (AGEs) and numerous prof-fibrotic cytokines, growth factors and metalloproteinases [16]. Of these, the pro-fibrotic cytokine TGF-β1 is a likely candidate in the development and progression of fibrotic complications observed in DN [17].

EMT and the pathogenesis of fibrosis in DN:

In diabetes, glomerular fibrosis is observed in the early progression from incipient to overt nephropathy [18]. Whilst tubulointerstitial fibrosis can also present itself in these early stages, a build up of fibrotic material in the tubular interstitium tends to accompany disease progression, correlating with a gradual decline in renal function [19]. Although the origin of fibroblasts in DN remains less clear, progressive renal fibrosis may, in part, be mediated by the phenotypic changes induced by EMT. Analysis of renal histology in both diabetic animals and from the kidneys of patients with DN, confirms the existence of EMT- induced changes. Markers of EMT have recently been observed in STZ treated Wistar Kyoto rats, Sprague Dawley rats and the STZ Ren-2 rat [20][21]. These studies suggest that EMT is closely correlated to a decline in renal function [15]. Recent studies by Yamaguchi *et al* have suggested that the existence of FSP-1 in podocytes from patients with diabetes is most likely to be associated with induction of podocyte detatchment through EMT, a potential catastrophic event since the depletion of glomerular podocytes is an important feature of progressive DN [22].

A role for TGF-β1, Smads and EMT in DN:

A broad spectrum cytokine, TGF-β regulates many biological processes [23][24]. Of its three isoforms (TGF-β1, β2 and β3) TGF-β1 is the principal mediator of diabetic complications and is key in the development of hypertophy and the accumulation of ECM [25][26]. In models of

renal disease and diabetes, TGF-β1 gene expression and secretion are increased [27][28], an up-regulation that is in part, due to, elevated glucose , AGEs, PKC and the MAPK pathway [29][30][31][32]. However, despite extensive data demonstrating TGF-β induced fibrosis, the underlying events mediating the development of the fibrotic lesion are poorly understood. In diabetes, TGF-β1 contributes to excessive deposition of fibrotic material through EMT, modulating the expression of several epithelial cell recognition and organizational proteins, including the cadherins, catenins as well as the actin cytoskeleton [33]. In human mesangial cells, TGF-β1 induces α-SMA, collagen type I expression and cell hypertrophy [34]. Furthermore, the downregulation of E-cadherin expression with the concomitant upregulation of α-SMA in tubular epithelial cells, strongly supports the case for TGF-β1 as a potent stimulus of EMT [35].

Predominantly mediated via Smad dependent pathways, TGF-β1 binds to a distinct receptor, TGF-β receptor II (TβRII), that activates the TGF-β receptor type I (TβRI)-kinase. This association results in the downstream phosphorylation of the Smad proteins. Smads are subdivided into three classes; Receptor regulated (R) Smads (Smad 1, 2,3,5 and 8), the Common (Co) Smads (Smad4) and the Inhibitory (I) Smads (Smad6 and 7). Following TβRII activation, R-Smads form oligomeric complexes with the common Smad (Co-Smad) prior to translocation and regulation of gene transcription [36]. Transfected cells with a mutant TβRI fail to exhibit TGF-β1 induced EMT in the absence of Smad activation, despite exhibiting full kinase activity [37]. The majority of TGF-β targeted genes regulated in EMT appear to be reliant on Smad3 dependent transcriptional regulation [38], though a role for Smad2 should not be discounted [39]. Differential roles for both Smad2 and Smad3 have been identified [40], and a Smad3 dependent reduction of E-cadherin in human proximal tubular cells (hPTC) is paralleled by a Smad2 dependent induction of metallomatrix proteinase 2 [41]. Microarray

