ERK-dependent Signaling Pathway and Transcriptional Factor Ets-1 Regulate Matrix Metalloproteinase-9 Production in Transforming Growth Factor- β 1 Stimulated Glomerular Podocytes

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Key Words

Transforming growth factor β 1 • Matrix metalloproteinase • Signaling pathway • Glomerulosclerosis • Ets-1

Abstract

The unregulated synthesis of glomerular basement membrane (GBM) components, extracellular matrix (ECM) proteins, or the secretion of ECM-degradation enzymes, matrix metalloproteinases (MMPs), by podocytes under pathological conditions might be major factors in GBM damage. The present study examined the effects and the underlying molecular mechanism of transforming growth factor β 1 (TGF β 1) on the production of gelatinase in cultured murine podocytes. Our results showed that TGF β 1 is the most potent inducer of MMP-9 secretion in both a dose- and time-dependent manner, but has very little effect on MMP-2 secretion. TGF β 1 upregulated MMP-9 mRNA levels, but did not affect the expression of matrix metalloproteinases TIMP-1 mRNA. TGF β 1 induced activation of both Smad2 and extracellular signal-regulated kinases (ERK1/2). However, blockade of Smad2 signaling pathway by Staurosporine did not affect the TGF β 1-stimulated secretion of MMP-9, whereas inhibition of activation

of ERK1/2 by PD98059 abolished TGF β 1-stimulated secretion of MMP-9 and expression of MMP-9 mRNA. Protein levels of the transcriptional factor Ets-1 increased and were sustained for 12 h by TGF β 1-stimulation. Our data also showed that blockage of ERK1/2 activation by PD98059 led to a reduction in the level of Ets-1 protein and to a consequent decrease in MMP-9 mRNA levels. These results demonstrate that TGF β 1 can induce the production of MMP-9 in podocytes through the ERK1/2 MAPK pathway, and suggested that an increase in MMP-9 enzymatic activities may be involved in the damage of the GBM in response to inflammatory factors, ultimately leading to glomerulosclerosis.

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Introduction

The glomerulus, the filtration apparatus of the kidney, is responsible for the generation of protein-free urine. The glomerular filter through which the ultrafiltrate has to pass consists of three layers: the fenestrated endothelium, the glomerular basement membrane (GBM)

and slit diaphragm connecting the glomerular epithelial cell (podocyte) foot processes. Podocytes are intrinsic components of the kidney glomerulus and play a key role in the maintenance of glomerular permselectivity [1, 2]. Several independent lines of research have implicated podocytes as key players in the initiation and propagation of pathways leading to glomerulosclerosis [3, 4]. Furthermore, morphological studies using animal models of glomerular diseases have identified the detachment of podocytes from the GBM as a critical step in the development and progression of glomerulosclerosis [5-7]. The underlying molecular mechanisms accounting for podocyte detachment could include apoptosis of podocytes [6, 8] or reorganization of the actin cytoskeleton in podocytes [9-11]. Proteinase mediated degradation of the GBM may also impair the adhesion of podocytes to the GBM. However, the role of podocytes in GBM degradation has not been extensively studied.

Matrix metalloproteinase-2 (MMP-2) and -9 (MMP-9) are gelatinases capable of degrading type IV collagen, the major component of the GBM. Both MMP-2 and MMP-9 play important roles in the balance between the matrix degradation and reconstruction of tissue homeostasis that is essential for maintenance of the integrity of the GBM. In the adult kidney, MMP-9 is primarily expressed in podocytes and collecting duct cells [12, 13]. In addition, both *in vivo* and *in vitro* studies have revealed that podocytes also secrete MMP-9 under pathological conditions [14, 15]. Recently, elevated concentrations of plasma MMP-9 and increased activity of urinary MMP-9 were observed in diabetic patients prior to the onset of microalbuminuria [16, 17]. Also, an increase in the glomerular expression and activity of MMP-9 was detected in human glomeronephritis [18]. Since local MMP-9 proteolytic activities are among the earliest processes to be observed in the pathophysiology of glomerular proteinuria [19], MMP-9 secretion and activity might be regulated directly by other molecules in the podocytes during glomerular disease.

