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Mitogen activated protein kinases in renal fibrosis

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1. ABSTRACT

The mitogen-activated protein (MAP) kinases are involved in both normal renal physiology and in the pathology of various forms of kidney injury, including renal fibrosis. *In vitro* studies have shown a role for all three MAP kinase (ERK, p38 and JNK) in the production of the major pro-fibrotic factor, transforming growth factor-beta1 (TGF-beta1) by intrinsic renal cell types. There is also considerable cross-talk between TGF-beta1 and MAP kinase signalling pathways in the synthesis and turnover of extracellular matrix by fibroblast-like cells in the kidney. In addition, MAP kinase signalling contributes to TGF-beta1 induced transition of tubular epithelial cells into myofibroblasts. Administration of specific inhibitors of individual MAP kinases has identified a pathogenic role for both p38 and JNK pathways in animal models of renal fibrosis. There is also evidence to suggest that MAP kinases are activated in human renal fibrosis. Thus, blockade of p38 and JNK pathways may have therapeutic potential for the treatment of chronic renal fibrosis.

2. INTRODUCTION

The kidney has a tremendous capacity for self-repair, as exemplified by the ability to recover normal structure and function following acute tubular necrosis. However, when the underlying renal insult cannot be switched-off, such as settings of chronic inflammation, diabetes or hypertension, then a pathological process in which the accumulation of extracellular matrix replaces the cellular structure results in progressive renal fibrosis leading to end-stage renal failure. The role of growth factors, such as transforming growth factor-beta1 (TGF-beta1) and angiotensin II, is well established in renal fibrosis although the mechanisms by which these factors promote renal fibrosis are still being elucidated. The MAP kinases represent a group of signalling pathways which regulate inflammation and apoptosis. There is also a growing appreciation of the role of MAP kinases in the development of renal fibrosis.

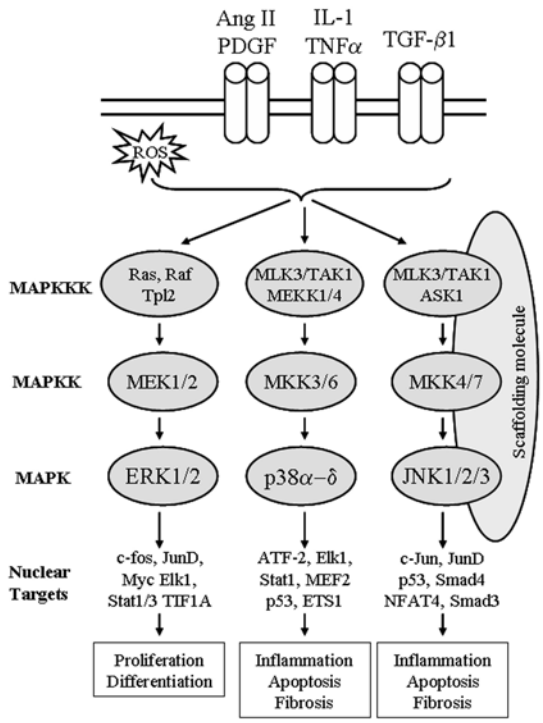

Figure 1. Overview of the mitogen-activated protein (MAP) kinase signalling pathways. Ligand binding to G-protein coupled receptors (e.g. angiotensin II) or receptor tyrosine kinases (e.g. PDGF, IL-1, TGF-beta1) leads to the activation of members of the p21-activated kinase family of enzymes that bind to and are activated by small GTPases of the Cdc42 and Rac families. This, in turn, results in phosphorylation of the upstream MAP kinase kinase kinases (MAPKKK). The MAPKKK can also be activated by reactive oxygen species (ROS) and UV irradiation through poorly defined intracellular mechanisms. Scaffold proteins bind members of the MAPKKK, MAPKK and MAPK families in close proximity via scaffold protein interaction domains. This facilitates a very rapid series of phosphorylation reactions between these kinases, resulting in phosphorylation and thus activation of the terminal MAP kinase in the pathway. There is a wide variety of MAPKKK, but a more restricted grouping of MAPKK which, in general, only phosphorylation a single MAP kinase. The activated MAP kinases can then translocate to the nucleus and phosphorylate a variety of transcription factors which mediates a change in the pattern of gene transcription that results in a biological response. This change in gene transcription is also mediated by MAP kinase phosphorylation of other kinases (known as MAP kinase-activated protein kinases) that modulate the function of a further series of transcription factors.

2.1. Overview of MAP kinase pathways

The MAP kinases are a family of serine/threonine kinases. There are at least 14 members of this family which have been identified and divided into groups based on sequence similarity: the extracellular signal-regulated kinase (ERK) 1 and 2; c-jun NH_2-terminal kinase (JNK) 1, 2, and 3; p38 kinase alpha, beta, gamma and delta; ERK5; and atypical MAP kinases (ERK3/4, ERK7/8, and NLK) (1). These kinases play an essential role in mediating the cell response to various external stimuli via modulating the pattern of gene transcription. MAP kinases regulate diverse biological functions including proliferation, differentiation, apoptosis, inflammation and fibrosis (2). As little information is available regarding ERK5 and the atypical MAP kinases in the kidney, or in renal fibrosis, this review will focus on the roles played by the three major MAP kinases; ERK, p38 and JNK.

MAP kinases are activated by phosphorylation of an activation loop in response to a wide range of stimuli such as growth factors, cytokines, ligands of the innate immune response, reactive oxygen species and osmotic stress. Activation of various cell surface receptors leads to activation of the MAP kinase pathways via the p21-activated kinase family of enzymes that bind to and are activated by small GTPases of the Cdc42 and Rac families. As illustrated in Figure 1, MAP kinase signalling operates via activation of a three-tiered cascade: MAPKKK, MAPKK and MAPK. This rapid sequence of phosphorylation events is facilitated by scaffolding proteins, such as the JNK-interacting proteins, that bind the kinases in close proximity (3). While the activation of ERK1/2 kinases is primarily due to the Ras-Raf-MEK1/2 cascade, there are many upstream MAPKKK (also called MAP3K) that can induce JNK and p38 signalling. The phosphorylation, and thus activation, of JNK is mediated by both MKK4 and MKK7, while p38 phosphorylation is mediated by MKK3 and MKK6 (4). Many transcription factor targets have been identified for each of the MAP kinases, with some individual transcription factors being phosphorylated at different sites by two different MAP kinases. This phosphorylation of transcription factors acts to facilitate or inhibit their association with other transcription factors, resulting in a change in the pattern of gene transcription. In addition to targeting transcription factors, MAP kinases can activate a group of kinases termed MAPK activated protein kinases (MAPKAPK, also abbreviated to MK) that provide a further level of regulation of the cellular response. Finally, an important mechanism regulating the duration of MAP kinase signalling is de-phosphorylation by a family of dual-specificity MAPK phosphatases (5).

