Transforming Growth Factor-β1 Stimulates Vascular Endothelial Growth Factor 164 via Mitogen-activated Protein Kinase Kinase 3-p38α and p38δ Mitogen-activated Protein Kinase-dependent Pathway in Murine Mesangial Cells*

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Transforming growth factor-β1 (TGF-β1) is a potent inducer of extracellular matrix synthesis leading to progressive glomerular fibrosis. The intracellular signaling mechanisms involved in this process remain incompletely understood. The p38 mitogen-activated protein kinase (MAPK) is a major stress signal transducing pathway that is rapidly activated by TGF-β1 in mesangial cells. We have previously demonstrated MKK3 as the immediate upstream MAPK kinase required for selective activation of p38 MAPK isoforms, p38α and p38δ, and stimulation of pro-α1(1) collagen by TGF-β1 in murine mesangial cells. In this study, we further sought to determine MAPK kinase 3 (MKK3)-dependent TGF-β1 responses by gene expression profiling analysis utilizing mesangial cells isolated from Mkk3(−/−) mice compared with Mkk3(+/+) controls. Interestingly, vascular endothelial growth factor (VEGF) was identified as a TGF-β1-induced gene affected by deletion of Mkk3. VEGF is a well known endothelial mitogen, whose actions in nonendothelial cell types are still not well understood. We confirmed that TGF-β1 increased VEGF mRNA and protein synthesis of VEGF164 and VEGF188 isoforms in wild-type mesangial cells. However, in the Mkk3(−/−) mesangial cells, both TGF-β1-induced VEGF mRNA and VEGF164 protein expression were inhibited, whereas TGF-β1-induced VEGF188 protein expression was unaffected. Furthermore, transfection of dominant negative mutants of p38α and p38δ resulted in marked inhibition of TGF-β1-induced VEGF164 expression but not VEGF188, and treatment with recombinant mouse VEGF164 increased collagen and fibronectin mRNA expression in mesangial cells. Taken together, our findings suggest a critical role for the Mkk3-p38α and p38δ MAPK pathway in mediating VEGF164 isomorph-specific stimulation by TGF-β1 in mesangial cells. Further, VEGF164 stimulates collagen and fibronectin expression in mesangial cells and thus in turn enhances TGF-β1-induced extracellular matrix and may play an important role in progressive glomerular fibrosis.

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Mitogen-activated protein kinases (MAPKs)1 constitute a family of serine/threonine kinases that are central in the signal transduction cascades regulating a wide array of intracellular processes such as cell growth, differentiation, apoptosis, and cellular responses to external stress signals (1). Three major subfamilies of MAPKs have been identified in mammalian cells and include the extracellular signal-regulated kinase (ERK), the c-Jun N-terminal kinase (JNK), and the p38 MAPK (2). These protein kinases share ~60–70% identity, but differ in the sequence and size of their activation loop, and are activated by different stimuli, and each MAPK subfamily consists of several isoforms and members, which often have distinct functions. Activation of the MAPK signaling cascade involves a series of three protein kinases consisting of a MAPK that is activated by the dual phosphorylation of Thr and Tyr residues in a TXY motif by specific upstream MAPK kinases (MKKs), which in turn are phosphorylated by MAPK kinase kinases. The TXY motif is unique to each of the MAPK subfamilies, where X is Glu, Pro, and Gly in ERK, JNK, and p38 MAPK, respectively (2). In general, ERK1 and ERK2, also known as p44 and p42 MAPKs, are prototypically activated by mitogenic stimuli and growth factors, whereas JNK and p38 MAPK are activated predominantly by environmental stresses such as osmotic changes, ultraviolet light, heat shock, and inflammation (2–5). More recent investigations including ours have revealed that p38 MAPK is also activated by a variety of cytokines including transforming growth factor-β1 (TGF-β1) (6–9).

