An imbalance between matrix metalloproteinase-2 and tissue inhibitor of matrix metalloproteinase-2 contributes to the development of early diabetic nephropathy

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Abstract

Background. High glucose and angiotensin-II (Ang-II) levels are the known important mediators of diabetic nephropathy. However, the effects of these mediators on matrix metalloproteinase-2 (MMP-2) and on tissue inhibitor of metalloproteinase-2 (TIMP-2) in proximal tubule cells have yet to be fully examined within the context of early stage diabetic nephropathy.

Methods. In this study, we attempted to characterize changes in MMP-2 and TIMP-2 in streptozotocin-induced diabetic rats. To further examine the molecular mechanisms involved, we evaluated the effects of high glucose (30 mM) or Ang-II on MMP-2, TIMP-2 and collagen synthesis in proximal tubule cells, and investigated whether MMP-2 and TIMP-2 are regulated via the TGF-β1 pathway.

Results. In streptozotocin-induced diabetic rats, TIMP-2 mRNA and protein levels were significantly higher than in controls. Urinary protein excretion also showed a significant positive correlation with glomerular and tubular TIMP-2 protein expressions, and a negative correlation with MMP-2 expression. In cultured cells, both high glucose and Ang-II induced significant increases in TGF-β1, TIMP-2, and in collagen synthesis, and significant decreases in MMP-2 gene expression and activity, and thus disrupted the balance between MMP-2 and TIMP-2. Moreover, treatment with a selective angiotensin type 1 (AT1) receptor antagonist significantly inhibited Ang-II mediated changes in TGF-β1, MMP-2, TIMP-2, and in collagen production, suggesting the role of the AT1 receptor. The addition of exogenous TGF-β1 produced an effect similar to those of high glucose and Ang-II. Furthermore, the inhibition of TGF-β1 prevented Ang-II-induced MMP-2 and TIMP-2 alterations, suggesting the involvement of a TGF-β1 pathway.

Conclusions. High glucose or Ang-II treatment induce alterations in MMP-2 and TIMP-2 balance, which favour TIMP-2 over-activity. Moreover, Ang-II mediated changes in the productions of MMP-2 and TIMP-2 occur via AT1 receptors and a TGF-β1-dependent mechanism. These results suggest that an imbalance between the MMP-2 and TIMP-2, caused primarily by an increase in TIMP-2 activity, contributes to the pathogenesis of diabetic nephropathy.

Keywords: angiotensin II; diabetic nephropathy; high glucose; matrix metalloproteinase-2; proximal tubule cell; tissue inhibitor of matrix metalloproteinase-2

Introduction

Imbalances between the syntheses and degradations of glomerular extracellular matrix (ECM) proteins are thought to play important roles in the progression of glomerular sclerosis in diabetic nephropathy. Both tissue inhibitor metalloproteinase-2 (TIMP-2) and plasminogen activator inhibitor 1 inhibit the degradation of matrix proteins like collagen. Alternatively, matrix metalloproteinase-2 (MMP-2), tissue plasminogen activator and urine plasminogen activator all degrade matrix proteins [1].

The roles of MMP and TIMP within the context of diabetes remain controversial. The expression of glomerular MMP-2 was found to be reduced, and TIMP-2 to be increased, in the kidney tissues of early diabetic patients and in those of experimental rats [2,3]. However, TIMP-2 levels remained unchanged in obese Zucker rats [4], although the stimulation of cultured...
mesangial cells with high levels of glucose increased MMP-2 expression in cultured mesangial cells [5].

A number of observations suggest that alterations in the tubulointerstitium of diabetic kidneys are important for the ultimate development of renal insufficiency [6–8]. A strong correlation exists between interstitial expansion and glomerulosclerosis via alterations in renal blood flow, altered haemodynamics, and the direct effect of glucose on tubular structures, which all act to promote collagen deposition and the activations of profibrogenic cytokines and growth factors [9]. Furthermore, increasing evidence suggests that tubular epithelial cells participate in epithelial–mesenchymal transdifferentiation, and ultimately interstitial fibrosis [10], which suggests that tubular cells also participate in the progression of diabetic nephropathy. Moreover, of the tubule cell types, proximal tubule cells contact and reabsorb a variety of glomerular filtrates, including glucose and protein.

