Gelatinase A (MMP-2) Is Necessary and Sufficient for Renal Tubular Cell Epithelial-Mesenchymal Transformation

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Progressive renal interstitial fibrosis and tubular atrophy represent the final injury pathway for all commonly encountered forms of renal disease that lead to end-stage renal failure. It has been recently recognized that myofibroblastic cells are the major contributors to the deposition of interstitial collagens. While there are several potential cellular sources of myofibroblasts, attention has focused on the transformation of the organized tubular epithelium to the myofibroblastic phenotype, a process potentially driven both *in vitro* and *in vivo* by transforming growth factor- β 1 (TGF- β 1). Integrity of the underlying basal lamina provides cellular signals that maintain the epithelial phenotype, and disruption by discrete proteases could potentially initiate the transformation process. We demonstrate that TGF- β 1 coordinately stimulates the synthesis of a specific matrix metalloproteinase, gelatinase A (MMP-2), and its activator protease, MT1-MMP (MMP-14), and that active gelatinase A is absolutely required for epithelial-mesenchymal transformation induced by TGF- β 1. In addition, purified active gelatinase A alone is sufficient to induce epithelial-mesenchymal transformation in the absence of exogenous TGF- β 1. Gelatinase A may also mediate epithelial-mesenchymal transformation in a paracrine manner through the proteolytic generation of active TGF- β 1 peptide. MT1-MMP and gelatinase A were co-localized to sites of active epithelial-mesenchymal transformation and basal lamina disruption in the rat remnant kidney model of progressive renal fibrosis. These studies indicate that a discrete matrix metalloproteinase, gelatinase A, is capable of inducing the complex genetic rearrangements that characterize renal tubular epithelial-mesenchymal transformation. (*Am J Pathol* 2003, 162:1937-1949)

Progressive fibrosis and tubular atrophy are the key determinants of end-stage renal disease, regardless of the primary disease process. Additionally, it is well established that interstitial fibrosis has a greater impact on the

progression of chronic renal disease than glomerulosclerosis.¹ Tubular injury from primary glomerular disease ensues from interstitial microvascular injury, adaptive tubular hypermetabolism, or proteinuria. Systemic hypertension, hyperfiltration, and glomerular capillary obstruction in glomerular injury also lead to post-glomerular capillary injury and hypoxia.²⁻⁴ In addition, tubular atrophy and loss in glomerular disease results in functional hypermetabolism by the remaining tubules, with increased oxygen consumption.⁵

These tubular insults create a hypoxic environment that further contributes to matrix production by tubules and fibroblasts.⁶⁻⁸ In addition to stimulating collagen synthesis, hypoxia also stimulates expression of growth factors and cytokines including transforming growth factor- β 1 (TGF- β 1), vascular endothelial growth factor, platelet-derived growth factor, endothelin-1, and angiotensin II, of which TGF- β 1 is the most studied.^{3,6,9} In cultured renal fibroblasts, TGF- β 1 stimulates production of fibronectin and types I, III, and V collagens, while tubular epithelial cells are stimulated to produce proteoglycans and type I, III, IV, and V collagen.^{10,11} Numerous studies have demonstrated that renal fibrosis *in vivo* is associated with elevated TGF- β 1 expression.¹² In the Thy1.1 rat model of glomerulonephritis, renal fibrosis was abrogated by administration of anti-TGF- β 1 antibody, antisense oligonucleotides, and decorin, a proteoglycan associated with the interstitial matrix and shown to bind TGF- β 1.¹³⁻¹⁶

It has become increasingly evident that myofibroblasts play a central role in the development of renal interstitial fibrosis. Myofibroblasts are mesenchymal cells that express α -smooth muscle actin and are thought to be the predominant source of types I and III collagen in fibrosis.¹⁷ Increased myofibroblast expression in both human disease and animal models has been associated with matrix accumulation and progression of renal disease.¹⁸⁻²⁸

Despite their importance, the cellular source(s) of renal interstitial myofibroblasts has not been entirely elucidated. While renal myofibroblasts may derive from the intrinsic fibroblastic population or vascular pericytic cells, considerable attention has been devoted recently to the process of tubular epithelial cell (TEC) transformation. In

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essence, TEC transformation represents a reversal of the mesenchymal-epithelial cell differentiation process characteristic of nephrogenesis. In a study of 5/6 nephrectomized rats, proximal tubule cells were shown to undergo stepwise transformation into α -smooth muscle actin-positive myofibroblasts.²⁹ Tubular cell expression of α -smooth muscle actin was associated with basement membrane disruption and eventual loss of epithelial morphology with migration into the stroma. Myofibroblasts appeared in areas of fibrosis and adjacent to α -smooth muscle actin-positive tubular cells.