2.2. MAP kinase pathways in renal physiology

MAP kinases play a critical role during foetal development as demonstrated by the embryonic death of mice with genetic deletion of ERK2 or p38alpha (6). In contrast, there is considerable redundancy between the two widely expressed JNK isoforms, JNK1 and JNK2, as illustrated by the viability of mice with deletion of a single JNK isoform compared to foetal death of combined JNK1/2 knock-out mice (6). However, these studies have provided little information regarding MAP kinases and the kidney since embryonic death occurs at an early stage of renal development. Tissue analysis has shown activation of all three MAP kinases during kidney development in the rat (7), and the addition of MAP kinase inhibitors to cultures of rat metanephroi has demonstrated a role for ERK in nephrogenesis and a role for p38 in kidney growth and nephrogenesis (8).

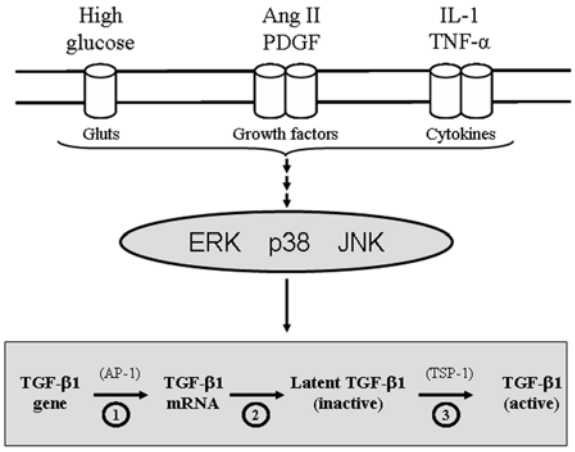

Figure 2. Role of MAP kinases in TGF-beta1 synthesis and activation. As described in Figure 1, cell stimulation by cytokines and growth factors can induce MAP kinase activation. In addition, hyperglycaemia can result in an increased intracellular glucose concentration via glucose transporters (Gluts). Several studies have shown that activation of the transcription factor, activator protein-1 (AP-1), through ERK and JNK signalling is required for increased TGF-beta1 gene transcription. In addition, ERK signalling has been shown to stabilise TGF-beta1 mRNA resulting in enhanced protein production. Finally, activation of the latent form of TGF-beta1 operates through proteases such as thrombospondin-1 (TSP-1). Angioteinsin II-induced TSP-1 production by mesangial cells resulting in TGF-beta1 activation is dependent upon p38 and JNK signalling.

The MAP kinases are widely expressed in many cell types in most tissues, with the kidney being no exception. There is little or no activation of these kinases in most normal tissues, with kinase activation being largely restricted to tissue stress or injury. Low levels of MAP kinase activation has been described in the normal human and rodent kidney based upon Western blotting and kinase assays (7, 9, 10). The use of immunohistochemistry to identify the cell types in which these kinases are activated have produced some conflicting results. While there is general agreement that phosphorylated-ERK (p-ERK) is restricted to collecting ducts, and possibly distal tubules, in normal human and rodent kidney (7, 10, 11), some disparate results have been obtained regarding the cellular localisation of p-p38 and p-JNK which is likely to be due to differences in the method of antigen retrieval employed or antibody cross-reactivity with phosphorylated peptides in other proteins. There is reasonable agreement that p38 and JNK activation is evident in collecting ducts and some tubules in normal kidney, with varying results described for glomerular cell activation of p38 and JNK (7, 12-16)

The function of MAP kinases in normal renal physiology is poorly understood and represents a potential limitation for therapeutic blockade of these pathways. A common theme of activation of all three MAP kinases in the collecting duct may represent a response to osmotic stress. The p38 kinase can be activated by hypertonicity and a functional role has been identified in protection against hypertonic damage. Mice heterozygote for the p38alpha gene (p38alpha +/-) appear normal but exhibit increased water intake and a higher kidney weight to total body weight ratio. These kidneys showed dilation of proximal convoluted tubules, vacuolar degeneration, focal interstitial fibrosis, and inflammation and enlargement of Bowman's capsule with advancing age (17). In response to hypertonic cell shrinkage, cells of the rat kidney medullary thick ascending limb of Henle's loop undergo regulatory volume increase through a p38 kinase-dependent reorganisation of the actin cytoskeleton (18). In addition, hypotonicity has been shown to stimulate renal epithelial sodium transport by activating JNK via receptor tyrosine kinases (19), and that synthesis of the Na-K-ATPase gamma-subunit is regulated at the transcriptional levels by JNK in inner medullary collecting duct cells (20). These studies point towards distinct roles for the different MAP kinases in renal physiology; however, an 8 week period of administration of a p38 kinase inhibitor to normal animals found no detrimental effects (21), although more subtle effects upon renal physiology may have been overlooked.

3. *IN VITRO* STUDIES OF THE PRO-FIBROTIC ACTIONS OF MAP KINASE SIGNALLING

It is well established that TGF-beta1 is a pri ncipal driver of renal fibrosis in most, if not all, forms of kidney disease (22). Control of TGF-beta1 function is complex involving regulation at multiple levels, including: gene transcription; enzymic activation of the inactive, latent form of TGF-beta1; expression of the cell surface receptors for TGF-beta1, and; receptor-driven activation of different intracellular signalling pathways (23, 24). MAP kinases can promote the fibrotic response through a number of different mechanisms, including; production and activation of TGF-beta1, regulating TGF-beta1-Smad signalling; and TGF-beta1 independent pro-fibrotic actions. These mechanisms are described below.