In the present study, we attempted to characterize the effects of high glucose or angiotensin-II (Ang-II) stimulation on the synthesis of MMP-2, TIMP-2, TGF-β1 and collagen in human proximal tubule cells. We focused on MMP-2 and TIMP-2 in these experiments, because these are the main regulators of type IV collagen metabolism, which is the most important ECM protein in diabetic nephropathy. To further examine the mechanism underlying the regulatory effects of Ang-II on MMP-2 and TIMP-2, we examined the effects of selective angiotensin type 1 and type 2 receptor antagonists on the Ang-II-induced regulations of MMP-2 and TIMP-2 and on collagen production. In addition, we examined whether the high glucose or Ang-II-induced imbalance between MMP-2 and TIMP-2 is mediated through a TGF-β1-dependent mechanism. Finally, we also investigated whether changes in MMP-2 and TIMP-2 expression are associated with type IV collagen synthesis during the 4-week post-diabetes induction period. This period represents early stage nephropathy, during which diabetes-specific morphological changes in streptozotocin (STZ)-induced diabetic rats remain minimal.

Subjects and methods

Human proximal tubule cell culture

Human kidney-2 (HK-2) cells, a line of immortalized proximal tubular epithelial cells derived from normal adult human kidneys, were acquired from the American Type Culture Collection (Rockville, MD, USA). These cells were cultivated in DMEM:F12 medium, supplemented with 5% heat-inactivated fetal bovine serum (FBS), 100 U/ml of penicillin, 100 μg/ml of streptomycin, 2 mM of L-glutamine, 5 ng/ml of sodium selenite, 5 μg/ml of transferrin, 5 μg/ml of thyroxine(T3), 5 ng/ml of hydrocortisone, 5 pg/ml of prosta-glandin E1 (PGE1) and 10 ng/ml of epidermal growth factor. In this experiment, the cells used were in the 5th, 6th or 7th passage. Subconflucent cell monolayers were then cultured for 24 h in serum and growth factor-free media, containing 5 mmol/l of d-glucose and 1% FBS. The study involved two experimental groups. The normal glucose group (NG group) was composed of confluent cell monolayers cultured with 5 mmol/l of d-glucose, whereas the high glucose group (HG group) was treated with 30 mmol/l of d-glucose. Mannitol was used as an osmotic control, i.e. a medium containing 5 mmol/l of d-glucose and 25 mmol/l of mannitol. In the Ang-II stimulation group, Ang-II was added to the culture media, at final concentrations of 1 nM or 10 nM, after 24 h of serum deprivation. To define the mechanism of Ang-II-mediated MMP-2 and TIMP-2 synthesis, we used both Ang-II receptor type 1 (AT1) antagonist (L158809) and Ang-II receptor type 2 (AT2) antagonist (PD123319). These drugs were added to tubular epithelial cells 1 h before Ang-II treatment at a final concentration of 100 nM. In addition, to evaluate whether high glucose (30 mM) or Ang-II-induced MMP-2 and TIMP-2 regulations are mediated by transforming growth factor (TGF)-β1, TGF-β1 gene expression and protein synthesis were examined in response to treatments with high glucose or Ang-II. To further confirm the dependencies of MMP-2 and TIMP-2 syntheses on the TGF-β1 pathway, anti-TGF-β1 neutralizing antibody (Genzyme, Cambridge, MA, USA) was administered at a final concentration of 10 μg/ml 1 h prior to HG or Ang-II treatment. To evaluate the direct effect of TGF-β1 on the syntheses of MMP-2 and TIMP-2, we treated cells with recombinant human TGF-β1 (R & D Systems, Minneapolis, MN, USA) in NG medium at a concentration of 2 ng/ml. All experimental groups were cultured in triplicate for 72 h, and were then harvested for total RNA and protein extraction. In order to preclude any serum-associated confounding effects on MMP-2 and TIMP-2 synthesis, all experiments were performed in serum-free media. The results are representative of the three independent experiments.