Under normal conditions, when GEC are in contact with the extracellular matrix of the GBM and are exposed to trivial concentrations of growth factors, there appears to be little turnover of the GBM. Recent *in vitro* studies have shown that GEC are capable of secreting TGF β 1 [20], PDGF [21] and Angiotensin II [22], factors that may mediate glomerular injury. Of these three, TGF β 1 is the most potent profibrogenic factor in glomerular cells. *In vitro* studies have shown that a range of stimuli (e.g. hyperglycaemia, AGEs, lipids, and various products of

oxidative stress), all of which are relevant to the progression of glomerulosclerosis, increase TGF β 1 expression [23]. It is likely that this multi-functional cytokine has a range of direct as well as indirect effects that mediate functional and structural damage of the GBM. Thus, it is hypothesized that growth factors may induce podocyte production of MMP-2 and MMP-9. TGF β 1 has been shown to be capable of stimulating the secretion of the matrix degrading proteinases MMP-2 and MMP-9 in podocytes [24-26]. However, the signaling pathway that mediates the TGF β 1-induced MMP production in podocytes has not yet been deciphered.

Several transcription factors, including Ets-1, have also been implicated in MMP-9 gene expression [27]. Ets transcription factors bind a GGA (A/T) consensus sequence called the Ets binding site (EBS) or PEA3 element via an 80-aa C-terminal domain. EBSs are present in the promoters of many genes involved in cellular proliferation, differentiation, development, hematopoiesis, apoptosis, metastasis, tissue remodeling and angiogenesis [28].

The aim of this study was to investigate the effect produced by Ets1 and two signaling pathways, MAPK and Smad2, on MMP-9 production. Our results showed that TGF β 1-stimulated MMP-9 production occurs through a MAPK-dependent, Smad2-independent signaling pathway, and that the protein level of the Ets-1 transcription factor was upregulated prior to MMP-9 expression.

Materials and Methods

Reagents and antibodies

TGF β 1 was purchased from R&D system (Minneapolis, MN, USA). The ERK activation inhibitor PD98059 was purchased from BIOMOL (Plymouth Meeting, PA, USA). A more general serine/threonine kinase inhibitor, Staurosporine, was purchased from Sigma (St. Louis, MO, USA). Anti-phosphorylated-Smad2 antibody was purchased from Upstate USA, Inc. (Charlottesville, VA, USA). Antibodies against Smad2, phosphorylated-ERK1/2, ERK1/2, and Ets-1 were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Anti-GAPDH antibody was purchased from Biodesign Int (Saco, ME, USA).

Cell culture

A conditionally immortalized mouse podocyte cell line was kindly provided by Dr. Peter Mundel at Albert Einstein College of Medicine in New York, NY. They were cultured following documented instructions [29]. The cells were first

maintained and propagated in RPMI 1640 that was supplemented with 5% fetal bovine serum (FBS) and 20 U/ml γ -IFN at 33°C. They were then differentiated in RPMI 1640 with 5% FBS but without γ -IFN at 37°C for 2 weeks and used in the following experiments.

Gelatin zymography

The supernatants from different treatment groups were used to measure the activity of MMP-2 and MMP-9 by gelatin zymography. Briefly, equal volumes of samples were loaded onto a 10% SDS-PAGE gel containing 0.01% gelatin. After electrophoresis, gels were washed in buffer containing 50 mM Tris (pH 7.5), 5 mM Ca^{2+} , 1 μM Zn^{2+} , 0.02% NaN_3 and 2.5% Triton X-100 for 1 h and incubated in collagenase buffer (50 mM Tris (pH 7.5), 5 mM Ca^{2+} , 1 μM Zn^{2+} , 0.02% NaN_3 and 1% Triton X-100 overnight at 37°C. The gels were subsequently stained with 0.35% Coomassie blue R-250 (Sigma), 20% acetic acid and 50% methanol, and then destained with 25% methanol and 10% acetic acid. Areas of gelatinase activity appeared as nonstaining bands on the gels. Gelatinolytic bands at represent latent MMP2 and active MMP2 activity respectively. Bands were scanned and quantitated by densitometry using KODAK Image software (New Haven, CT, USA).