Recently, TGF- β 1 was shown to directly induce tubular epithelial-myofibroblast transformation in the NRK-52e normal rat kidney epithelioid cell line *in vitro*.³⁰ Yang and Liu³¹ subsequently demonstrated that TGF- β 1-mediated transformation of cultured tubular epithelial cells was temporally associated with a specifically enhanced expression of gelatinase A. Transgenic mice expressing TGF- β 1 develop progressive renal fibrosis with the characteristic features of epithelial-mesenchymal transformation, an event also associated with enhanced synthesis of gelatinase A.³² Other studies have shown that TGF- β 1 stimulates gelatinase A synthesis by cultured fibroblasts and glomerular mesangial cells at both the transcriptional and post-transcriptional levels.^{33,34}

Integrity of the underlying basal lamina is required for the maintenance of a polarized epithelial phenotype, and disruption of type IV collagen lattice assembly by addition of a dominant negative α 1NC1 domain results in epithelial-mesenchymal transformation.³⁵ Song et al³⁶ showed that the epithelial-mesenchymal transformation of endocardial cushions was dependent on gelatinase A activity for penetration and disruption of the underlying type IV collagen-rich basal lamina. Taken together, these observations suggest that the degradation and disruption of the underlying basal lamina by specific matrix metalloproteinases, such as gelatinase A, is a critical component of the epithelial-mesenchymal transformation process. In this report we demonstrate that gelatinase A, in association with the membrane-bound MT1-MMP (MMP-14), is absolutely required for the epithelial-mesenchymal transformation of NRK-52e cells induced by TGF- β 1 *in vitro*. In addition, purified active gelatinase A alone is sufficient to induce epithelial-mesenchymal transformation in these cells without the addition of TGF- β 1. Finally, in a model of renal epithelial-mesenchymal transformation and fibrosis, we demonstrate the co-localization of gelatinase A and MT1-MMP by the tubular epithelium at sites of ongoing myofibroblast formation and basal lamina disruption. Taken together, these observations indicate that a single matrix metalloproteinase, gelatinase A, is necessary and sufficient for the induction of the complex genetic rearrangements that characterize epithelial-mesenchymal transformation, a finding of considerable therapeutic potential.

Materials and Methods

Cell Culture

The NRK-52e normal rat tubular epithelioid cell line was obtained from the American Type Culture Collection and

maintained in DME-H21 (Gibco, Rockville, MD) supplemented with 10% fetal calf serum (FCS; Gibco), 100 units/ml penicillin and 100 μ g/ml streptomycin.

Stimulation Studies

Subconfluent cultures of NRK-52e cells were washed twice in warm phosphate-buffered saline (PBS) and given fresh DME-H 21 medium supplemented with 0.1% bovine serum albumin (BSA) and the indicated concentrations of TGF- β 1 (R&D Systems, Minneapolis, MN). For fluorescence-activated cell sorter (FACS) analysis, fresh serum-free medium containing TGF- β 1 was replaced after 3 days. Treated cells were analyzed by immunohistochemistry, FACS analysis, quantitative gelatinase zymography, and transfection with gelatinase A and MT1-MMP luciferase reporter constructs as detailed below.

For direct treatment with matrix metalloproteinases, NRK-52e cells were grown to subconfluency, washed with PBS and given fresh serum-free medium containing the denoted concentrations of active and latent gelatinase A or active gelatinase B. Latent gelatinase A was purified to homogeneity from the serum-free conditioned medium of cultured rat glomerular mesangial cells according to the protocol of Okada et al³⁷ Latent gelatinase B was purified to homogeneity by chromatography of conditioned medium over gelatin-Sepharose and *Lens culinaris* lectin-agarose as reported in detail.³⁸ Latent gelatinases were activated by incubation with 0.5 mmol/L p-aminophenylmercuric acetate (confirmed by zymography), dialyzed against PBS, and used at the indicated concentrations.

Inhibition Studies

These studies used a cyclic peptide gelatinase A inhibitor, CTTHWGFTLCGG, isolated by phage display, and a control non-inhibitory peptide, CRAVRALWRCGG.³⁹ Biotin was added during the synthesis to the terminal G residues to permit immunolocalization (see below). To block TGF- β 1-mediated transformation, cells were treated as detailed above in the presence or absence of the indicated concentrations of the inhibitory or control cyclic peptides.

Immunohistochemistry

For identification of myofibroblasts, cells cultured on etched glass coverslips were fixed for 20 minutes at 4°C with 4% buffered paraformaldehyde and permeabilized in acetone. The slips were blocked with 5% normal goat serum for 30 minutes, rinsed, and blocked with an avidin/biotin kit (Vector, Burlingame, MA). Rinsed coverslips were incubated with primary monoclonal mouse α -smooth muscle actin antibody (Sigma, St. Louis, MO; 6.5 mg/ml) at 1:50 in 0.1% BSA/PBS for 2 hours at room temperature, followed by biotinylated goat anti-mouse IgG (Zymed, San Francisco, CA; 0.4 mg/ml) at 1:20 in 0.1% BSA/PBS for 2 hours at room temperature. Rinsed slips were incubated with either streptavidin-rhodamine

or streptavidin-fluorescein (0.5 $\mu\text{g/ml}$, Jackson ImmunoResearch Laboratories, West Grove, PA) at 1:100 in 0.1% BSA/PBS for 30 minutes.