RNA extraction and analysis of gene expressions by real-time quantitative polymerase chain reaction (PCR) in tissues and cells

Total RNA was extracted from renal cortical tissues and cells using Trizol reagent and further purified using RNeasy Mini kits (Qiagen, Valencia, CA, USA). cDNA was synthesized via reverse transcription, using RNA PCR kits (Applied Biosystems, Roche Inc., Foster City, CA, USA), in a 20 μl mixture containing 1 μg RNA, 50 mM KCl, 10 mM Tris-HCl, 5 mM MgCl2, 1 mM of each deoxyribonucleotide triphosphate (dNTPs), oligo-(dT) primer, 20 units of RNase inhibitor and 50 units of MuLV reverse transcriptase. Reaction mixtures were then incubated for 1 h at 42°C, and then heated at 90°C for 7 min in a thermocycler (GeneAmp PCR system 9600, Perkin Elmer, Roche Molecular System, Branchburg, NJ, USA). Primer sequences are shown in Table 1. Gene expressions were quantified using a LightCycler (Roche, Mannheim, Germany) using SYBR Green technology. The specificity of each PCR product was evaluated by the melting curve analysis, followed by an agarose gel electrophoresis to confirm the presence of a single clean band. PCR efficiency was tested using serially diluted cDNA samples. For amplification, a 10 μl SYBR Green master mix was added to 1 μl of cDNA (corresponding to 50 ng of total RNA), 900 nM of forward and reverse primers in a reaction volume of 20 μl. The real-time reverse transcription–polymerase chain reaction (RT–PCR) was performed over 45 cycles of
transcribed mRNA sample was used as a negative control. The mRNA level of each sample was normalized PC-

ZnCl2 and 2.5% Triton X-100, and then incubated overnight

Human TGF-

Human TIMP-2, reverse AGA TGT AGC ACG GGA TCA TGG G

Human TIMP-2, forward CTC TGG AAA CGA CAT TTA TGG C 332

Human MMP-2, reverse GAA CCA TCA CTA TGT GGG

Human MMP-2, forward ATA ACC TGG ATG CCG TCG TG 778

MMP-2, matrix metalloproteinase-2; TIMP-2, tissue inhibitor of matrix metalloproteinase-2; TGF-

b

Rat PC

Rat TIMP-2, reverse CAA GAA CCA TCA CTT CTC TTG

Rat TIMP-2, forward ATT TAT CTA CAC GGC CCC 322

Rat MMP-2, reverse CAA TCT TTT CTG GGA GCT C

Rat MMP-2, forward GCT GAT ACT GAC ACT GGT ACT G 199

MMP-2 activity by gelatin zymography

in tissues and cells

To measure MMP-2 activity in kidneys, renal cortical tissues were homogenized in glass homogenizers using lysis buffer (150 mM NaCl, 50 mM Tris–HCl, pH 8.0, 1% Triton X-100, 1 mM phenylmethanesulfonylfluoride), and total protein concentrations were quantified with advanced protein assay reagent (Bio-Rad, Hercules, CA, USA) using a spectrophotometer. An aliquot of 40 μg of proteins were then electrophoresed on 12% SDS-polyacrylamide gels (Tefco Co., Tokyo, Japan) under denaturing conditions. Proteins were then transferred onto polyvinylidene difluoride membranes (Immobilon-P, Millipore, Bedford, MA, USA) for 120 min at 250 mA. After blocking in blocking solution (1 × PBS, 0.15% Tween-20, 5% non-fat milk) for 1 h at room temperature, membranes were hybridized overnight at 4°C using mouse monoclonal TIMP-2 antibody (Oncogene, Cambridge, MA) diluted to 1:1000. Membranes were then washed four times with phosphate buffered saline Tween-20 (PBST), and incubated for 60 min at room temperature with horseradish peroxidase-conjugated secondary antibody, diluted to 1:1000. Specific signals were detected using the ECL method (Amersham, Buckinghamshire, UK).

Western blotting for TIMP-2 protein

in tissues and cells

The cells and homogenized renal cortical tissues were lysed in lysis buffer (150 mM NaCl, 50 mM Tris–HCl, pH 8.0, 1% Triton X-100, 1 mM phenylmethanesulfonylfluoride), and total protein concentrations were quantified with advanced protein assay reagent (Bio-Rad, Hercules, CA, USA) using a spectrophotometer. An aliquot of 40 μg of proteins were then electrophoresed on 12% SDS-polyacrylamide gels (Tefco Co., Tokyo, Japan) under denaturing conditions. Proteins were then transferred onto polyvinylidene difluoride membranes (Immobilon-P, Millipore, Bedford, MA, USA) for 120 min at 250 mA. After blocking in blocking solution (1 × PBS, 0.15% Tween-20, 5% non-fat milk) for 1 h at room temperature, membranes were hybridized overnight at 4°C using mouse monoclonal TIMP-2 antibody (Oncogene, Cambridge, MA) diluted to 1:1000. Membranes were then washed four times with phosphate buffered saline Tween-20 (PBST), and incubated for 60 min at room temperature with horseradish peroxidase-conjugated secondary antibody, diluted to 1:1000. Specific signals were detected using the ECL method (Amersham, Buckinghamshire, UK).