Western blot analysis

Podocytes from different treatments were prepared in lysis buffer (50 mM Tris-Cl (pH8.0), 150 mM NaCl, 1% Nonidet P-40, 0.1% sodium dodecyl sulfate (SDS), 1% Triton X-100) containing protease inhibitors. The lysates were centrifuged at 10,000g at 4°C for 10 min. The supernatants were collected and protein concentration was quantified by Bradford assay. Immunoblot procedures were performed using a standard protocol, and the signals were visualized using a chemiluminescence reagent (ECL; Perkin Elmer, Boston, MA, USA). Quantitation was carried out by scanning and analyzing the intensity of the hybridization signals using the electrophoresis documentation and analysis system (EADS) 120 (Kodak). The complete procedure was repeated 3 times.

Reverse transcription-PCR

Total RNA of differentiated podocytes was extracted with Trizol reagent (Invitrogen, Carlsbad, CA, USA) after treatments, following the instructions of the manufacturer. First strand cDNA synthesis reverse-transcribed as instructed by the kit (Promega, Madison, WI, USA). An equal amount of cDNA products was amplified for 32-PCR cycles using a MMP-9 primer as documented [30]. The PCR products were separated on a 1.8% agarose gel. The complete RT-PCR procedure was repeated 3 times.

Statistical analysis

All data were expressed as means $\pm$ SD. Statistical analysis of the data was performed using SPSS 10.0 software. Comparison between groups was made using one-way analysis of variance (ANOVA). $P < 0.05$ was considered to be statistically significant in all cases.

Fig. 1. TGF β 1 significantly upregulates enzymatic activity of MMP-9, not MMP-2. Differentiated podocytes were serum starved for 24 h followed by treatment with Control (C), 2 ng/ml TGF β 1, 10^{-6} M Angiotensin II (Ang. II), 10 ng/ml PDGF cytokines for 24 h. Cell supernatants were used to measure activity of gelatinase by gelatin zymography. Results are representative of three independent experiments.

Results

Effect of growth factors on the activity of MMP-2 and MMP-9 in the supernatant of cultured podocytes

Although TGF β 1 is considered the major profibrogenic cytokine implicated in the progression of glomerulosclerosis, other cytokines such as Angiotensin II and PDGF may also act on podocytes. Therefore, we first tested the change in activity of MMP-2 and MMP-9 after cells were incubated with these factors. The podocytes were cultured for 2 d, serum starved for 24 h and then treated with 2 ng/ml TGF β 1 for 24 h. A significant increase in MMP-9 activity was seen after TGF β 1 stimulation when compared to the control culture. PDGF also induced activity, but to a lesser extent, while Angiotensin II had no affect on MMP-9 activity. We found that MMP-2 activity did not significantly change after treatment with any of these cytokines (Figure 1).

Dosage and time course of TGFβ1-stimulated change of MMP-9 activity in the supernatant of cultured podocytes

To further observe the stimulating effect of TGF β 1, different doses of TGF β 1 and various incubation times were applied. In the control group, where there was a low basal level of MMP-9 activity, 1 ng/ml TGF β 1 treatment for 24 h induced a marked increase of MMP-9 activity. MMP-9 activity continued to increase with 2 and 5 ng/ml treatments (Figure 2A). Since a significant increase in the level of activity can be obtained from cultures treated with 2 ng/ml TGF β 1, the dosage was

Fig. 2. Dosage and time dependent increase of MMP-9 activity induced by TGF β 1. Differentiated podocytes were serum starved for 24 h and treated with increasing concentrations of TGF β 1 for 24 h, or stimulated by 2 ng/ml TGF β 1 for the indicated time periods. Cell supernatants were used to measure gelatinase activity by gelatin zymography. (A) Dosage course of TGF β 1-stimulated increase of MMP-9 activity. (B) Time course of TGF β 1-stimulated increase of MMP-9 activity. Results are representative of three independent experiments.

Fig. 3. TGF β 1 upregulates levels of MMP-9 mRNA. Differentiated podocytes were serum starved for 24 h and treated with 2 ng/ml TGF β 1 for the indicated time periods. Total RNA was harvested and RT-PCR was performed to assess the level of MMP-9 mRNA, normalized to that of GAPDH. Corresponding densitometric analysis of intensity of mean MMP-9 mRNA/GAPDH ration from three independent experiments (low panel). * P <0.01, ** P <0.001, compared to 0 h.

chosen for a time course and the followed experiments. MMP-9 activity increased significantly when 2 ng/ml TGF β 1 was applied after 12 h, and levels remained elevated with 2ng/ml TGF β 1 treatment after 24 and 48 h (Figure 2B).