For co-localization of α -SMA and active gelatinase A, cells were fixed in 2% buffered paraformaldehyde for 20 minutes, blocked with avidin/biotin and incubated with the biotinylated cyclic peptides (inhibitory or control) at 5 $\mu\text{g/ml}$ for 1 hour at 4°C, followed by a 1:200 dilution of streptavidin-rhodamine for 30 minutes. Rinsed cells were re-fixed with 2% paraformaldehyde, permeabilized with 0.1% Triton X-100 for 90 seconds, and blocked with 5% normal donkey serum for 30 minutes (Vector). This was followed by monoclonal anti- α -SMA antibody at 1:50 for 2 hours at room temperature and fluorescein-conjugated donkey anti-mouse IgG (Jackson ImmunoResearch Laboratories) at 1:100 for 2 hours at room temperature.

To co-localize active gelatinase A and MT1-MMP, the same protocol as above was followed until after the second fixation. Cells were blocked with 5% normal goat serum, followed by avidin/biotin blockade. Murine monoclonal α -MT1-MMP antibody (Oncogene Research Products, San Diego, CA) was used at 1:50 dilution for 2 hours at room temperature, followed by biotinylated goat anti-mouse IgG (Zymed, 0.4 mg/ml) at 1:200 dilution for 2 hours, and finally with streptavidin-fluorescein (Vector) at 1:100 for 30 minutes at room temperature.

For co-localization of MT1-MMP and E-cadherin, cells were fixed with 4% paraformaldehyde, blocked with PBS/CMF with 5% BSA followed by avidin/biotin. Murine monoclonal α -MT1-MMP IgG3 and murine α -E-cadherin IgG2a (Transduction Laboratories, Lexington, KY) were used at 1:50 (2 $\mu\text{g/ml}$) and 1:50 (2.5 $\mu\text{g/ml}$), respectively, for 3 hours. The cells were then incubated with biotinylated rat α -IgG3 (CalTag, Burlingame, CA) and FITC-conjugated rat α -IgG2a (CalTag) at 1:200 (2 $\mu\text{g/ml}$) and 1:50 (8 $\mu\text{g/ml}$), respectively, followed by streptavidin-rhodamine at 1:200 (2 $\mu\text{g/ml}$). For control experiments, murine monoclonal α -MT1-MMP IgG3 was incubated with FITC-conjugated rat α -IgG2a, and murine α -E-cadherin IgG2a with biotinylated rat α -IgG3 and streptavidin-rhodamine.

Ionomycin Studies

NRK-52e cells were incubated with TGF- β 1 (2 ng/ml) along with 0, 100, or 500 nmol/L ionomycin (Sigma) for 48 hours in serum-free medium. Cells were then prepared and stained for α -SMA as described above.

Activation of Latent TGF- β 1 by Gelatinase A

Eighty percent confluent cultures of NRK-52e cells were incubated with the indicated concentrations of active gelatinase A for 3 days in Optimem (Invitrogen, Carlsbad, CA). The conditioned medium was harvested and measured for active TGF- β 1 by ELISA (R&D Systems). Data are expressed as means of quadruplicate determinations $\pm$ 1 SD of pg active TGF- β 1/100 μg cell layer protein.

Immunohistochemistry of Remnant Kidney

Male Munich Wistar rats (275 to 350 g) were subjected to 5/6 renal ablation ($n = 6$) or a sham operation ($n = 6$) consisting of laparotomy and manipulation of the renal pedicle as reported.⁴⁰ Kidneys were harvested for analysis at 10 weeks when segmental sclerosis and tubulointerstitial fibrosis are present. At the time of harvest, animals were anesthetized with pentobarbital, the kidneys perfusion-fixed with 4% paraformaldehyde in PBS, and embedded in paraffin. Deparaffinized 5-micron sections were hydrated, endogenous peroxidase blocked by incubation for 30 minutes with 0.1% H_2O_2 , followed by incubation with 5% normal goat serum and avidin/biotin blocking solution. Monoclonal anti- α -smooth muscle actin antibody (1:400), anti-gelatinase A antibody (1:500, Oncogene Research), or anti-MT1-MMP (1:500, Oncogene Research) were applied for 60 minutes at room temperature, followed by the Vectastain Elite ABC kit (Vector) according to the manufacturer's instructions. Development with DAB/ NiCl_2 and counterstaining with methyl green were performed using standard methodology.

FACS Analysis

Cells in the respective treatment groups were released with trypsin, fixed with 4% paraformaldehyde for 20 minutes, and permeabilized with 0.2% saponin for 10 minutes at 4°C. Cells were then stained with monoclonal anti- α -SMA antibody and fluorescein-conjugated donkey anti-mouse IgG as detailed above and analyzed by FACS (Becton Dickinson, San Jose, CA) with histograms of 10,000 counts, using excitation of 488 nm and emission of 530 nm.