3.1. MAP kinase regulation of TGF-beta1 production and activity

A wide variety of factors implicated in causing renal injury can induce up-regulation of TGF-beta1 gene transcription, including angiotensin II, interleukin-1 (IL-1) and hyperglycemia. While a number of different signalling pathways are involved in the up-regulation of TGF-beta1 mRNA levels in renal cell types, there is good evidence that MAP kinases play an important role in TGF-beta1 production (see Figure 2). Indeed, the TGF-beta1 gene promoter contains functionally important binding sites for the transcription factor, activator protein-1 (AP-1) (25, 26). AP-1 is a dimeric transcription factor complex consisting of homo- and heterodimers of the Jun, Fos and activating transcription factor (ATF) family members (27). ERK and JNK play critical roles in the up-regulation and phosphorylation of c-fos and c-Jun to make active AP-1 (27), making these two kinases important regulators of TGF-beta1 gene transcription.

Angiotensin II promotes renal fibrosis though the up-regulation and activation of TGF-beta1 (28). All three

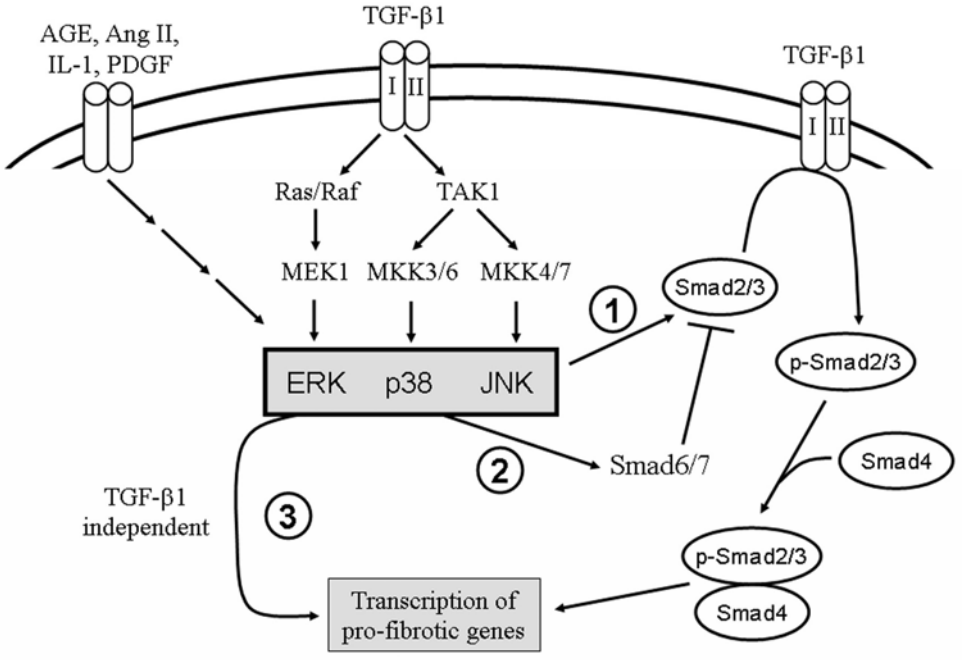

Figure 3. Cross-talk between MAP kinase and Smad signalling pathways. TGF-beta1 can directly activate all three MAP kinases via the TGF-beta1 type I receptor. TGF-beta1 activation of ERK operates via the conventional Ras/Raf pathway, while activation of p38 and JNK operates via TGF-beta activated kinase-1 (TAK1), a member of the MAPKKK family. TGF-beta1 induces phosphorylation of the receptor Smads (Smad2 and 3) which then combine with Smad4 and translocate to the nucleus and promote transcription of pro-fibrotic genes (e.g. collagen alpha (2)I, fibronectin, PAI-1). There are two main mechanisms of MAP kinase cross-talk with the Smad signalling pathway. (1) MAP kinases can phosphorylate the linker region of Smad2 and Smad3 which modifies the ability of Smad2/3 to combine with Smad4 and promote transcriptional activity. Alternatively, phosphorylated forms of c-Jun and JunB (via JNK activition) can bind to Smad3 and down-regulate Smad3-mediated gene transactivation. (2) MAP kinases are involved in up-regulation of the inhibitory Smads (Smad 6 and 7) which act to suppress TGF-beta1-Smad2/3 signalling. Finally, (3) factors such as advanced glycation end-products (AGE), angiotensin II, IL-1 and PDGF can activate MAP kinases and promote transcription of pro-fibrotic genes in a TGF-beta1 independent fashion through the action of transcription factors such as AP-1.

MAP kinase pathways, in addition to protein kinase C (PKC) and phosphoinositide-3 kinase (PI3K), are involved in angiotensin II induced TGF-beta production by glomerular mesangial cells (29, 30). In addition, the ability of angiotensin II to induce activation of latent TGF-beta1 in human mesangial cells operates through JNK and p38 dependent induction of thrombospondin-1 (29). Furthermore, angiotensin II-induced TGF-beta secretion by tubular epithelial cells is, in part, dependent upon JNK signalling (16).

Hyperglycaemia is an important inducer of TGF-beta1 and glomerulosclerosis in diabetic nephropathy and *in vitro* studies have identified a role for ERK and p38 kinases in high glucose induced up-regulation of TGF-beta1 mRNA in mesangial cells (31, 32). Pro-inflammatory cytokines, such as IL-1 and tumour necrosis factor-alpha (TNF-alpha), can also induce up-regulation of TGF-beta1 production, in part, via JNK signalling (33). In addition, TNF-alpha increases TGF-beta1 production in fibroblasts through an ERK-dependent mechanism involving stabilisation of TGF-beta1 mRNA (34). Furthermore, the ability of TGF-beta1 to up-regulate its own production (auto-induction) in tubular epithelial cells operates via distinct transcriptional and translational mechanisms involving ERK and p38, respectively (35).