Measurements of the amounts of TGF-β1

protein in culture supernatant

TGF-β1 amounts secreted into the culture medium were quantified using a commercially available ELISA kit (R & D systems, Abingdon, OX, UK). Total (active plus latent fractions) TGF-β1 was measured after acid activating with 1N HCl for 10 min at room temperature and neutralizing with 1.2N NaOH/0.5M HEPES. Samples were then applied to microtitre plates pre-coated with TGF-β1, incubated for 2h, washed three times, and horseradish peroxidase (HRP)-conjugated anti-TGF-β1 antibody was then applied to each well for 2h at room temperature. The plates were then washed five times, and developed with 100μl of colour reagent per well. Colour intensities were measured using an

Table 1. Primer sequences for real-time quantitative PCR

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<th>Target gene</th>
<th>Primer sequence (5’ to 3’)</th>
<th>Amplicon length (bp)</th>
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<tr>
<td>Human MMP-2, forward</td>
<td>ATA ACC TGG ATG CCG TCG TG</td>
<td>778</td>
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<td>Human MMP-2, reverse</td>
<td>GAA CCA TCA CTA TGT GGG</td>
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<tr>
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<td>332</td>
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<tr>
<td>Human TIMP-2, reverse</td>
<td>AGA TGT AGC ACG GGA TCA TGG G</td>
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<tr>
<td>Human TGF-β1, forward</td>
<td>GGA GGG GAA ATT GAG GCC TTT CGC</td>
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<tr>
<td>Human TGF-β1, reverse</td>
<td>TTA TGC TGG TGT TAC AGG GC</td>
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<tr>
<td>Human β-actin, forward</td>
<td>CCC CAG GCA CCA GGG CGT GAT</td>
<td>263</td>
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<td>Human β-actin, reverse</td>
<td>GGT CAT CTT CTC GCG GTT GGC CTT GGG GT</td>
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<tr>
<td>Rat MMP-2, forward</td>
<td>GCT GAT ACT GAC ACT GGT ACT G</td>
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<td>Rat MMP-2, reverse</td>
<td>CAA TCT TTT CTG GGA GCT C</td>
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</tr>
<tr>
<td>Rat TIMP-2, forward</td>
<td>ATT TAT CTA CAC GGC CCC</td>
<td>322</td>
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<tr>
<td>Rat TIMP-2, reverse</td>
<td>CAA GAA CCA TCA CTT CTC TTG</td>
<td></td>
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<tr>
<td>Rat PC2(l,IV), forward</td>
<td>TAG GTG TCA GCA ATT AGG CAG G</td>
<td>484</td>
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<td>Rat PC2(l,IV), reverse</td>
<td>CGG ACC ACT ATG CTT GAA GTG A</td>
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<tr>
<td>Rat β-actin, reverse</td>
<td>TCA TGA GGT AGT CCG TCA GG</td>
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enzyme-linked immunosorbent assay (ELISA) reader at 450 nm. To control for differences in cell numbers in wells, concentrations were corrected vs total cell protein.

Measurement of secreted collagen in cultured proximal tubule cells

Total soluble collagen was measured in culture supernatants using Sircol™ soluble collagen assay kits (Biocolor, Belfast, N. Ireland). Briefly, 1 ml of Sirius red reagent was added to 10 μl of test sample and mixed for 60 min at room temperature. The collagen-dye complex formed was precipitated by centrifugation at 14,000 g for 10 min. To release the bound dye, 1 ml of alkaline reagent (0.5 M NaOH) was added to the precipitate and absorbance was then measured at 540 nm using an ELISA reader.