The effect of TGF β 1 on the expression of MMP-9 mRNA in podocytes

To evaluate the effect of TGF β 1 on the levels of cellular MMP-9 mRNA, cells were cultured and serum starved for 24 h before treatment with 2 ng/ml TGF β 1. As shown in Figure 3, TGF β 1 was a potent stimulator of MMP-9 mRNA expression. The mRNA levels were

significantly up-regulated at 6 h by 2 ng/ml TGF β 1 compared to the control. Levels remained elevated after 12 h and 24 h.

The effect of TGF β 1 on the expression of TIMP-1 mRNA in podocytes

Since TIMP-1 acts as the natural inhibitor of MMP-9, we tested whether TGF β 1 affects cellular TIMP-1 mRNA expression. The levels of TIMP-1 mRNA in podocytes stimulated by 2 ng/ml TGF β 1 for 12 h were comparable to that in untreated cells, as assessed by RT-PCR (Figure 4).

Fig. 4. TGF β 1 had no effect on mRNA levels of TIMP. Differentiated podocytes were serum starved for 24 h and treated with 2 ng/ml TGF β 1 for the indicated time periods. Total RNA was harvested and RT-PCR was performed to assess the level of TIMP-1 mRNA, normalized to that of GAPDH. Results are representative of three independent experiments.

Fig. 5. TGF β 1 activates ERK1/2 and Smad signaling pathways. Differentiated podocytes were serum starved for 24 h and treated with 2 ng/ml TGF β 1 for the indicated time periods. Cell extracts were subjected to Western blot analysis using phosphor-specific antibodies. Results are representative of three independent experiments.

Activation of ERK1/2 and phosphorylation of Smad2 signal pathways in TGF β 1-stimulated cells

To assess the effect of TGF β 1 on activation of both the Smad and MAPK signaling pathways, podocytes were cultured for 2 days, serum starved for 24 h and then treated with 2 ng/ml TGF β 1. The cytokine induced Smad2 phosphorylation as early as 5 min after treatment. Peak levels had been attained by 30 min and persisted for at least 240 min (Fig. 5A). Following TGF β 1 treatment, activation of ERK1/2 occurred at 5 min, was at its maximum by 30 min, and had returned to basal level by 240 min (Fig. 5B). Two other MAPK members, phosphorylation of p38 and JNK were undetectable (data not shown).

Effect on TGF β 1-stimulated MMP-9 activity of blocking either TGF β -Smad or the ERK1/2 signal pathway

To demonstrate the role of ERK and Smad signaling on TGF β 1-induced MMP-9 activity, podocytes were

pretreated with PD98059, a specific MEK1 inhibitor, or with Staurosporine, a general serine/threonine kinase inhibitor [31] before TGF β 1 treatment. As shown in Figure 6, blockage of ERK activation abolished TGF β 1-stimulated MMP-9 activity, whereas inhibition of TGF β -Smad had no effect on TGF β 1-stimulated MMP-9 activity.

The cross effect of Staurosporine and PD98059 on the activation of ERK1/2 and Smad2 phosphorylation

To determine the inhibitory effect of either a serine/threonine kinase inhibitor or a MEK1 inhibitor on the activation of ERK1/2 and Smad2 phosphorylation, cells were pretreated with Staurosporine or PD98059 before TGF β 1 incubation. Staurosporine pretreatment suppressed TGF β 1-mediated Smad2 phosphorylation, but PD98059 pretreatment had no effect (Fig 7A). Activation of ERK1/2 was inhibited by PD98059 pretreatment, while Staurosporine pretreatment did not inhibit TGF β 1-mediated activation of ERK1/2 (Fig 7B).