Quantitative Gelatin Zymography

Crude (gelatinase A) and Triton-X114-extracted (MT1-MMP) microsomes from control and TGF- β 1-treated cells were prepared as reported in detail.⁴¹ Equal concentrations of microsomal protein (15 $\mu\text{g/lane}$) were loaded on 7.5% SDS-polyacrylamide gels containing 2 mg/ml gelatin and separated by electrophoresis. Processing of gels was as reported.³⁴ Experiments were performed in triplicate and repeated at least three times. The major enzymatic activities at 66 (gelatinase A) and 62 (MT1-MMP) kd were quantified by laser densitometry and standardized using serial dilution of known quantities of purified gelatinase A or MT1-MMP.

Transient Transfection with Gelatinase A and MT1-MMP Luciferase Reporter Constructs

Subconfluent cultures of NRK-52e cells were washed and transfected with FuGene (Roche, Indianapolis, IN) using 1 μg of plasmid DNA from the control pGL2-Basic luciferase reporter plasmid (Promega, Madison, WI), plasmid pT4-Luc1686 (composed of the first 1686 bp of the rat gelatinase A 5' flanking region cloned into pGL2-Basic), or plasmid pMT1-Luc3280 (composed of the first 3280

Figure 1. TGF- β 1 induces the myofibroblastic phenotype with concurrent expression of MT1-MMP and active gelatinase A. Cultured NRK-52e cells were incubated in the presence (panels B–H) or absence (A) of 2 ng/ml TGF- β 1 for 3 days followed by immunohistochemical analysis. **A:** Control cells stained for α -SMA. **B:** TGF- β 1-treated cells stained for α -SMA showing intense expression at the migratory front. **C and D:** Dual immunohistochemical staining for α -SMA (C) and MT1-MMP (D) of TGF- β 1-treated cells showing co localization of expression in the migratory front. **E and F:** Dual immunohistochemical staining for MT1-MMP (E) and active gelatinase A (F) of TGF- β 1-treated cells showing co localization of enzyme proteins. **G and H:** Normarski optics (G) of TGF- β 1-treated cells demonstrating migratory phenotype with acquisition of myofibroblastic morphology. **H:** Same cells demonstrating localization of active gelatinase A protein on the leading and trailing edges of the cells. (Magnification, $\times 500$).

bp of the murine MT1-MMP 5' flanking region). Sixteen to 24 hours after transfection, fresh medium was added in the presence or absence of 2 ng/ml TGF- β 1 and the incubation continued for a further 24 hours. Cells were washed, extracted with 400 μ l Triton lysis buffer (1% Triton X-100, 1 mmol/L dithiothreitol, 25 mmol/L glycylglycine at pH 7.8, 15 mmol/L MgSO_4), and followed by measurement of luciferase and galactosidase activities as reported.⁴² All transfections were performed in quadruplicate and repeated at least three times. Transfection results are graphed and expressed as means $\pm$ 1 SD.

Statistical Analyses

Statistical significance was determined for paired comparisons using Student's *t*-test or by analysis of variance for multiple comparisons where appropriate.

Results

These studies used the well-characterized rat renal tubular epithelial cell line, NRK-52e, which is generally considered to be of proximal tubular origin.^{43,44} Prior reports by Fan et al³⁰ demonstrated that TGF- β 1 induces transdifferentiation of NRK-52e epithelial cells to a mesenchymal phenotype characterized by α -smooth muscle actin (α -SMA) expression and migration. The effects of TGF- β 1 are time and concentration dependent. As summarized in Figure 1, treatment of NRK-52e cells with 2 ng/ml TGF- β 1 for 3 days induced α -SMA expression and a migratory

phenotype (Figure 1, A and B). Notably, α -SMA expression is limited to the subset of cells at the periphery of the epithelial clusters of NRK-52e cells. As detailed in Figure 1, C and D, the subset of α -SMA+ cells also concurrently expressed MT1-MMP protein as determined by immunohistochemistry. Using a biotinylated cyclic peptide, CT-THWGFTLCGG, as a probe for activated gelatinase A, dual immunohistochemical techniques demonstrated the co-localization of the MT1-MMP and active gelatinase A proteins on the cells at the migratory front of the TGF- β 1-treated cells (Figure 1, E and F). As shown in Figure 1, G and H, the active gelatinase A protein is clustered at specific sites on the leading and trailing edges of the transdifferentiated cells, consistent with a direct role in cellular migration.

The extent of cell-cell contact appeared to regulate the expression of MT1-MMP induced by TGF- β 1. As shown in Figure 2, cells with intact E-cadherin complexes did not express MT1-MMP, while cells with disrupted complexes did.