Animal study and experimental design

Male Sprague-Dawley rats, each weighing 200–220 g, were divided into two groups: the control group (n = 10) and the STZ-induced diabetic group (n = 14). Diabetes mellitus was induced by a single intraperitoneal (i.p.) injection of STZ, 65 mg/kg body weight (Sigma-Aldrich, St Louis, MO). STZ rats were confirmed to have diabetes when tail blood sugar concentrations were >300 mg/dl, 48 h after i.p. STZ treatment. Animals were given free access to rat chow and water, and caged individually. Body weights were recorded, and 24h urine samples were collected in a metabolic cage. Blood samples were obtained when the animals were sacrificed 2 days or 4 weeks after STZ treatment, under anesthesia by injecting sodium pentobarbital i.p. at 50 mg/kg body weight. Plasma glucose levels were measured by a method predicated on glucose oxidase. Using a nephelometric method, 24h urine protein levels were measured using sulfosalicylic acid. Creatinine levels were assessed using a modified version of the Jaffe method. All experimental kidneys were perfused with normal saline prior to removal, and stored at −70°C for analysis. This study was performed in accordance with our institutional guidelines for animal research.

Immunohistochemical staining for MMP-2, TIMP-2 and type IV collagen

After perfusion with normal saline, one kidney/per animal was processed for periodic acid-Schiff (PAS) and immunohistochemical study, and the other was immediately frozen in liquid nitrogen for subsequent RNA extraction and zymographic analysis. After paraffin removal with xylene and dehydration in a graded alcohol series, slides were immersed in distilled water. For immunohistochemical staining, renal tissues were immediately fixed in 10% neutral buffered formalin, cast in paraffin, sliced into 4 μm-thick sections, and positioned onto microscope slides. Kidney sections were transferred to a 10 mmol/l D-mannitol as an osmotic control solution at a pH of 6.0, and then heated at 80°C for 30 min to retrieve antigens for MMP-2 and TIMP-2 staining, or transferred to a 1 M EDTA buffer solution (pH 8.0) and microwaved for 10 min for antigen retrieval prior to type IV collagen staining. After washing in water, 3.0% H2O2 in methanol was applied for 20 min, in order to block endogenous peroxidase activity. To prevent non-specific staining, slides were incubated with normal goat serum at room temperature for 20 min. Mouse monoclonal MMP-2 and TIMP-2 antibodies (1:500, Oncogene, Cambridge, MA) and rabbit polyclonal antitype IV collagen (1:200, Santa Cruz Biotechnology, Santa Cruz, CA, USA) were added for 1 h at room temperature. Negative control sections were then stained under identical conditions by substituting primary antibody for equivalent concentrations of normal mouse or rabbit IgG. Using an LASB2 kit/HRP (DAKO, Carpantaria, CA, USA), kidney sections were treated sequentially with normal goat serum, primary antibody, biotinylated secondary antibody, streptavidin–horseradish peroxidase-conjugated tertiary antibody and diaminobenzidine. Sections were then counterstained with Mayer’s haematoxylin. To evaluate MMP-2, TIMP-2 and type IV collagen staining, each glomerular or tubulo-interstitial grid field measuring 0.245 mm² was graded semi-quantitatively. The scores reflect changes in the extent rather than in the intensity of staining, and were defined on the basis of the percentage of positive grid fields or glomeruli. Cases in which more than 5% of the resident cells showed positive staining were regarded as positive. Four scores were awarded: 0, absent or <25% positive staining; 1, 25–50% positive staining; 2, 50–75% positive staining and 3, >75% positive staining. For each sample, 50–60 glomeruli and 20 consecutive interstitial fields, including tubular segments, were examined, and average scores were calculated. Slides were scored by an observer unaware of the experimental details.

Statistical analysis

We used non-parametric analysis due to the small number of samples. Results were expressed as mean ± SEM. The Kruskal–Wallis test was used to compare more than two groups, and was followed by the Mann–Whitney U test in SPSS for Windows 10.0 (SPSS Inc., Chicago, IL, USA). Correlations between urinary protein excretion and renal MMP-2 and TIMP-2 immunostaining scores were examined using Spearman’s rank correlation test. P-values of <0.05 were considered statistically significant.