TGF-β1 is a pleiotropic cytokine that regulates multiple cellular functions including cell proliferation, differentiation, and apoptosis (10, 11). TGF-β1 also plays a key role in progressive diseases as a potent inducer of extracellular matrix (ECM) protein synthesis and progressive tissue fibrosis, including the kidney (12–14). There is now increasing body of evidence to provide support for the involvement of p38 MAPK pathway in mediating TGF-β1 signals. For instance, activation of p38 MAPK has been demonstrated in various disease models such as inflammation, septic shock, ischemia, ischemia-reperfusion, vascular injury, and pulmonary fibrosis (15–17). Moreover, specific inhibitors of p38 MAPK were shown to attenuate disease severity in these studies. In the kidney, increased p38 MAPK activation has been demonstrated in ischemic and ischec-
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reperfused rat kidneys and in glomeruli isolated from rats with experimental proliferative glomerulonephritis and diabetic nephropathy, and administration of a specific p38 MAPK inhibitor ameliorated acute glomerulonephritis (18–21).

We have previously demonstrated that TGF-β1 rapidly and strongly activated p38 MAPK in cultured glomerular mesangial cells from the rat and mouse (8, 9). Activation of the p38 MAPK involves phosphorylation by upstream MKKs in the protein kinase Cε and includes MKK3, MKK6, and possibly MKK4 (22). Our previous studies have also demonstrated MKK3 as the immediate upstream MAPK kinase required for selective activation of p38 MAPK isoforms, p38α and p38β, and stimulation of pro-α(I) collagen by TGF-β1 in mouse mesangial cells (9). In the present study, we further sought to determine MKK3-dependent TGF-β1 responses by gene expression profiling analysis utilizing mesangial cells isolated from MKK3-null (Mkk3−/−) mice compared with wild-type (Mkk3+/+) control mice. Interestingly, vascular endothelial growth factor (VEGF) was identified as a TGF-β1-induced gene affected by the deletion of Mkk3 in mesangial cells. VEGF is a well-known endothelial mitogen that plays an important role in angiogenesis, wound repair, tumorigenesis, and vascular diseases (23, 24). However, its actions in nonendothelial cell types are still not well understood. Moreover, the molecular mechanism responsible for isoform-specific VEGF gene regulation are not known. Our data provide novel evidence that this occurs through activation of distinct intracellular signaling molecules. Here we report that the MKK3-p38α and p38β MAPK pathway is required in mediating VEGF₁₄₄ isoform-specific stimulation by TGF-β1 in mouse mesangial cells. Further, VEGF₁₄₄ induces collagen and fibronectin expression in mouse mesangial cells and thus in turn enhances TGF-β1-induced ECM and may play an important role in progressive glomerular fibrosis.

EXPERIMENTAL PROCEDURES

Reagents—Recombinant human TGF-β1 was purchased from R & D Systems (Minneapolis, MN). VEGF (147) rabbit polyclonal antibodies and α-tubulin antibodies were obtained from Santa Cruz Biotechnology. Mouse recombinant VEGF₁₄₄ was purchased from Sigma. The specific inhibitor of p38 MAPK, SB203580, was purchased from Calbiochem (San Diego, CA). Geneticin® (G418 sulfate) and LipofectAMINE Plus™ reagent were obtained from Invitrogen.

Murine Mesangial Cell Culture—Glomerular mesangial cells were isolated and characterized as previously described, from glomeruli of MKK3-null (Mkk3−/−) mice and wild-type (Mkk3+/+) control mice and from C57BL/6 mice, using differential sieving technique with the following modifications (9). Following collagenase digestion, the cells were plated in RPMI 1640 medium (Mediatech) supplemented with 20% FBS (Bio-Whittaker), insulin (10 μg/ml), 5 units/ml penicillin, and 5 μg/ml streptomycin and incubated in a humidified atmosphere of 5% CO₂ and 95% air at 37 °C. Using this technique, we have been successful in establishing homogeneous cultures of glomerular mesangial cells that immunostain for anti-vimentin (Dako) and anti-myosin antibodies (Zymed Laboratories Inc. and negative staining for cytokeratin (Roche Applied Science) and von Willebrand’s factor (Dianova) as well as negative fluorescent acetylated low density lipoprotein uptake (Biomedical Technologies Inc.). Cells between 7 and 16 passages were used for the experiments. The targeted disruption of the Mkk3 gene by homologous recombination and the generation of Mkk3−/− mice were as previously described (9).

To identify the cells established in culture, they were maintained in RPMI 1640 medium with 15% FBS, 5 units/ml penicillin, and 5 μg/ml streptomycin. In experiments involving treatment with exogenous TGF-β1 (2 ng/ml) or VEGF (100 ng/ml), the cells grown to confluence were rendered quiescent in serum-free medium for 24 h, followed by treatment with human TGF-β1 (2 ng/ml) (R & D Systems) or VEGF (100 ng/ml) (Sigma). In experiments using the p38 MAPK inhibitors, the cells were incubated for 1 h in the absence or presence or pretreatment with 10 μM SB203580, prior to treatment with or without exogenous TGF-β1 or VEGF₁₄₄.