To quantify the extent of gelatinase A and MT1-MMP induction by TGF- β 1, NRK-52e cells were incubated for 3 days with 2 ng/ml TGF- β 1 in serum-free medium, followed by quantitative gelatin zymography of the culture supernatants (gelatinase A) or Triton-X114 membrane extracts (MT1-MMP). Under these conditions, TGF- β 1 increased the synthesis of both gelatinase A and MT1-MMP by nearly threefold. (Figure 3, A). The increases in gelatinase A and MT1-MMP protein synthesis are, at least in part, due to increased rates of transcription following

Figure 2. Cell-cell contact regulates TGF- β 1-mediated induction of MT1-MMP. NRK-52e cells were cultured for 3 days with TGF- β 1 (2 ng/ml) followed by dual immunohistochemical staining for E-cadherin (**A**) and MT1-MMP (**B**). **Arrows** in **A** denote sites of cell-cell contact with cadherin complexes. (Magnification, $\times 700$).

treatment with TGF- β 1. To demonstrate this, NRK-52e cells were transiently transfected with luciferase reporter constructs containing 1686 or 3280 bp of the 5' flanking regions of the gelatinase A or MT1-MMP promoters, respectively (Figure 3B). Gelatinase A transcription rates under these conditions were stimulated over twofold by TGF- β 1, while MT1-MMP transcription rates were increased by nearly fivefold.

The localization of active gelatinase A to the specific cellular sites of NRK-52e transformation induced by TGF- β 1 suggested that gelatinase A may be the final

common mediator of this process. To directly test this hypothesis, NRK-52e cells were incubated for 72 hours with varying concentrations of active or latent purified gelatinase A, followed by assessment of transformation by staining for α -SMA. The results of these studies are summarized in Figure 4. Concentrations of active gelatinase A as low as 10 nmol/L were sufficient to induce NRK-52e transformation and affected virtually 100% of the cells when a concentration of 50 nmol/L was used (Figure 4, B-D). In contrast, 50 nmol/L of latent gelatinase A had no effect on NRK-52e transformation (Figure 4E),

Figure 3. TGF- β 1 induces MT1-MMP and gelatinase A synthesis and transcription. NRK-52e cells were treated with 2 ng/ml TGF- β 1 for 3 days followed by quantitative analysis of MT1-MMP and gelatinase A protein synthesis and transcription as detailed in Materials and Methods. **A:** Quantitative zymography of controls and TGF- β 1-treated cells demonstrated an approximate threefold increase in both MT1-MMP and gelatinase A protein synthesis. **B:** TGF- β 1 induces an approximate twofold increase in gelatinase A transcriptional activity as measured with the promoter-luciferase construct, pT4-Luc1686, and a nearly fivefold increase in MT1-MMP transcriptional activity using the promoter-luciferase construct pMT1-Luc3280.

Figure 4. Active gelatinase A induces NRK-52e transdifferentiation. Purified active gelatinase A in concentrations ranging from 0, 10, 25, and 50 nmol/L (**A–D**, respectively) were added to cultured NRK-52e cells for 72 hours, followed by immunohistochemical staining for α -SMA. There is an evident concentration-dependent increase in α -SMA expression. Addition of 50 nmol/L of latent gelatinase A (**E**) did not induce α -SMA expression, nor did addition of 50 nmol/L active gelatinase B (**F**). (Magnification, $\times 500$).

consistent with a requirement for enzymatic activity. Zymographic analysis of NRK-52e culture supernates indicated that the only other MMP family member synthesized by this cell type is gelatinase B or MMP-9 (not shown). Addition of 50 nmol/L activated purified gelatinase B had no effect on NRK-52e transdifferentiation (Figure 4F), indicating a specific requirement for a gelatinase A-specific enzymatic activity for this process.

To further demonstrate the absolute requirement of gelatinase A activity for TGF- β 1-mediated transformation, NRK-52e cells were incubated for 72 hours with TGF- β 1 (2 ng/ml) in the presence of increasing concentrations of the gelatinase A cyclic peptide inhibitor, CTTHWGFTL-CGG, or with a control cyclic peptide, CRAVRAL-WRCGG, followed by staining for α -SMA. The results of these experiments are summarized in Figure 5. Addition of increasing concentrations of the gelatinase A cyclic peptide inhibitor significantly decreased cellular staining for α -SMA beginning at concentrations of 25 μ mol/L and greater, while the control cyclic peptide at 100 μ mol/L concentration had no effect on TGF- β 1-mediated α -SMA expression. The concentrations of the cyclic peptide gelatinase A inhibitor required for blockade of α -SMA expression are in the same range required for inhibition of enzymatic activity ($IC_{50} \sim 10 \mu$ mol/L) as determined in Koivunen et al³⁹. It should be noted in this context that the cyclic peptide gelatinase A inhibitor has no effect on the activity of MT1-MMP.³⁹