Results

MMP-2 changes in response to high glucose or angiotensin II stimulation in cultured human proximal tubule cells

In this experiment, the expression of MMP-2 mRNA was reduced under high glucose conditions. Cells exposed to 25 mmol/l D-mannitol as an osmotic control exhibited no differences with regard to MMP-2 expression vs the normal glucose group. Ang-II was shown to significantly suppress MMP-2 gene expression in a dose-dependent manner. To define the roles of angiotensin type 1 and type 2 receptors in Ang-II-mediated MMP-2 mRNA down-regulation, tubule cells were pre-incubated with L158809 (a selective AT1 receptor antagonist) and PD123319 (a selective AT2 receptor antagonist). Supplementation with 100 nM L158809 significantly prevented the down-regulation of MMP-2 mRNA expression induced by...
Ang-II. However, PD123319 did not have a significant effect on MMP-2 mRNA expression (Figure 1A). In order to verify that this decrease in gene expression was associated with the enzymatic activity of MMP-2, we conducted zymographic analysis in conditioned media, and witnessed a similar pattern of the degradative capacity of tubule cells, such as gene expression. Both high glucose and Ang-II treatment suppressed MMP-2 activity. Pre-treatment with L158809 completely restored degradative capacity to control level under Ang-II stimulation. However, PD123319 pre-treatment did not change MMP-2 activity vs the Ang-II treatment group (Figure 1B).

TIMP-2 expression changes in response to high glucose or angiotensin II stimulation in cultured human proximal tubule cells

TIMP-2 gene expression tended to increase after stimulation with high glucose, although this tendency did not reach statistical significance. Cells exposed to 25 mmol/l D-mannitol as an osmotic control showed no TIMP-2 expressional differences vs the normal glucose group. Treatment with 1 nM Ang-II increased TIMP-2 gene expression, although this was not significant. However, supplementation with 10 nM of Ang-II significantly up-regulated TIMP-2 mRNA expression (Figure 1C). TIMP-2 protein synthesis also markedly increased after Ang-II stimulation in a dose-dependent manner (Figure 1D). Similar to MMP-2 gene expression, angiotensin II-induced TIMP mRNA expression was abolished by pre-treatment with L158809, but not by PD123319 (Figure 1C). As for TIMP-2 gene expression, TIMP-2 protein expression also demonstrated that L158809 pre-treatment prevented Ang-II-induced TIMP-2 protein production (Figure 1D).

Roles of TGF-β1 in glucose and Ang-II-induced MMP-2 and TIMP-2 changes associated with collagen synthesis in cultured human proximal tubule cell

Since high glucose and Ang-II both induced MMP-2 and TIMP-2 dysregulation, favouring overactivity of...
TIMP-2, we further examined whether alteration of MMP-2 and TIMP-2, induced by high glucose or Ang-II, increased collagen content in culture supernatant. As shown in Figure 2A, 10 nM Ang-II significantly increased collagen content in the supernatant, and this was abolished by L158809. Because TGF-β1 is an important factor that mediates the actions of high glucose and Ang-II, it may be that high glucose or Ang-II affect MMP-2 and TIMP-2 regulation through the TGF-β1 pathway. To determine whether high glucose and Ang-II-induced MMP-2 and TIMP-2 regulations were mediated by TGF-β1, TGF-β1 gene expression and protein synthesis were examined in response to high glucose or Ang-II. As expected, both high glucose and Ang-II, partially through the AT1 receptor, markedly up-regulated TGF-β1 gene expression and protein synthesis (Figure 2B and C). Next, we examined the effect of exogenous TGF-β1 on the productions of MMP-2 and TIMP-2 to elucidate the role of TGF-β1 in the regulation of MMP-2 and TIMP-2. Exogenous TGF-β1 dramatically reduced MMP-2 gene expression and its activity (Figure 1A and B), and increased TIMP-2 gene expression and protein synthesis in the same manner as high glucose or angiotensin II (Figure 1C and D). To further confirm the dependencies of MMP-2 and TIMP-2 synthesis on the TGF-β1 pathway, anti-TGF-β1 neutralizing antibody was administered 1 h before high glucose or Ang-II. It was found that the inhibition of TGF-β1 by anti-TGF-β1 significantly prevented Ang-II-induced MMP-2 and TIMP-2 changes. However, this inhibitory effect was not observed on the effect of high glucose (Figures 1A, C, D and 2D).

Changes in MMP-2, TIMP-2 and type IV collagen gene expressions in experimental animals

Blood glucose concentrations were found to be higher at 2 days post-induction in STZ-induced diabetic rats than in control rats, and this persisted at 4 weeks.
At 2 days post-induction, no differences in 24 h proteinuria were noted between control and diabetic rats. However, proteinuria increased significantly in diabetic rats ($0.58 \pm 0.54$ vs $1.25 \pm 0.69$ mg/mg Cr, $P < 0.001$) at 4 weeks (Table 2). The renal expressions of the MMP-2 gene were similar in diabetic and control rats (Figure 3A). The activity of MMP-2, determined zymographically, did not show significant change in diabetic and control rats which was similar to gene expression (Figure 3B). In contrast, TIMP-2 gene expression increased by more than 3-fold at 4 weeks vs the controls (Figure 3C). As was observed for its gene expression, the synthesis of TIMP-2 protein was higher in diabetic rats than in control rats at 4 weeks.