analysis of TGF-β1-induced EMT in mouse and human epithelial cells demonstrates a critical requirement for Smad signalling in the regulation of all tested target genes [42]. Smad signalling is stringently controlled in order to protect the cells from an unwanted TGF-β response, and a safeguard mechanism exits in the form of both inhibitory Smads and transcriptional co-repressors [43]. The inhibitory Smads (Smad6 and Smad7) inhibit R-Smad phosphorylation by blocking their access to TβRI, and/or by promoting the degradation of the receptor complexes. Co-repressors SnoN (Ski-related novel gene, non Alu-containing), Ski (Sloan-Kettering Institute proto-oncogene), and TGIF (TG-interacting factor) prevent gene transcription through inhibition of R-Smads [43]. These antagonists are critical in ensuring the regulation of Smad-mediated gene transcription, therfore a fine balance must be achieved in order to match the demands of the cell. It is no surprise, that diminished levels of co-repressors SnoN, Ski and TGIF are observed in animal models of obstructive nephropathy and diabetes [44]. Smad ubiquitination regulatory factor-2 (Smurf2) is an ubiquitin ligase that specifically targets certain members of Smad proteins for degradation, including Ski, SnoN and TGIF [45]. The close association between Smurf2 expression and enhanced SnoN degradation [44] suggests that the dysregulation of Smurf2 is most likely to affect profibrotic TGF-β/Smad signalling and may contribute to the development and progression of human kidney fibrotic diseases [45].

TGF-β, SGK1 and EMT in the collecting duct:

The role of TGF-β in more distal regions of the nephron is yet to be established. In the collecting duct, TGF-β promotes increased expression of the serum and glucocorticoid inducible kinase-1 (SGK1), a serine/threonine kinase that regulates sodium re-absorption through control of the epithelial sodium channel (ENaC) [46]. SGK1 is elevated in models of diabetes [47], suggesting that it may contribute to the development of secondary hypertension.

Interestingly SGK1 is expressed in numerous tissues that exhibit fibrosis including cases of Crohn's disease, lung fibrosis, liver cirrhosis, fibrosing pancreatitis, DN and glomerulonephritis [48]. These studies suggest that SGK1 may mediate some TGF-β-induced fibrotic effects. In diabetic mice SGK1 potentiates the effect of high glucose on fibronectin formation [49], a response that in human fibroblasts has been found to be dependent on the epidermal growth factor receptor (EGFR) [50]. EMT has been confirmed in the collecting duct in a model of fetal UUO [51] and both insulin growth factor 1 (IGF1) and TGF-β1 have been shown to induce classic EMT-like changes in mouse inner medullary collecting duct cells [52], despite initial studies suggesting that these cells could not undergo phenotypic conversion in response to TGF-β1 [53]. How TGF-β1 induced EMT contributes to fibrosis in the distal nephron is unclear. In 2009 Aldehni F et al. demonstrated that TGF-β1 induced EMT in mouse renal collecting duct increased levels of Bestrophin 1 (Best1), a compound which controls intracellular Ca^{2+} concentration and increases cellular proliferation [54]. Suppression of Best1 by RNAi inhibited proliferation and down-regulated markers of EMT, suggesting that Best1 may function as a downstream mediator of TGF-β1 induced EMT and renal fibrosis in the CD.

Therapeutic intervention:

Although TGF-β1 is regarded as the major isoform involved in fibrosis, improved renal function coincides with reduced expression of both TGF-β1 and β2 [55]. Furthermore, a pro-fibrotic role for all 3 isoforms has been observed in diabetes [56]. The pro-fibrotic actions of TGF-β make it an ideal therapeutic target for reno-protective agents. Neutralizing TGF-β1, TGF-β2 or TGF-β3 reduces renal scarring and diminishes a loss of kidney function [57][58]. Inhibition of TGF-β in this way also prevents glomerular enlargement and suppresses the expression of genes encoding for the ECM in models of chemically induced diabetes [57].

These findings were corroborated in *db/db* mice, where both TGF-β and TGF-β receptor mRNA expression were increased [58]. Chronic treatment of these animals with TGF-β-neutralizing antibodies markedly diminished the expression of collagen and fibronectin and reduced mesangial matrix expansion. Several anti-fibrotic and reno-protective agents have been shown to partially alleviate TGF-β induced fibrosis and include, Bone Morphogenic Protein 7 (BMP-7) and Hepatocyte Growth Factor (HGF).