The data summarized in Figures 1 through 5 are consistent with a TGF- β 1-mediated coordinated synthesis of MT1-MMP and gelatinase A, membrane assembly of the MT1-MMP/gelatinase A complex, and induction of transformation through the localized proteolytic action of gelatinase A. This suggests that the presentation of active MT1-MMP on the cell surface is a critical regulatory step in the assembly of the complete proteolytic complex. Yu et al⁴⁵ and Lehti et al⁴⁶ demonstrated that calcium influx mediated by ionomycin blocked the conversion of pro-MT1-MMP to the active 60 kd form. To evaluate the importance of assembly of the MT1-MMP/gelatinase A complex for induction of transformation, NRK-52e cells were incubated with TGF- β 1 (2 ng/ml) along with ionomycin (0, 100, and 500 nmol/L) for 48 hours, followed by assessment of α -SMA expression. The results of these experiments are summarized in Figure 6 and demonstrate that inclusion of ionomycin effectively blocks the development of TGF- β 1-mediated transformation, as assessed by α -SMA expression. These experiments demonstrate that TGF- β 1-mediated transformation is dependent on the coordinated presentation of active MT1-MMP on the cell surface, thereby permitting assembly of the MT1-MMP/gelatinase A proteolytic complex.

As several of the studies summarized above are expressed in terms of qualitative histochemistry, a further set of experiments was performed using FACS measurement of α -SMA to assess in a quantitative manner the transformation status of the NRK-52e cells. As summarized in

Figure 5. A specific gelatinase A inhibitor blocks TGF- β 1-mediated NRK-52e transformation. NRK-52e cells were treated with 2 ng/ml TGF- β 1 in the presence of concentrations of the cyclic peptide inhibitor, CTTHWGFTLCGG, ranging from 0, 10, 25, 50, and 100 μ mol/L (**A–E**, respectively). Cells in **F** were treated with 100 μ mol/L of the control cyclic peptide, CRAVRALWRCGG. Expression of α -SMA is significantly inhibited by concentrations of the cyclic peptide gelatinase A inhibitor above 25 μ mol/L, while the control peptide had no significant effect on α -SMA expression. (Magnification, $\times 500$).

Figure 7, treatment of NRK-52e cells with 50 nmol/L active gelatinase A results in a significant increase in measured α -SMA expression, while latent gelatinase A had no effect (Figure 7A). This effect of gelatinase A on α -SMA expression is concentration-dependent (Figure 7B), and could not be reproduced by incubation with 50 nmol/L acti-

vated gelatinase B (not shown). Finally, inclusion of 100 μ mol/L of the specific gelatinase A cyclic peptide inhibitor completely blocked TGF- β 1-mediated α -SMA expression (Figure 7C).

Gelatinase A secreted by cultured hepatocellular carcinoma cells has been recently shown to convert latent

Figure 6. Calcium ionophore blocks TGF- β 1-mediated NRK-52e transformation. Cells were treated with TGF- β 1 (2 ng/ml) for 48 hours in the absence (**A**) or presence of ionomycin (100 and 500 nmol/L, **B** and **C**, respectively) followed by staining for α -SMA. (Magnification, $\times 600$).

Figure 7. Quantitative FACS analysis of α -SMA expression by NRK-52e cells. **A:** Cells were treated for 72 hours with control medium, 50 nmol/L latent gelatinase A or 50 nmol/L active gelatinase A. FACS analysis demonstrates a highly significant increase in α -SMA expression in the group treated with active gelatinase A, but not in the controls or in cells treated with latent gelatinase A. **B:** A gelatinase A concentration-dependent increase in α -SMA expression is demonstrated. **C:** Inclusion of 100 μ mol/L of the gelatinase A cyclic peptide inhibitor blocked TGF- β 1-mediated NRK-52e transdifferentiation as determined by α -SMA expression.

TGF- β 1 to active TGF- β 1 by cleavage of latency-associated peptide.⁴⁷ To determine whether a similar process was operative within the context of NRK52e cells, cultures were incubated for 72 hours in serum-free medium with active gelatinase A. Exposure to 10 nmol/L and 50 nmol/L active gelatinase A increased the content of active TGF- β 1 peptide in the supernates by 28 and 94%, respectively (Figure 8). Thus, gelatinase A may further facilitate the development of the transformation process by the paracrine generation of additional biologically active TGF- β 1 peptide.