Results

Identification of VEGF as a TGF-β1-induced Gene Affected by the Deletion of Mkk3 in Murine Mesangial Cells—To identify
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TGF-β1 stimulates VEGF<sub>164</sub> via MKK3-p38-dependent pathway. We performed gene expression profiling analysis utilizing mesangial cells isolated from <i>Mkk3<sup>−/−</sup></i> mice compared with <i>Mkk3<sup>+/+</sup></i> controls. We screened a high density cDNA array (Clontech Atlas<sup>TM</sup> mouse 1.2 array) with labeled cDNA obtained from MKK3-deficient (<i>Mkk3<sup>−/−</sup></i>) and wild-type (<i>Mkk3<sup>+/+</sup></i>) mesangial cells incubated in the absence or presence of exogenous TGF-β1 (2 ng/ml). One gene that was observed to be consistently induced by TGF-β1 in wild-type (<i>Mkk3<sup>+/+</sup></i>) mesangial cells but not in MKK3-deficient (<i>Mkk3<sup>−/−</sup></i>) mesangial cells was VEGF (Fig. 1).

**FIG. 1. Effect of deletion of Mkk3 on TGF-β1 induced gene expression in murine mesangial cells.** cDNA microarray analysis of the relative levels of gene expression in wild-type (<i>Mkk3<sup>+/+</sup></i>) and MKK3-deficient (<i>Mkk3<sup>−/−</sup></i>) mouse mesangial cells treated with exogenous TGF-β1 (2 ng/ml) for 6 h. 32P-Labeled cDNA probes generated from reverse transcription of DNase-treated RNA were hybridized to the Atlas<sup>TM</sup> mouse 1.2 arrays containing 1176 mouse genes (Clontech) as described under “Experimental Procedures.” The position of VEGF cDNA is indicated by the arrow. The relative expression of housekeeping genes served to normalize gene expression levels, and one (glycerol-aldehyde-3-phosphate dehydrogenase) is indicated by the arrowhead.

To confirm that the <i>Mkk3<sup>−/−</sup></i> mesangial cells were completely abrogated in MKK3-deficient (<i>Mkk3<sup>−/−</sup></i>) mesangial cells (data not shown).

**FIG. 2. Stimulation of VEGF mRNA expression by TGF-β1 in murine mesangial cells.** Wild-type mouse mesangial cells were incubated in the absence (<i>Ctl</i>) or in the presence of exogenous TGF-β1 (2 ng/ml) for the indicated time periods. Total RNA was extracted and subjected to Northern blot hybridization with 32P-labeled cDNA probe corresponding to VEGF, as described under “Experimental Procedures.” 18 S rRNA hybridization signals served as normalization for RNA loading.

**FIG. 3. Time course of TGF-β1-stimulated VEGF protein synthesis in murine mesangial cells.** Wild-type mouse mesangial cells were incubated in the absence (<i>Ctl</i>) or in the presence of exogenous TGF-β1 (2 ng/ml) for the indicated time periods. Total cell lysates were isolated and subjected to Western blot analyses using polyclonal anti-VEGF antibodies, as described under “Experimental Procedures.” As loading controls, the same cell lysates were subjected to immunoblotting with mouse monoclonal anti-α-tubulin antibodies.

**FIG. 4. Inhibition of TGF-β1-stimulated VEGF gene expression in MKK3-deficient murine mesangial cells.** Total RNA isolated from wild-type (<i>Mkk3<sup>+/+</sup></i>) and MKK3-deficient (<i>Mkk3<sup>−/−</sup></i>) mouse mesangial cells incubated in the absence (<i>Ctl</i>) or in the presence of exogenous TGF-β1 (2 ng/ml) for the indicated time periods were subjected to Northern blot hybridization with 32P-labeled cDNA probe corresponding to VEGF. 18 S rRNA hybridization signals served as normalization for RNA loading.