3.2. Cross-talk between MAP kinases and TGF-beta1 signalling

3.2.1. TGF-beta1 activates MAP kinases

There is now ample evidence that all three major MAP kinases can be activated downstream of the TGF-β1 receptor and that MAP kinases can interact directly with Smad signalling, or act independently to promote the pro-fibrotic actions of TGF-beta1 (see Figure 3). The addition of TGF-beta1 to cultured cells results in a very rapid activation of all three MAP kinases. This rapid activation of MAP kinases can operate independent of Smad signalling based upon studies using cell types deficient in different Smad components or expressing a mutated TGF-beta1 type I receptor that cannot activate receptor Smads (36-38). Recent studies have shown that activation of TGF-beta type I receptor leads to the recruitment and direct phosphorylation of the adaptor protein ShcA, which is then able to associate with Grb2 adaptor and the Sos GTP-exchange factor, and thereby initiate the well-characterised

Ras/Raf pathway linking receptor tyrosine kinases with ERK activation (39). TGF-beta1 activation of the p38 and JNK pathways operates via an upstream MKKK family member called TGF-beta1 activated kinase-1 (TAK1) (40), although the intermediate steps involved in TAK1 activation by the TGF-beta type I receptor are unclear at present. The ability of TAK1 to phosphorylate MKK6 is directly regulated by polyubiquitination on lysine 63 of TAK1 (41). In addition, TAK1 binding proteins (TAB1 and TAB2) have been shown to enhance TAK1 activity (42). An important consideration is that TAK1 can also be activated by other cell surface receptors. For example, ligand binding to the IL-1 receptor or Toll-like receptor 4 leads to TAK1 dependent activation of p38, JNK and nuclear factor kappaB (43), linking TAK1 to the innate immune system.

3.2.2. MAP kinase modulation of Smad signalling

Interaction of receptor Smads (Smad2/3) with the activated TGF-beta1 type I receptor results in direct phosphorylation of these receptor Smads at the C-terminus. Phosphorylated Smad2/3 can then bind with Smad4 to make a complex which translocates to the nucleus and regulates gene transcription through physical interaction and functional cooperation with DNA binding transcription factors and coactivators. There is also negative feedback of this signaling pathway as TGF-beta1 induces up-regulation of the inhibitory Smads (Smad6/7) (38). MAP kinases can directly modify this signaling pathway by phosphorylation of specific residues in the linker region of Smad2/3 which regulate complex formation between p-Smad2/3 and Smad4 and subsequent nuclear translocation in a cell type dependent fashion. ERK-dependent phosphorylation of Smad2 increases Smad2 transcriptional activity (44), while ERK-dependent phosphorylation of Smad3 enhances TGF-beta1 dependent responses in human mesangial cells (45). TGF-beta1 induced p38 activation results in phosphorylation of Smad3 and an increase in matrix production by murine embryonic fibroblasts (46). Several studies have shown that TGF-beta1 activation of JNK results in phosphorylation of the linker region of Smad3 (36, 47, 48), and this JNK-dependent Smad3 phosphorylation has been implicated in TGF-beta1 induced migration of hepatic stellate cells (48). However, JNK activation can also be anti-fibrotic. The ability of pro-inflammatory cytokines IL-1 and TNF-alpha to inhibit TGF-beta/Smad signaling is JNK-dependent. This operates by JNK-mediated c-Jun and JunB phosphorylation which binds to Smad3, such that Jun family members down-regulate Smad3-mediated gene transactivation (49, 50). An additional mechanism of MAPK/Smad cross-talk is the recent demonstration that advanced glycation end-products and angiotensin II can induce Smad signaling independent of TGF-beta through the induction of ERK and p38 signaling (51, 52).

MAP kinase signaling can operate in an indirect fashion to modulate the TGF-beta1-Smad signaling pathway through at least two distinct mechanisms. Firstly, TGF-beta1 induced up-regulation of the inhibitory Smad7 is stimulated by JNK activity but inhibited by ERK activity (53). Secondly, the function of Smad4 is regulated in a post-translational fashion by sumoylation at lysine 159 located in the linker region. This sumoylation of Smad4 is strongly enhanced by TGF-beta-induced activation of the p38 kinase and is independent of Smad signalling (54).

3.3. MAP kinase regulation of fibroblast recruitment and differentiation

An initial step in the fibrotic response is the accumulation of fibroblasts at a site of tissue injury. This often involves fibroblast migration and proliferation. These cells can then undergo differentiation into myofibroblasts expressing alpha-SMA which are considered to play a key role in remodelling of the extracellular matrix (55). There are several potential sources of fibroblasts in the development of interstitial fibrosis; circulating fibrocytes, resident interstitial fibroblasts, pericytes and epithelial-mesenchymal transition (EMT) of tubular epithelial cells into myofibroblasts (56, 57). In addition, glomerular mesangial cells have many fibroblast-like characteristics, including extracellular matrix production, and undergo differentiation into alpha-SMA+ myofibroblasts following glomerular injury. There is good evidence to argue that mesangial cell proliferation and extracellular matrix production is an important early step leading to glomerulosclerosis (58, 59), leading to extensive study of the pro-fibrotic responses of cultured mesangial cells.

Fibroblast migration is a complex process dependent upon chemotactic stimuli, matrix-integrin interactions, cytoskeletal reorganisation, and degradation and remodelling of extracellular matrix (60). *In vitro*, platelet-derived growth factor (PDGF) induced migration of hepatic myofibroblasts operates via JNK signalling (61). Similarly, c-Jun phosphorylation by JNK is critical for fibroblast migration in cell wounding assays (62), while MEKK1-/- fibroblasts have reduced JNK activation and a reduced response in migration assays (63). In a different assay system, connective tissue growth factor (CTGF) induced ERK activation contributes to syndecan-4 dependent migration of murine embryonic fibroblasts (64).

Fibroblast proliferation is a prominent feature in active fibrosing lesions in different types of renal injury, including crescentic glomerulonephritis, the remnant kidney and the obstructed kidney (11, 65, 66). The signalling pathways regulating fibroblast proliferation *in vitro* are cell type and stimulus dependent with PDGF-induced proliferation of hepatic myofibroblasts operating via ERK but not JNK signalling (61), whereas plasmin-induced fibroblast proliferation operates via ERK and p38 kinases (67).