Ng et al²⁹ recently demonstrated tubular epithelial-myofibroblast transformation in the rat 5/6 nephrectomy

Figure 8. Gelatinase A generates active TGF- β 1 peptide. Cultured NRK-52e cells were maintained for 3 days in serum-free medium with concentrations of active gelatinase A ranging from 0 to 50 nmol/L. Levels of active TGF- β 1 peptide were determined by ELISA and expressed as the mean $\pm$ 1 SD/100 μ g cell layer protein. (*, $P < 0.05$; **, $P < 0.01$)

model of renal fibrosis. To correlate our *in vitro* studies with NRK-52e cells to this model, immunohistochemical staining for α -SMA, gelatinase A, and MT1-MMP was performed using specific monoclonal antibodies. Representative results ($n = 6$ for each group) are shown in Figures 9–11. At 10 weeks following sham surgery, there was minimal observable staining for α -SMA in renal cortical sections (Figure 9A), as well as gelatinase A and MT1-MMP (Figure 9, B and C). In contrast, renal cortical sections from rats subjected to 5/6 nephrectomy for 10 weeks revealed dramatic changes in structure, with extensive glomerulosclerosis, interstitial fibrosis, tubular dropout, and dilation of remnant tubular structures. Intense staining for α -SMA was evident within the glomeruli and sites of interstitial fibrosis, and was particularly prominent in the tubular epithelial cells lining the dilated tubules (Figure 10A). Intense staining in tubular epithelia of dilated tubules was also observed for both MT1-MMP and gelatinase A proteins (Figure 10, B and C). Staining for all three proteins was particularly intense at foci of myofibroblastic proliferation and migration through disrupted tubular basement membranes (Figure 11, A–C, respectively). The results of the histochemical studies were confirmed by Western blots of renal cortical extracts, in which levels of SMA-actin, gelatinase A, and MT1-MMP proteins were greatly increased at 10 weeks

Figure 9. Immunohistochemical staining of sham-operated rat renal cortical tissues. There is no significant staining for α -SMA, MT1-MMP, or gelatinase A (A–C, respectively). (Magnification, $\times 200$).

as compared to controls (not shown). In conjunction with our *in vitro* data, the 5/6 nephrectomy studies provide compelling evidence that during the acquisition of the α -SMA+ phenotype, tubular epithelial cells co-express MT1-MMP and gelatinase A, thereby driving the transition to the proliferative and migratory myofibroblast.

Discussion

The transformation (or transdifferentiation) of epithelial cells to a mesenchymal or fibroblastic phenotype is a critical feature of tissue morphogenesis.⁴⁸ Epithelial-mesenchymal transformation also plays a central role in the development of neoplasia in epithelial tissues and has been extensively analyzed in the evolution of breast and colonic carcinomas.^{49–53} Tissue fibrosis occurring as a consequence of epithelial-mesenchymal transformation has been demonstrated for pulmonary, hepatic, and renal diseases, and an extensive body of evidence has

highlighted TGF- β 1 as the dominant peptide mediator of this process.^{17,30,48,54–57} The conversion of highly polarized epithelial cell structures with prominent cell-cell and cell-matrix attachments to a migratory, non-polarized phenotype characterized by limited cell-cell interaction is associated with profound rearrangements in multiple cellular systems. For example, a transcriptome screen of TGF- β 1-treated epidermal cells undergoing epithelial-mesenchymal transition suggested that nearly 10% of all expressed genes (~ 4000) manifested significant changes in expression levels.⁵⁸ Pathway mapping and functional module assessment demonstrated major changes in the expression levels of genes in signaling cascades, cell-cell and cell-matrix interactions, motility, and mesenchymal development.⁵⁸ The remarkable complexity of the genetic consequences of TGF- β 1-mediated epithelial-mesenchymal transformation underscores the significance of this study, in which a single, TGF- β 1-dependent gene product, gelatinase A, was found to be

Figure 10. Immunohistochemical staining at 6 weeks following 5/6 nephrectomy. There is extensive tubular atrophy and dropout, associated with interstitial fibrosis and glomerular segmental sclerosis. Staining for α -SMA (A) is notable within the sclerotic glomeruli, interstitium and in the tubular epithelial cells of the dilated tubules (arrowheads). There is a virtually identical pattern of staining for MT1-MMP (B), and gelatinase A (C), which is very intense within the tubular epithelial cells of dilated tubules (arrowheads). (Magnification, $\times 200$).

Figure 11. Concordant expression of α -SMA, MT1-MMP, and gelatinase A at sites of tubular epithelial cell proliferation and basal lamina disruption at 6 weeks following 5/6 nephrectomy. Clusters of proliferating tubular epithelial cells undergoing transdifferentiation concordantly express α -SMA (A), MT1-MMP (B), and gelatinase A (C). (Magnification, $\times 350$).

both necessary and sufficient for the epithelial-mesenchymal transformation of renal tubular epithelial cells.

Enzymatically active gelatinase A was required for the induction of tubular epithelial cell transformation and this effect could not be recapitulated using the closely related gelatinase B (MMP-9). While both enzymes are similar in terms of protein structure, they manifest discrete patterns of substrate specificity. Gelatinase B has a higher degree of activity for types IV and V collagens, while gelatinase A can additionally degrade fibronectin and laminins.^{59,60} MT1-MMP, first identified as the cell-surface associated activator of gelatinase A, also exhibits catalytic activity against several extracellular matrix proteins, including native types I and III collagens, laminin, and fibronectin.^{61–66} Notably, recombinant MT1-MMP has no activity against type IV collagen.⁶⁷ While all three enzymes cleave the canonical Pro-X-X-/X_H-Y peptide sequence characteristic of collagenous substrates, Smith and colleagues^{68–70} have recently demonstrated that gelatinase A, gelatinase B, and MT1-MMP have additional defined substrate recognition motifs unique to each enzyme. These observations suggest that the absolute requirement for gelatinase A in renal tubular epithelial-mesenchymal transformation, as opposed to gelatinase B or MT1-MMP (note the cyclic peptide inhibitor used in this study does not inhibit MT1-MMP), presumably reflects enzyme-specific cleavage of heretofore undefined target substrates. Gelatinase A may also amplify the transformation process in a paracrine manner through the proteolytic generation of active TGF- β 1 peptide.