Fig. 6. Effect of blocking either Smad2 phosphorylation or the ERK1/2 signal pathway on TGF β 1-stimulated MMP-9 activity. Differentiated podocytes were serum starved for 24 h and incubated with 2 ng/ml TGF β 1 alone for 24 h, or were treated with 25 μ M PD98059 or staurosporine prior to the TGF β 1 addition. Cell supernatants were used to measure gelatinase activity by gelatin zymography. (A) PD98059 blockade of ERK1/2 signaling. Densitometric analysis of intensity of mean MMP-9 activity from three independent experiments (low panel). * P <0.01, compared to control, NS: no significance, compared to TGF β 1 treatment. (B) Blockade of Smad2 signaling. Results are representative of three independent experiments. Densitometric analysis of intensity of mean MMP-9 activity from three independent experiments (low panel). * P <0.01, compared to control, # P <0.01, compared to TGF β 1 treatment.

The expression of transcriptional factor Ets-1 protein by TGF β 1

Because the maximal levels of MMP-9 mRNA were present 12 h after treatment, we assessed the protein levels of the Ets-1 transcriptional factor in cells exposed to different dosages of TGF β 1 for 12 h. A dose as low as 0.5 ng/ml of TGF β 1 resulted in an increase in the expression of Ets-1, with maximal levels achieved by 2 ng/ml of TGF β 1 (Fig.8A). Also, the 2 ng/ml treatment significantly increased expression of Ets-1 protein by 4 h, a timepoint that precedes the increase in MMP-9 mRNA (6 h). The high levels of Ets-1 protein were sustained for 24 h (Fig.8B).

The effect of inhibition of ERK1/2 activation on the expression of Ets-1 protein and MMP-9 mRNA stimulated by TGF β 1

Again, PD98059 pretreatment completely inhibited TGF β 1-stimulated ERK activation (Fig.9A). This inhibition also produced a down-regulation of TGF β 1-stimulated expression of Ets-1 protein (Fig.9B), resulting in a decrease in the levels of MMP-9 mRNA at 12 h (Fig.9C).

Discussion

Development, cell migration, wound healing, and tissue remodeling are physiological processes in which matrix metalloproteinases (MMPs) play crucial roles [32]. Both of the MMP-2 and MMP-9 gelatinases are reportedly synthesized and secreted by glomerular epithelia cells *in vivo* and *in vitro* [25, 26, 33, 34], and could be involved in normal and/or aberrant degradation

or remodeling of the glomerular membrane. However, MMP-9 has garnered special attention for its potential involvement in kidney development and disease [26, 35].

MMP-9 is expressed at high levels by podocytes during rat Heymann nephritis [36]. This observation is consistent with the view that this enzyme is capable of GBM remodeling, since excessive gelatinase activity could promote GBM degradation and thereby disrupt the filtration barrier. Although several cytokines elicit MMP-9 expression, TGF β 1 is particularly potent for podocytes. The passage of excess plasma proteins across the glomerular capillary wall could possibly trigger the overexpression of TGF β 1 in podocytes [37]. In the present study, our data showed that among the growth

Fig. 7. The cross effect of Staurosporine and PD98059 on the activation of ERK1/2 and Smad2 phosphorylation. Differentiated podocytes were serum starved for 24 h and incubated with 2 ng/ml TGF β 1 for 24 h alone, or were treated with 25 μ M PD98059 or Staurosporine prior to the addition of TGF β 1. Cell extracts were subjected to Western blot analysis using phosphor-specific antibodies. Results are representative of three independent experiments. (A & B) Effect of Staurosporine and PD98059 treatments on Smad2 phosphorylation. (C & D) Effect of Staurosporine and PD98059 treatments on ERK1/2 activation.

factors (PDGF, angiotensin II and TGF β 1) that lead to glomerular damage, TGF β 1 is the only one with the ability to stimulate MMP-9 mRNA expression and secretion, although it had a smaller effect on MMP-2 activity. However, the Tomino lab observed that MMP-2 activity in podocytes significantly increased when a high dose (10 ng/ml) of TGF β 1 was applied [26].