The differentiation of fibroblasts into myofibroblasts that express alpha-smooth muscle actin (alpha-SMA) and produce increased amounts of collagen is considered to be an important mechanism driving renal fibrosis (55). TGF-beta1 is a potent inducer of alpha-SMA in rat renal fibroblasts, via CTGF, leading to a cell type with increased synthesis of collagen I and III, but little cell proliferation (68). Studies in hepatic stellate cells show that TGF-beta1 induced expression of alpha-SMA operates via the p38 kinase (69), while all three MAP kinases are

involved in TGF-beta1 induced alpha-SMA expression in human fetal lung fibroblasts (70). TGF-beta1 induced differentiation of human gingival fibroblasts into alpha-SMA+ myofibroblasts is dependent upon JNK signalling (71). In another *in vitro* system, TGF-beta1 induced alpha-SMA expression in murine embryonic fibroblasts operates via p38 activation of its downstream target, MAP kinase-activated protein kinase-2 (MK-2) (72). Although, rather unexpectedly, MK-2 gene deficient mice develop more severe lung fibrosis in response to Bleomycin despite the presence of fewer alpha-SMA+ cells in the lung, with MK-2 deficient fibroblasts showing enhanced collagen production and proliferation, but reduced migration (73).

3.4. MAP kinase signalling in epithelial-mesenchymal transition

Epithelial cells have considerable phenotypic plasticity and undergo epithelial-mesenchymal transition (EMT) and mesenchymal-epithelial transition during foetal development. The EMT process can also be recapitulated in the adult in various pathologies, such as the invasive behaviour of epithelial tumours and in fibrotic diseases. EMT has been implicated in the pathogenesis of interstitial fibrosis in both human and experimental kidney disease (74-78), although some studies have failed to detect EMT *in vivo*. In addition, the transition of parietal epithelial cells into myofibroblasts has been implicated in fibrous progression of glomerular crescents (79, 80).

The growth factor TGF-beta1 plays a pivotal role in the induction of EMT. The addition of TGF-beta1 to cultured tubular epithelial cells is sufficient to induce phenotypic and morphologic transition to alpha-SMA+ myofibroblasts that are elongated, invasive with a new front-end back-end polarity (81). Other factors such as IL-1 and advanced glycation end-products can induce EMT in tubular epithelial cells in a TGF-beta1 dependent fashion (77, 82), while the TGF-beta1 inducible pro-fibrotic factor, CTGF, can directly induce EMT in tubular epithelial cells (83).

Given the well described ability of TGF-beta1 to activate all the major MAP kinases, it is not surprising that these kinases have been implicated in the process of EMT in tubular epithelial cells. TGF-beta1 induced EMT in tubular epithelial cell lines is dependent upon signalling through both ERK and p38 MAP kinase pathways (84, 85), while EMT induced in cultured tubular epithelial cells by myeloma light chains was shown to be independent of TGF-beta1, but dependent upon the p38 kinase (86). In addition, advanced glycation end-products and Oncostatin M can induce EMT in cultured tubular epithelial cells through activation of ERK signalling (87, 88). Studies using non-renal epithelial cells have also shown an important role for ERK and p38 kinases in TGF-beta1 induced EMT (37, 85, 89, 90). The JNK signalling pathway has also been implicated in TGF-beta1 induced EMT, although there is little data available in tubular epithelial cells. Blockade of JNK function via anti-sense oligonucleotides or the SP600125 drug was found to suppress TGF-beta1 induced EMT in mouse keratinocytes (91).

3.5. MAP kinase regulation of extracellular matrix production and deposition

The deposition of extracellular matrix seen in progressive glomerulosclerosis and interstitial fibrosis is the net result of increased matrix synthesis and modulation of the rate of matrix turnover. There is general agreement that mRNA and protein synthesis of extracellular matrix proteins is increased during the active phase of renal fibrosis, whereas the contribution of individual matrix metalloproteinases (MMPs) and tissue inhibitors of matrix metalloproteinases (TIMPs) to matrix turnover is a somewhat controversial topic.

The ability of TGF-beta1 to up-regulate extracellular matrix production by mesangial cells, renal fibroblasts and tubular epithelial cells *in vitro* is well established. This is directly related to the presence of functional AP-1 binding sites in the promoter regions of collagen alpha (2)I and fibronectin genes which are important for both basal and TGF-beta1 inducible matrix gene transcription (92-94). A series of studies have shown that both ERK and p38 kinase signalling contributes to TGF-beta1 stimulated fibronectin and collagen production by glomerular mesangial cells and renal fibroblasts (95-100). In HK2 tubular epithelial cells, TGF-beta-induced procollagen-1 alpha gene expression is dependent upon JNK signalling (101). Studies in cultured pulmonary fibroblasts have identified that pro-fibrotic actions of TGF-beta1 are dependent upon down-regulation of caveolin-1 (102). This reduction in caveolin-1 was blocked by the JNK inhibitor SP600125, but not in fibroblasts from *Jnk1* gene deficient mice, demonstrating redundancy between JNK1 and JNK2 in this novel mechanism of TGF-beta1 induced matrix production (102). In addition, some of the pro-fibrotic effects of TGF-beta1 are thought to be mediated via CTGF (103). Of relevance here is the finding that TGF-beta1 induced CTGF production in human renal proximal tubule epithelial cells requires both Ras/MEK/ERK and Smad signalling (104). Furthermore, TGF-beta induced CTGF production in human lung fibroblasts is suppressed by JNK blockade via anti-sense oligonucleotides (105). However, in contrast to the above studies, TGF-beta1 induced activation of the CTGF promoter in skin fibroblasts was found to be dependent upon ERK and Smad signaling and inhibited by c-Jun, identifying an anti-fibrotic action of JNK signaling (106).

The ability of other stimuli such as angiotensin II, high glucose, stretch and glycated albumin to increase fibronectin production by mesangial cells and renal fibroblasts is also dependent upon signalling via ERK and p38 pathways (31, 95, 100, 107-109), although in some of these studies it is difficult to distinguish between a direct action of MAP kinases and an indirect mechanism through the production of TGF-beta1. In addition, angiotensin II can induce Smad signalling in vascular smooth muscle cells via the p38 kinase and independent of endogenous TGF-beta1 (52).