exogenous TGF-β1 (2 ng/ml) resulted in increased protein expression of VEGF isoforms VEGF<sub>164</sub> and VEGF<sub>188</sub> as expected in wild-type (<i>Mkk3<sup>+/+</sup></i>) mesangial cells. In contrast, in MKK3-deficient (<i>Mkk3<sup>−/−</sup></i>) mesangial cells, TGF-β1 treatment resulted in increased expression of the VEGF<sub>188</sub> protein but not the VEGF<sub>164</sub> isof orm. The previously observed TGF-β1 stimulated VEGF<sub>164</sub> isoform expression in the wild-type mesangial cells was completely abrogated in MKK3-deficient (<i>Mkk3<sup>−/−</sup></i>) mesangial cells. A higher concentration of exogenous TGF-β1 (10 ng/ml) also failed to induce VEGF<sub>164</sub> isoform expression in MKK3-deficient (<i>Mkk3<sup>−/−</sup></i>) mesangial cells (data not shown).

**Deletion of Mkk3 Inhibits TGF-β1-induced VEGF Gene Expression in Murine Mesangial Cells**—To confirm that the TGF-β1 induction of VEGF is a MKK3-dependent TGF-β1 response, we examined whether TGF-β1-induced VEGF gene expression was affected by targeted disruption of the <i>Mkk3</i> gene utilizing MKK3-deficient (<i>Mkk3<sup>−/−</sup></i>) and wild-type (<i>Mkk3<sup>+/+</sup></i>) mesangial cells incubated in the absence or presence of exogenous TGF-β1. As shown in Fig. 4, treatment with exogenous TGF-β1 (2 ng/ml) resulted in increased VEGF mRNA expression within 4 h in wild-type (<i>Mkk3<sup>+/+</sup></i>) mesangial cells. However, in MKK3-deficient (<i>Mkk3<sup>−/−</sup></i>) mesangial cells, TGF-β1-induced VEGF mRNA expression was markedly inhibited.

**MKK3 Is Required for Induction of VEGF<sub>164</sub> Isoform by TGF-β1 in Murine Mesangial Cells**—We also examined the effects of MKK3 deficiency on TGF-β1-stimulated protein synthesis of VEGF isoforms. As shown in Fig. 5, treatment with TGF-β1 (2 ng/ml) for 6 h resulted in increased VEGF<sub>164</sub> expression as early as after 4 h of treatment in mesangial cells and up to 24 h after TGF-β1 treatment.

**FIG. 5. Time course of TGF-β1-stimulated VEGF protein synthesis in murine mesangial cells.** Wild-type mouse mesangial cells were incubated in the absence (<i>Ctl</i>) or in the presence of exogenous TGF-β1 (2 ng/ml) for the indicated time periods. Total cell lysates were isolated and subjected to Western blot analyses using polyclonal anti-VEGF antibodies, as described under “Experimental Procedures.” As loading controls, the same cell lysates were subjected to immunoblotting with mouse monoclonal anti-α-tubulin antibodies.

Deletion of <i>Mkk3</i> Inhibits TGF-β1-induced VEGF Gene Expression in Murine Mesangial Cells—To confirm that the TGF-β1 induction of VEGF is a MKK3-dependent TGF-β1 response, we examined whether TGF-β1-induced VEGF gene expression was affected by targeted disruption of the <i>Mkk3</i> gene utilizing MKK3-deficient (<i>Mkk3<sup>−/−</sup></i>) and wild-type (<i>Mkk3<sup>+/+</sup></i>) mesangial cells incubated in the absence or presence of exogenous TGF-β1. As shown in Fig. 4, treatment with exogenous TGF-β1 (2 ng/ml) resulted in increased VEGF mRNA expression within 4 h in wild-type (<i>Mkk3<sup>+/+</sup></i>) mesangial cells. However, in MKK3-deficient (<i>Mkk3<sup>−/−</sup></i>) mesangial cells, TGF-β1-induced VEGF mRNA expression was markedly inhibited.