Treatment of defined extracellular matrix substrates with purified or recombinant matrix metalloproteinases has demonstrated the release of cryptic, biologically active cleavage products. For example, Xu et al⁷¹ exposed a cryptic site with type IV collagen by digestion with gelatinase A. The cryptic fragment induced mobility of cultured endothelial cells and co-localized with active gelatinase A at sites of angiogenesis. Giannelli et al⁶⁰ demonstrated that gelatinase A cleavage of laminin-5, a

major component of the tubular basement membrane, releases a cryptic domain from the γ 2 subunit that promotes breast epithelial cell migration. Recently, Gilles et al⁴⁹ found that MT1-MMP was also capable of releasing the γ 2 subunit fragment. In each of these studies, the γ 2 subunit stimulated cellular migration without evidence for a complete epithelial-mesenchymal transformation. Thus, one potential mechanism of action for gelatinase A-mediated epithelial-mesenchymal transformation could be the generation of specific biologically active extracellular matrix cleavage products.

Enzymatic disruption of cell-cell or cell-matrix attachments has been suggested as an alternative mechanism for induction of epithelial-mesenchymal transformation. Induction of stromelysin-1 expression in the murine mammary epithelial cell line SCp2 triggers a mesenchymal conversion associated with loss of E-cadherin and disruption of cell-cell contacts.⁷² In contrast, integrin alterations were relatively insignificant, suggesting that within the cellular context of this system that disruption of cell-matrix interactions is not a major driving force in the transformation process. Ho et al⁷³ used matrix metalloproteinase inhibition to investigate the relative roles of cell-cell and cell-matrix interaction. MMP inhibition resulted in stabilization of cell-cell contracts with redistribution of p125^{FAK} to sites of contact. In addition, MMP inhibition resulted in increased cadherin levels and augmented calcium-dependent cellular aggregation. Based on these findings, it was proposed that MMP inhibitors act to stabilize both cell-cell and cell-matrix interactions.

TGF- β 1 induced the synthesis of both gelatinase A and MT1-MMP, and transcriptional reporter studies indicate that at least one component of the enhanced synthesis of these enzymes is the consequence of transcriptional activation. The proximal promoters of both these genes include functional overlapping Sp1/Sp3 (gelatinase A)⁷⁴ and Sp1/Egr-1 (MT1-MMP)⁷⁵ sites and the increases in transcriptional activity of these promoters by TGF- β 1 may be the consequence of the recently reported interaction

of Smad3/Smad4 proteins with Sp-1.^{76–78} This interaction, as a coordinated mechanism for TGF- β 1 transcriptional regulation of these two genes, is currently under investigation.

Notably, only a subset of the NRK52e cells exposed to TGF- β 1 manifested the changes characteristic of the epithelial-mesenchymal transformation. These cells were located on the periphery of the typical epithelial growth islands of the NRK52e cells at sites of active proliferation and had diminished staining for E-cadherin and loss of cell-cell contacts, as opposed to the cells within the middle of the growth islands. E-cadherin has been recently shown to suppress MT1-MMP transcription by suppression of MAP kinase (ERK) activity, suggesting that stabilization of cadherin-dependent cell-cell interaction prevents MT1-MMP expression, gelatinase A activation, and initiation of the epithelial-mesenchymal transformation.⁷⁹

Given the fact that the epithelial-mesenchymal transformation of tubular epithelial cells displays an absolute requirement for enzymatically active gelatinase A and the recent demonstration that there are uniquely specific cleavage consensus motifs for this enzyme,⁷⁰ we are examining defined subsets of cell-associated and extracellular matrix proteins for the existence of such motifs to assemble a list of candidate target proteins. These efforts may be expected to provide further insight into the precise means by which gelatinase A triggers the epithelial-mesenchymal transformation. Nonetheless, the determination that the blockade of a single matrix metalloproteinase is sufficient to halt the renal tubular epithelial cell transformation program has major clinical implications. The crystallization of gelatinase A and the determination of enzyme-specific substrates will facilitate the development of specific gelatinase A inhibitors suitable for testing *in vitro* and in animal models of renal transdifferentiation.^{70,80} Gelatinase A-specific inhibition may potentially offer a novel approach for the treatment of progressive renal fibrosis.

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