Active fibrotic lesions feature not only an increase in matrix synthesis and deposition, but there is also an increase in matrix turnover although the relative

contribution of this matrix turnover to the pathogenesis of renal fibrosis is still poorly understood (110). Matrix is degraded by various types of protease enzymes, including the large family of MMPs and plasmin which are regulated by TIMPs and plasminogen activator inhibitor-1 (PAI-1), respectively. TGF-beta1 is a potent inducer of PAI-1 gene transcription in mesangial cells which operates via the ERK and JNK pathways and an AP-1 site in the PAI-1 gene promoter region (111, 112). PAI-1 gene transcription can also be induced by hypoxia through the p38 and JNK, but not ERK, signalling pathways (113). A pro-fibrotic role for PAI-1 has been proposed since PAI-1 gene deficient mice exhibit reduced fibrosis in the unilateral ureteric obstruction (UUO) model and in the early phase of diabetic nephropathy (114, 115). However, plasminogen gene deficient mice are unexpectedly protected from fibrosis in the UUO model, a finding attributed to plasmin induction of EMT in tubular epithelial cells via ERK signalling (116). Similarly, the lack of protection of TIMP-1 gene deficient mice in the UUO model argues for redundancy between different TIMP family members or the possibility that inhibition of intrinsic MMP activity does not constitute a profibrogenic event in the kidney (117).

While many *in vitro* studies have identified a requirement for MAP kinase signaling in the up-regulation of individual MMPs in mesangial cells, fibroblasts and macrophages (118-121), investigation of the function of the individual MMPs in renal fibrosis has thrown up some unexpected findings. For example, the expression levels of some MMPs are increased while others are decreased in models of renal fibrosis (110). MMP9 was found to play a protective role in experimental anti-GBM glomerulonephritis (122), although this may be due functions other than matrix degradation. In addition, over-expression of MMP2 in tubular epithelial cells induces chronic kidney disease with glomerulosclerosis and interstitial fibrosis (123), although the precise mechanisms by which this operates are yet to be delineated. Thus, on one hand MAP kinase signalling promoting MMP9 expression may be protective in renal fibrosis, while on the other hand MAP kinase induced MMP2 production may be detrimental (121, 124).

4. *IN VIVO* STUDIES OF MAP KINASE SIGNALLING IN ANIMAL MODELS OF RENAL FIBROSIS

In vitro studies have provided molecular insights into the mechanisms by which MAP kinase signalling modulates many different aspects of the fibrotic response, as summarised above. However, the findings in these studies are highly dependent upon the cell type examined, culture conditions selected and the stimulus employed. Therefore, it is critical to test these *in vitro* findings by modulating the function of the MAP kinases in experimental models involving glomerulosclerosis and interstitial fibrosis.

When examining animal models of kidney disease, it is important to remember that renal fibrosis is a response to an ongoing renal insult. In many situations the ongoing insult involves chronic inflammation, thus any treatment which can suppress the underlying inflammation will inevitably reduce fibrosis in an indirect fashion. This makes it difficult to examine the role of the p38 and JNK pathways in renal fibrosis since they play a pivotal role in the inflammatory response (2). Thus, the ability of p38 inhibitor drug treatment to suppress glomerulosclerosis and interstitial fibrosis in anti-GBM glomerulonephritis and adriamycin-induced nephropathy (13, 125-127), could be due to a direct anti-fibrotic effect or to an indirect consequence of blocking the inflammatory response. In this regard, one of the most useful models in which to study the role of MAP kinases in renal fibrosis is UUO. The increased hydrostatic pressure in the ureter results in rapid tubular dilatation, damage and atrophy. This is associated with the onset of tubular apoptosis and the development of significant interstitial myofibroblast accumulation and fibrosis within one week (128). Of importance, the factors implicated in the development of renal fibrosis in human kidney disease and in other experimental models of renal fibrosis are also involved in the UUO model, including angiotensin II, TGF-beta1 and CTGF (129-131). Renal fibrosis in this model is induced by a non-immune insult and is independent of the acquired immune response (132), although the potential role of macrophages in the development of renal fibrosis in this model is a controversial topic with highly disparate results depending upon the experimental strategy employed (129, 133-135). On this basis, we will mainly focus on the UUO model in our discussion on the role of MAP kinases in experimental renal fibrosis.

4.1. p38 MAP kinase in experimental renal fibrosis

There is a progressive increase in p38 activity in the obstructed kidney as quantified by Western blotting, while immunostaining identified p38 activation in both tubular epithelial cells and interstitial myofibroblasts (136). The role of the p38 MAP kinase in the rat UUO model was investigated using a specific inhibitor of p38alpha, NPC 31169. Treatment with this drug significantly reduced renal fibrosis which was associated with a reduction in collagen IV mRNA levels and a reduction in interstitial myofibroblast accumulation. Of note, p38 blockade did not affect the up-regulation of renal TGF-beta1 mRNA or protein levels in the UUO model; however, up-regulation of CTGF mRNA levels was prevented, arguing that the p38 kinase acts downstream of TGF-beta1 to drive matrix production in this model. In addition, p38 blockade had no effect upon interstitial macrophage infiltration (136). To examine which of the upstream kinases contribute to p38 signalling in renal fibrosis, we examined the UUO model in mice deficient for MKK3 (137). Despite a lower basal level of p38 activity in *Mkk3-/-* mice compared to wild type mice, there was still a significant increase in p38 activation in the *Mkk3-/-* obstructed kidney which was attributed to the compensatory increase in MKK6 expression seen in the *Mkk3-/-* kidney. Apoptosis of tubular and interstitial cells in the obstructed kidney was reduced by 50% in *Mkk3-/-* mice. However, the up-regulation of TGF-beta1 and CTGF mRNA and development of renal fibrosis in the UUO model was not altered in *Mkk3-/-* mice indicating redundancy between MKK3 and MKK6 in this response (137).

An increase in glomerular p38 activation has been described in the early phase of streptozotocin-induced diabetic nephropathy in rats (138), while an increase in p38 activation is evident in tubular and interstitial cells in the progressive phase of disease (15). Administration of a p38 inhibitor drug was shown to suppress albuminuria in rat diabetic nephropathy, although the effect on renal fibrosis was not examined (139).