**MKK3 Is Required for Induction of VEGF<sub>164</sub> Isoform by TGF-β1 in Murine Mesangial Cells**—We also examined the effects of MKK3 deficiency on TGF-β1-stimulated protein synthesis of VEGF isoforms. As shown in Fig. 5, treatment with TGF-β1 (2 ng/ml) resulted in increased VEGF<sub>164</sub> expression as early as after 4 h of treatment in mesangial cells and up to 24 h after TGF-β1 treatment. **FIG. 6. Inhibition of TGF-β1-stimulated VEGF gene expression in MKK3-deficient murine mesangial cells.** Total RNA isolated from wild-type (<i>Mkk3<sup>+/+</sup></i>) and MKK3-deficient (<i>Mkk3<sup>−/−</sup></i>) mouse mesangial cells incubated in the absence (<i>Ctl</i>) or in the presence of exogenous TGF-β1 (2 ng/ml) for the indicated time periods were subjected to Northern blot hybridization with 32P-labeled cDNA probe corresponding to VEGF. 18 S rRNA hybridization signals served as normalization for RNA loading.

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inhibition of VEGF164 isoform induction by TGF-β1, an inhibitor of p38 signaling pathway through chemical inhibition using exogenous TGF-β1 in murine mesangial cells. As shown in Fig. 6, following treatment with exogenous TGF-β1 (2 ng/ml) increased protein levels of VEGF164 were observed in mesangial cells stably transfected with p38α dnm or p38β dnm, as well as in empty vector (pcDNA3.1) transfected control cells. In contrast, TGF-β1-stimulated VEGF164 isoform observed in the empty vector (pcDNA3.1) control cells was markedly inhibited in mesangial cells stably transfected with p38α dnm or p38β dnm.

**Dominant Negative Mutants of p38α and p38β Inhibits Induction of VEGF164 Isoform by TGF-β1 in Murine Mesangial Cells**—Our previous study had demonstrated that MKK3 is the upstream MAPK kinase for the selective activation of p38α and p38β MAPK isoforms by TGF-β1 in murine mesangial cells (9). Here we further investigated whether p38α and/or p38β were involved in mediating the TGF-β1-stimulated VEGF expression in murine mesangial cells. We employed an approach of stable transfection of dominant negative mutants of p38α or p38β (p38α dnm and p38β dnm) to block the p38 MAPK signaling pathway, in an isoform-specific fashion, in wild-type murine mesangial cells. As shown in Fig. 6, following treatment with exogenous TGF-β1 (2 ng/ml) increased protein levels of VEGF164 were observed in mesangial cells stably transated with p38α dnm or p38β dnm, as well as in empty vector (pcDNA3.1) transfected control cells. In contrast, TGF-β1-stimulated VEGF164 isoform observed in the empty vector (pcDNA3.1) control cells was markedly inhibited in mesangial cells stably transfected with p38α dnm or p38β dnm.

**p38 MAPK Inhibitor SB 203580 Inhibits Induction of VEGF164 Isoform by TGF-β1 in Murine Mesangial Cells**—We next utilized an alternative approach to block the p38 MAPK signaling pathway through chemical inhibition using SB203580, an inhibitor of p38α and p38β activation. As seen in Fig. 7, in wild-type murine mesangial cells, dose-dependent inhibition of the TGF-β1-stimulated VEGF164 isoform was observed after pretreatment with p38 MAPK inhibitor SB203580. Given that SB203580 specifically inhibits p38α and p38β, but the other p38 isoforms including p38δ are insensitive to inhibition by SB203580, we examined the combined effects of SB203580 and overexpression of dominant negative mutant p38δ in cultured wild-type murine mesangial cells. Fig. 8 demonstrates that pretreatment of SB203580 in mesangial cells stably transfected with p38δ dnm resulted in a more complete inhibition of VEGF164 isoform induction by TGF-β1, compared with similar pretreatment of SB203580 in empty vector (pcDNA3.1)-transfected control cells.

**Recombinant Mouse VEGF164 Induces ECM Genes in Murine Mesangial Cells**—It is well known that VEGF is an endothelial mitogen, but little is known about its actions in nonendothelial cell types, including glomerular mesangial cells. Here we used recombinant mouse VEGF164 to investigate its effects in murine mesangial cells. The effects of VEGF164 on the expression of ECM genes, namely collagen α1(I) and fibronectin and PAI-1, were examined by Northern analyses. As shown in Fig. 9, treatment with recombinant mouse VEGF164 (100 ng/ml) resulted in increased expression of collagen α1(I) and fibronectin mRNA in cultured wild-type murine mesangial cells. Both collagen and fibronectin are known to be potently induced by TGF-β1 in mesangial cells.