Identified as an osteogenic factor, BMP-7 plays an important role in kidney development and the regulation of nephrogenesis associated with MET. Renal fibrosis in diabetes is inhibited by BMP-7. Following EMT, increased levels of TGF-β are paralleled by a reduction in expression of BMP-7, as a potential consequence of increased gremlin levels [59]. Gremlin limits BMP-7 availability and is markedly elevated in humans with DN [60]. This reciprocal relationship accounts for low BMP-7 concentrations in models of acute and chronic renal injury [61], and explains how exogenous BMP-7 restores renal function through blockade of EMT [62]. Aside from the glomerular reno-protective effects observed in both STZ rats and *db/db* mice, BMP-7 is capable of intercepting at the level of TGF-β signalling. In the adult, BMP-7 alleviates TGF-β-induced renal fibrosis [63] and antagonises TGF-β-induced Smad3-dependent EMT [64]. However, the mechanism remains elusive since BMP-7 is unable to negate TGF-β induced EMT in human PTC, suggesting that the effects of BMP-7 are region specific [65]. The extent by which BMP-7 blocks EMT requires further clarification.

A key anti-fibrotic cytokine, HGF prevents renal tissue fibrosis after chronic injury [66]. Administration of HGF reduces loss of kidney function, whilst blockade of HGF signalling further exacerbates the extent and progression of renal fibrosis [67]. As with BMP-7, HGF and TGF-β have a reciprocal relationship. HGF inhibits TGF-β induced EMT, and

ameliorates renal fibrotic lesions in numerous renal disease models [67]. The underlying molecular mechanisms mediating these reno-protective effects are unknown. The anti-fibrotic activity of HGF appears to stem from up-regulation of the transcriptional co-repressor SnoN. Binding of this co-repressor to Smad-2 results in formation of a transcriptionally inactive complex, preventing the activation of Smad mediated genes thereby blocking TGF-β induced EMT [68]. Administration of HGF has been shown to alleviate renal complications in DN, including the reversal of glomerulosclerosis [69], a reduction in albuminuria [70] and blockade of fibrosis with a concomitant improvement in renal function [71]. However, a number of studies contradict these findings, suggesting that chronically elevated HGF promotes the progression of nephropathy in *db/db* mice [72]. Furthermore, potential proto-oncogenic actions of HGF raises questions as to the potential therapeutic use of this growth factor in alleviating EMT induced complications in DN. Further studies are essential if HGF is to be considered as a future therapeutic intervention for fibrosis in DN.

EMT is a complex process involving numerous downstream signalling cascades. Strategies to disrupt any one of these may negate EMT and prevent fibrosis. Identifying other reno-protective agents may unearth future therapeutic interventions. Potential candidates recently identified upstream of HGF include, 9-cis retinoic acid, 1,25-dihyrdoxyvitamin D3, the PPARγ agonist troglitazone and C-peptide [33][69][70][71]. A cleavage product of pro-insulin, C-peptide exerts a number of protective affects against the micro-vascular and macro-vascular complications associated with hyperglycaemia [72] and in patients with DN, C-peptide is reno-protective [73]. Furthermore, C-peptide has been shown to negate TGF-β induced EMT in cells of the proximal tubule [33]. As an adjunct to insulin therapy, C-peptide could be used to alleviate some renal complications of diabetes. However, like BMP-7 and

HGF these findings are preliminary and whilst encouraging, need to be fully corroborated in the clinical scenario.

Conclusion:

TGF-β induced EMT is a key contributor to fibrotic scar formation as seen in DN. Manipulating downstream TGF-β signalling represents a viable therapeutic target to alleviate fibrosis and restore renal function. Administration of the anti-fibrotic growth factors HGF or BMP-7 can reverse the fibrogenic response and growing evidence supports the use of novel reno-protective agents to assist in alleviating complications of CKD, including DN.

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