In a rat model of established chronic allograft nephropathy, daily treatment with a p38 inhibitor was added to a suboptimal dose of cyclosporine therapy. This treatment suppressed glomerulosclerosis and interstitial fibrosis in parallel with a reduction in MCP-1 mRNA levels and macrophage infiltration, resulting in preserved renal function and prolonged survival (140). While this study demonstrates the utility of p38 inhibitor treatment in a clinically relevant model, it cannot differentiate whether the beneficial outcome of p38 blockade was due to an anti-inflammatory action or an anti-fibrotic action, or a combination of both.

Despite the promising effects of p38 inhibitor drugs in a wide range of kidney disease models, a note of caution is warranted as two studies have reported a detrimental effect of p38 blockade. Administration of a p38 inhibitor in a rat model of Heymann's nephritis exacerbated proteinuria, an effect attributed to a protective role for p38 signalling in podocyte injury induced by antibody plus complement (141). In the rat model of subtotal nephrectomy, treatment with a p38 inhibitor exacerbated renal dysfunction, proteinuria and glomerulosclerosis in association with increased activation of the ERK pathway (21).

4.2. JNK in experimental renal fibrosis

Activation of the JNK signalling pathway has been described in a number of kidney disease models (9, 142-144), but its role in renal fibrosis is only just beginning to be explored. A marked increase in JNK activation is evident in the UUO model based upon phosphorylation of JNK and phosphorylation of c-Jun at Serine 63, a specific JNK target (16). Immunostaining identified JNK activation in tubular epithelial cells and in interstitial cells. Administration of a JNK inhibitor, CC-401, selectively inhibited JNK signalling in the obstructed kidney and significantly inhibited renal fibrosis in terms of interstitial myofibroblast accumulation and collagen IV deposition. This effect was attributed to suppression of renal TGF-beta1 and CTGF mRNA levels. This provides an important contrast with the results of p38 kinase inhibition in this model (136), indicating that JNK but not p38 signalling is required for up-regulation of TGF-beta1 mRNA levels in the obstructed kidney. In addition, neither p38 or JNK blockade affected interstitial macrophage accumulation in the UUO model despite a reduction in interstitial fibrosis. JNK blockade also reduced tubular apoptosis in the obstructed kidney (16).

Both JNK1 and JNK2 isoforms are widely expressed in the kidney. Genetic deletion of either *Jnk1* or *Jnk2* did not prevent the marked activation of JNK signalling seen in the obstructed kidney, demonstrating redundancy between the two JNK isoforms. Deletion of either *Jnk1* or *Jnk2* failed to protect against the development of renal fibrosis in the UUO model; however, Jnk1 deletion did significantly reduce tubular cell apoptosis in the obstructed kidney (16). This is consistent with the finding that blockade of all JNK isoforms with CC-401 also reduced apoptosis of tubular epithelial cells in the UUO model (16).

An interesting finding is that genetic deletion of JunD, an inhibitory component of the AP-1 transcription factor that is phosphorylated by both JNK and ERK, makes mice susceptible to developing glomerulosclerosis and interstitial fibrosis following surgical ablation of 75% of the renal tissue (145). However, the physiological importance of this finding is unclear given that JNK inhibition has been shown to suppress renal injury and inflammation in a rat model of anti-GBM glomerulonephritis (144).

4.3. ERK in experimental renal fibrosis

An increase in ERK signalling has been described in a wide variety of animal models of kidney disease, including anti-GBM glomerulonephritis and Thy-1 mesangial proliferative nephritis (9, 146). Administration of the MEK1 inhibitor, U0126, suppressed ERK activation and mesangial proliferation in the rat Thy-1 model but failed to significantly reduce the increase in glomerular fibronectin deposition (146).

There is a dramatic increase in ERK activation in the obstructed kidney (11, 147). ERK activation (phosphorylation) is evident in tubular epithelial cells prior to the onset of tubular cell proliferation. In addition, ERK activation is seen in the many interstitial myofibroblasts suggesting a possible role for ERK in both cell proliferation and interstitial fibrosis (11). Administration of the MEK1 inhibitor, U0126, over days 2 to 5 in the UUO model was effective in suppressing ERK activation. This was associated with a significant reduction in the proliferation and accumulation of interstitial macrophages. However, U0126 treatment failed to suppress myofibroblast accumulation and the development of interstitial fibrosis in this model (148), arguing that ERK signalling does not play an important role in the pathogenesis of interstitial fibrosis *in vivo*.

5. MAPK SIGNALLING IN HUMAN RENAL FIBROSIS

All three of the major MAP kinases have been examined in cross-sectional studies of human kidney disease. These studies have utilised immunostaining to identify the phosphorylated (activated) forms of the individual kinases, or their down stream targets (phospho-c-Jun). These data provide clinical relevance for the *in vitro* and *in vivo* studies.

In normal human kidney, p-p38 immunostaining was found to be restricted to the nuclei of a small number of podocytes, parietal epithelial cells, and

tubular cells (14). There was a dramatic increase in the number of p-p38-positive cells in glomeruli and tubules in both non-proliferative and proliferative forms of glomerulonephritis, and a substantial increase in the number of interstitial p-p38-positive cells in proliferative glomerulonephritis. Double immunostaining identified p38 activation in intrinsic renal cells and infiltrating macrophages. In particular, p38 activation was evident in alpha-SMA+ myofibroblasts in the glomerular tuft, in fibrocellular crescents and in areas of interstitial fibrosis in diseases such as focal and segmental glomerulosclerosis (FGS) and lupus nephritis (14). The degree of renal dysfunction and proteinuria correlated with the number of p-p38+ cells. Furthermore, glomerular p38 activation correlated with segmental proliferative and necrotic lesions, and interstitial p38 activation correlated with the degree of interstitial inflammation. However, p38 activation in interstitial cells did not correlate with interstitial fibrosis which probably reflects the chronic, acellular nature of the fibrosis in many of these biopsies (14). In a separate study of human crescentic glomerulonephritis, it was found that p-p38 MAPK-positive cells were mainly detected in crescentic lesions, tubular epithelial cells, and interstitial mononuclear infiltrates. The number of p-p38 MAPK-positive glomerular cells correlated with glomerular crescents and macrophage accumulation (149), although renal fibrosis was not specifically examined.