TGF β 1 signals through transmembrane serine/threonine kinase receptors that proceed to induce activation of the cytoplasmic molecules Smad2 or/and Smad3. They have two serine residues in the SS[V/M]S motif at their C terminals that are phosphorylated by activated type I receptor kinases. Phosphorylated Smad2 and Smad3 then form a complex with Smad4 [38] that translocates into the nucleus and binds to specific DNA elements of TGF β 1 targeted genes [39]. In addition to

the differential contribution of Smads, the specific outcome of a TGF β 1-induced cellular response seems to also be dependent on the activation of non-Smad signaling MAPK pathways that include ERKs, C-jun N-terminal kinases (JNKs), and p38 MAPK [40, 41]. However, a direct link between MAPK pathways to the receptors bound by TGF β 1 has not yet been made. Transforming growth factor-beta-activated kinase 1 (TAK1) has been proposed as the upstream kinase activator of MAPKs [42, 43].

In this study, we investigated the relative contribution of ERK and Smad2 signaling in regulating MMP-9 secretion and gene expression. Our results showed that inhibition of ERK activation by PD98059 abolishes the TGF β 1-induced production of MMP-9, whereas a block of Smad2 phosphorylation by a general serine/threonine kinase inhibitor, Staurosporine, did not suppress TGF β 1-

Fig. 8. Dosage and time dependent increase of Ets-1 protein induced by TGF β 1. Differentiated podocytes were serum starved for 24 h and treated with increasing concentrations of TGF β 1 for 12 h, or stimulated by 2 ng/ml TGF β 1 for the indicated time periods. Cell extracts were subjected to Western blot analysis. (A) Dosage course of TGF β 1-stimulated increase of ETS-1 protein. (B) Time course of TGF β 1-stimulated increase of ETS-1 protein. To correct for variations in the protein loading, the blot was stripped and reincubated with an anti-GAPDH antibody (lower panel). Results are representative of three independent experiments.

induced production of MMP-9. These results are in agreement with data indicating that sustained ERK activation is required for growth factor-dependent MMP-9 production [44] and TGF β 1-modulated cellular production of MMP-9 in transformed keratinocytes [45]. MAPK has also been found to play an important role in mediating the prosclerotic effect of this cytokine [46, 47]. Together these results demonstrated that these two TGF β 1-mediated signaling pathways, the Smad signaling pathway and the MAPK pathways, are independent of each other, and that the MAPK pathway is involved in the production of MMP-9 by TGF β 1 in podocytes.

MMP-9 induction by growth factors has been shown to operate primarily through transcriptional stimulation that targets the MMP-9 gene promoter [48]. Several transcriptional factors have been implicated in MMP-9 gene expression, including Ets-1 and NF- κ B [15, 49, 50]. Ets transcription factor binds to a GGA(A/T) consensus sequence, called the Ets binding site (EBS) or PEA3 element, via its C-terminal domain [51]. Ets-1 is known to be localized predominantly to glomerular epithelial cells, endothelial cells and interstitial fibroblasts in the kidney, and the levels of Ets-1 mRNA and protein are markedly increased in kidney disease [52, 53]. Ets-1 is the target of the Ras-MAPK signaling pathway, and TNF α can induce expression of Ets-1 through the ERK pathway in vascular smooth muscle cells [54]. We showed that an increase in protein levels of the Ets-1 transcription factor precedes the upregulation of MMP-9 mRNA induced by TGF β 1, and a blockade of ERK activation can abrogate TGF β 1-stimulated Ets-1 protein expression and MMP-9 mRNA in podocytes. The implication is that Ets-1

Fig. 9. Inhibition of ERK1/2 activation abrogates expression of Ets-1 protein and of MMP-9 mRNA stimulated by TGF β 1. Differentiated podocytes were serum starved for 24 h and treated with 2 ng/ml TGF β 1 alone, or were treated with 25 μ M PD98059 prior to the addition of TGF β 1. Cell extracts were subjected to Western blot analysis using specific antibodies. (A) Effect of PD98059 pretreatment on TGF β 1-stimulated ERK activation. (B) Effect of PD98059 pretreatment on the TGF β 1-stimulated expression of Ets-1 protein. (C) Effect of PD98059 pretreatment on the level of TGF β 1-stimulated MMP-9 mRNA (after 12 h). Results are representative of three independent experiments.

contributes to the TGF β 1-stimulated production of MMP-9 by driving MMP-9 gene expression.

In summary, MMP-9 is regulated by TGF β 1 at the level of gene transcription through ERK1/2 MAPK signaling and via upregulation of the expression of Ets-1 in kidney podocytes.

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