**DISCUSSION**

Although it is known that TGF-β1 is a potent inducer of ECM synthesis and is largely regarded as a key mediator in the pathogenesis of renal fibrosis, the intracellular signaling mechanisms involved in this process remain incompletely understood. The MKK3-p38 MAPK is emerging as a major stress signal transducing pathway activated by TGF-β1 and implicating an important role in mediating TGF-β1 signals. Our previous studies have demonstrated that TGF-β1 rapidly and strongly activated p38 MAPK and its immediate upstream MAPK kinase, MKK3, in cultured glomerular mesangial cells, a major target cell type is a variety of renal glomerular injury (8, 9). In the present study, gene expression profiling analysis, utilizing mesangial cells isolated from Mkk3−/− mice compared with Mkk3+/+ controls, have identified VEGF as a MKK3-dependent TGF-β1 target gene in mesangial cells (Fig. 1).

VEGF was initially described as a vascular permeability factor and subsequently as an endothelial mitogen (26–29). VEGF has been shown to act a key regulator of physiological angiogenesis, such as in embryogenesis, as well as pathological...
angiogenesis, most notably neovascularization in tumorigenesis (24, 30). However, besides these well documented activities, VEGF can also exert other certain effects such as promoting atherosclerotic plaque development and inflammatory response through monocyte activation and migration (31, 32). Indeed, although endothelial cells are generally thought to be the primary target of VEGF, nonendothelial cell types express VEGF and its receptors and can be targets of VEGF actions. Aortic smooth muscle cells, which play a pivotal role in the pathogenesis of atherosclerosis, express and secrete VEGF, and hypoxia and hypoglycemia have been demonstrated to stimulate VEGF expression in these cells (33, 34). Enhanced VEGF expression was also observed in retinal pigment epithelial cells stimulated by hypoxia and by advanced glycation products and in osteoblasts that are thought to promote bone formation (35–37). Renal glomerular expression of VEGF has also been demonstrated, for instance, in glomerular visceral epithelial cells where VEGF may have a role in the induction of proteinuria in renal diseases (38). Both VEGF and VEGF receptors were up-regulated in kidneys of diabetic rats, and administration of monoclonal anti-VEGF antibodies improved early renal dysfunction, as assessed by decreased hyperfiltration, albuminuria, and glomerular hypertrophy, in experimental diabetes, suggesting a role in the pathogenesis of diabetic nephropathy (39, 40). Up-regulation of VEGF expression in the glomerular mesangium has also been reported in human and experimental mesangioproliferative glomerulonephritis (41, 42). It is noteworthy that in both of these pathological processes, TGF-β1 is thought to be a key mediator. Our present findings confirm that glomerular mesangial cells express VEGF mRNA and, further, that stimulation with exogenous TGF-β1 strongly induces the expression of VEGF in a time-dependent fashion (Fig. 2).

VEGF, also known as VEGF-A, in its native form is a disulfide-linked homodimeric glycoprotein of 34–46 kDa in molecular size (30). At least six human VEGF isoforms (VEGF121, VEGF145, VEGF165, VEGF183, VEGF189, and VEGF206) generated from alternative splicing of the VEGF mRNA have been identified (30, 43). VEGF121 is a freely soluble protein; VEGF165, the major isoform, is partially secreted, whereas VEGF189 and VEGF206 are almost completely sequestered in the extracellular matrix. VEGF145 and VEGF183 are less frequent splice variants (44). Murine VEGF is shorter than human VEGF by one amino acid. Analysis of exons suggests the generation of three isoforms, VEGF120, VEGF164, and VEGF188, in mice (26, 43, 44). In our studies, we clearly demonstrate increases in protein expression of VEGF isoforms, VEGF164 and VEGF188, in glomerular mesangial cells (Fig. 3). In agreement with our results of gene expression profiling analysis, Northern analyses confirmed that in Mkk3-deficient mice (Mkk3–/–) mesangial cells the TGF-β1-induced VEGF mRNA was notably inhibited (Fig. 4), and moreover, TGF-β1 treat-
ment failed to induce the VEGF164 isoform (Fig. 5), suggesting a critical role of the MKK3 pathway. We have previously established the specific absence of activated MKK3 protein in the MKK3−/− mesangial cells and that in the absence of MKK3 activation, TGF-β1 was unable to induce downstream p38 MAPK phosphorylation, specifically the isoforms p38α and p38β MAPK (9). Accordingly, we examined whether either or both of the downstream p38 MAPK isoforms were involved in inducing VEGF expression by TGF-β1 in mesangial cells. By isoform-specific dominant negative inhibition of p38α or p38β, and additionally by chemical inhibition of p38α MAPK pathway using SB203580, we show the involvement of both isoforms in mediating TGF-β1-stimulated VEGF164 expression in murine mesangial cells (Figs. 6 and 7). However, dominant negative mutant of p38α or p38β, or SB203580, individually did not completely inhibit TGF-β1-stimulated VEGF164 expression but required simultaneous blockade of both p38α and p38β (Fig. 8), indicating that both isoforms contributed to TGF-β1-stimulated VEGF164 expression in mouse mesangial cells.