Immunostaining in biopsy specimens identified an increase in the number of p-p38+ cells in glomeruli, tubules and the interstitium in human diabetic nephropathy (15). Most interstitial p-p38+ cells were identified as alpha-SMA+ myofibroblasts. The number of patients examined was too small to allow correlation analysis; however, studies in two different mouse models of diabetic nephropathy found a significant correlation between interstitial p-p38+ cells and interstitial myofibroblasts accumulation and deposition of collagen IV (15). In another study of diabetic nephropathy, Sakai *et al* (150) used immunostaining to show p-38 activation in glomerular cells, tubular epithelial cells, and mononuclear infiltrates in the interstitium. The number of p-p38+ glomerular cells did not correlate with glomerular lesions. However, the number of tubulointerstitial p-p38+ cells reflected the severity of tubulointerstitial lesions in patients with diabetic nephropathy (150). These two studies suggest that activation of p38 kinase signalling promotes the development of interstitial fibrosis in diabetic nephropathy.

Activation of JNK signalling has been examined in normal and diseased human kidney using immunostaining for p-c-Jun (101). Staining for p-c-Jun was largely absent from normal human kidney but was present in many glomerular and tubular cells in a wide range of human kidney diseases, including minimal change disease, FGS and diabetic nephropathy. The number of p-c-Jun+ glomerular cells correlated with the degree of glomerulosclerosis, while tubulointerstitial p-c-Jun staining correlated with interstitial fibrosis and renal dysfunction (101). These data, taken together with the ability of a JNK inhibitor to suppress interstitial fibrosis in the UUO model (16), provide a strong argument for JNK signalling as a major mechanism driving renal fibrosis.

A study of ERK activation in renal biopsies found that p-ERK immunostaining was restricted to the collecting ducts in normal human kidney (10). In glomerulopathies, glomerular ERK activation was highly variable. However, there was co-localization of cell proliferation and ERK activation in the glomerular tuft and crescents. ERK activation was prominent in tubules and interstitial cells in areas of tubulointerstitial damage. In particular, ERK activation was observed in glomerular and interstitial alpha-SMA+ myofibroblasts. There was a significant correlation between ERK activation and cell proliferation in the glomerulus and in tubules, while tubular ERK activation correlated with renal dysfunction and interstitial fibrosis and tubular atrophy (10). These results argue that ERK signaling may contribute to the development of renal fibrosis through the proliferation of glomerular and interstitial myofibroblasts.

6. THERAPEUTIC POTENTIAL FOR TARGETING MAP KINASES IN RENAL FIBROSIS

The ability of small molecule inhibitors of JNK and p38 kinases to suppress renal fibrosis in animal disease models, together with evidence implicating these two pathways in the development of renal fibrosis in human kidney disease, provides a strong rationale for therapeutic targeting of these kinases. In addition to the systemic administration of small molecule kinase inhibitors, which is considered below, emerging technologies may allow alternative therapeutic strategies. Administration of RNA interference (RNAi) either as naked RNA, conjugated/complexed RNA or RNAi in plasmid or viral vectors has been shown to knock-down expression of selected molecules in a variety of animal disease models (151). Although there are a number of hurdles to overcome for clinical use, the rapidly moving technology of RNAi does suggest the potential for targeted knock-down of specific MAPK in individual cell types as a strategy to inhibit renal fibrosis.

The role of MAP kinases in normal renal physiology remains unclear at present. Short term administration of ERK, p38 of JNK inhibitors to normal rats and mice has no deleterious effects, but long term administration of the drugs beyond 8 weeks has not been reported. This is important since renal fibrosis is a chronic disease progress which will require relatively long term treatment. In addition, the foetal death of MAP kinase gene knock-out mice makes it imperative to avoid MAP kinase inhibitors during pregnancy. One other concern is that most MAP kinase inhibitors currently in use are reversible, competitive inhibitors of the ATP-binding pocket of the activated kinase which interfere with phosphorylation of specific targets. However, the similarity between the ATP binding pockets of different kinases makes it difficult to develop a drug that selectively targets just one of the more than 500 kinases in the genome (152).

There are a number of limitations which make it unlikely that ERK inhibitors will be used in the treatment of renal fibrosis. First, there is a lack of clear animal data to functionally link ERK signalling to progressive renal fibrosis. Second, most current ERK inhibitors have poor solubility and bioavailability which severely limits their study in animal disease models. Third, the well established role of ERK in cell proliferation makes it unclear whether this will be beneficial in terms of suppressing inflammation and fibrosis, or detrimental if blocking ERK suppresses the repair response in the injured kidney. However, there are other potential therapeutic applications for ERK inhibitors such as in the treatment of polycystic kidney disease based upon recent successful studies in the pcy mouse model (153).

A number of companies have developed specific p38 inhibitors which are soluble with good bioavailability. A series of studies have shown highly beneficial effects of p38 inhibitors in animal models which make therapeutic targeting of the p38 kinase an attractive option. However, this needs to be tempered with this some negative findings in which detrimental effects of p38 blockade were identified in animal models of Heymannn's nephritis and subtotal nephrectomy (21, 141), and the development of renal abnormalities in p38alpha+/- mice (17). Currently, p38 inhibitors are in clinical trials of rheumatoid arthritis and Crohn's disease, but some toxicity (increased liver enzyme levels and dizziness) has been reported which has slowed clinical development (154). Thus, clinical application of p38 inhibitors in renal fibrosis may be some years away.

The development of specific JNK inhibitors has lagged behind that of ERK and p38 inhibitors. CEP1347 is a drug that inhibits MLK3 that results in suppression of JNK, and to a lesser extent, p38 kinase signaling (155). CEP1347 has been successful in suppressing various non-renal models of inflammation (154). Importantly, this drug was well tolerated in a phase I study of Parkinson's Disease and subsequently was well tolerated in a 21.4 month clinical trial in Parkinson's disease with no adverse effects identified (156), which provides important data on the potential for long term JNK blockade in patients. The development of a soluble and specific JNK inhibitor, CC-401 (157), that can suppress animal models of renal inflammation and renal fibrosis (16, 144) is an exciting prospect for therapeutic treatment of renal fibrosis; however, this drug and other specific JNK inhibitors will need to be proven safe and effective in clinical trials.

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