Although the molecular mechanism of regulation of VEGF gene expression remains incompletely understood, an emerging body of evidence suggests that, indeed, the gene expression of VEGF isoforms derived from specific splice variants is cell type- and stimulus-specific. For example, VEGF isoforms are differentially transcribed by specific cell types within the mammary gland during mouse mammary gland development (45). Analysis of VEGF transcription by reverse transcriptase-PCR revealed mRNA for all three isoforms VEGF120, VEGF164, and VEGF188 within the mammary gland of nulliparous females, but during pregnancy VEGF188 levels declined and became undetectable during lactation in association with the increased abundance of VEGF164 and VEGF188 mRNAs. VEGF188 mRNA transcription occurs as a late event during lobogenesis distinct from earlier induction of VEGF120 and VEGF164 mRNAs during differentiation (45). In the human uterus, 17β-estradiol increases endometrial expression of all VEGF isoforms, whereas progesterone selectively increases the expression of the VEGF188 isoform (46). Studies in hyperoxic acute lung injury by Corne et al. (47) show that interleukin-13 selectively stimulates VEGF164, whereas interleukin-13 plus hyperoxia stimulate VEGF120 and VEGF188. Although the precise molecular mechanism responsible for such isoform-specific differential VEGF gene regulation is not known, our data provide novel evidence that this occurs through activation of distinct intracellular signaling molecules. In glomerular mesangial cells, TGF-β1 selectively stimulates VEGF164 (and not VEGF188) via the MKK3-p38α and p38β MAPK-dependent pathway.

The expression of multiple VEGF isoforms in mammalian cells, and the different profiles of VEGF isoforms expressed in different cell types suggest that these VEGF isoforms may differ in their physiological function. Compared with VEGF164, VEGF120 binds with lower affinity to VEGFR-1 and is less potent in stimulating endothelial growth (44). VEGF164 is at least twice as potent as VEGF120 in inducing intercellular adhesion molecule 1 (ICAM-1)-mediated leukocyte stasis within the retinal vasculature and blood-retinal barrier breakdown in diabetic retinal (48). To date, little is known regarding the role of the distinct VEGF isoforms in glomerular mesangial cells. Demonstration of all three VEGF receptor types, VEGFR-1 (Flt-1), VEGFR-2 (KDR), and neuropilin-1, expressed in cultured glomerular mesangial cells indicate that the mesangial cells are potential targets of VEGF actions (49). In the present study, we have demonstrated that MKK3 mediates isoform-specific stimulation of VEGF164 by TGF-β1 in mesangial cells, and given our previous studies demonstrating role of MKK3 in TGF-β1-stimulated collagen expression, we explored the effects of VEGF164 on ECM gene expression. Interestingly, treatment with recombinant mouse VEGF164 increased the expression of two major ECM genes, collagen α1(1) and fibronectin, known to be potently induced by TGF-β1 in mesangial cells (Fig. 9). Other known inducers of ECM such as angiotensin II, high glucose, and mechanical stretch also have been reported to stimulate VEGF in mesangial cells (50–52). Thus taken together, our findings suggest a critical role of MKK3-p38α and p38β MAPK pathway in mediating VEGF164 isoform-specific stimulation by TGF-β1 in mesangial cells. Further, VEGF164 stimulates collagen and fibronectin expression in mesangial cells. Both collagen and fibronectin are known to be potently induced by TGF-β1 in mesangial cells. Thus, TGF-β1 stimulation of VEGF164 can in turn further enhance the effects of the TGF-β1-induced ECM and may play an important role in progressive glomerular fibrosis.
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