**Table 2. Basic characteristics of control and diabetic rats**

<table>
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<tr>
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<th>2 days</th>
<th>4 weeks</th>
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<tbody>
<tr>
<td></td>
<td>DM(−)</td>
<td>DM(+)</td>
</tr>
<tr>
<td>Blood glucose (mg/dl)</td>
<td>111.3 ± 9.29</td>
<td>554.2 ± 79.1*</td>
</tr>
<tr>
<td>Urine amount (ml/day)</td>
<td>8.5 ± 2.12</td>
<td>51.5 ± 4.43*</td>
</tr>
<tr>
<td>Proteinuria (mg/mg Cr)</td>
<td>0.62 ± 0.23</td>
<td>0.91 ± 0.43</td>
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</table>

Values are expressed as mean ± SEM. Statistical differences were determined between groups at the same time of post-diabetes induction with the same duration of experimental period. *$P < 0.001$ vs control rats.

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**Fig. 3. Changes in MMP-2 and TIMP-2 mRNA and protein, and in type IV collagen mRNA in the experimental animals.**

(A) MMP-2 mRNA expression in renal cortices was measured by real-time quantitative RT–PCR. (B) MMP-2 activity in the renal cortical tissues in the experimental animals. (C) Expressions of TIMP-2 mRNA in renal cortices were measured by real-time quantitative reverse RT–PCR. (D) Representative western blot analysis of TIMP-2 protein from renal cortical tissues. (E) Expressions of type IV collagen mRNA in renal cortices were measured by real-time quantitative RT–PCR. Total RNA and protein were extracted from the renal cortices of normal controls and streptozotocin-induced diabetic rats at different times after diabetes induction. Gelatinolytic MMP-2 activity is shown as a 72 kDa band. TIMP-2 protein was detected as a single band of ~21 kDa. *$P < 0.05$, **$P < 0.01$ vs control rats.
post-induction (Figure 3D). Moreover, renal cortical
type IV collagen mRNA expression in diabetic rats was
significantly up-regulated to almost three times that of
the control level (Figure 3E).

**Histological changes and immunostaining for MMP-2, TIMP-2 and type IV collagen in experimental animals**

Figure 4 shows representative renal pathologies of the
experimental rats at each time point. No significant
changes in glomerular and tubulointerstitial morpho-
ologies were observed between the three groups
(Figure 4A–C). The expressions of MMP-2 and
TIMP-2 proteins were also evaluated by immuno-
histochemical staining. The MMP-2 staining occurred
only at a low level in control rat kidneys. Positive
staining was observed in the glomerular capillary tuft,
the apical region of proximal tubules, and in
the basolateral region of distal tubules (Figure 4D).
In the diabetic kidneys, MMP-2 immunostaining
was elevated at 2 days post-induction, and was then
reduced to the control levels at 4 weeks (Figure 4E–F).
In diabetic glomeruli, MMP-2 immunoreactivity
was mainly up-regulated in both visceral and
parietal epithelial cells at 2 days post-induction.

MMP-2 staining was also observed in apical borders
of proximal tubules, suggesting that substrates of
this enzyme are located within the tubular lumen
(Figure 4D–F). Semi-quantitative analysis of glomer-
ular MMP-2 protein expression showed a 2.5-fold
increase at 2 days post-induction vs controls, and a
decrease to the control level at 4 weeks. In the proximal
tubules of diabetic kidneys, MMP-2 expression was
increased 6-fold at 2 days, and decreased to the control
level at 4 weeks. A similar tendency was observed for
MMP-2 expression in distal tubules (Figure 5A).

Western blotting showed that TIMP-2 expression
was elevated in diabetic kidney vs control kidneys.
However, the pattern of MMP-2 distribution was
similar to the pattern shown by its specific inhibitor,
TIMP-2 (Figure 4G–I). Semi-quantitative analysis of
glomerular TIMP-2 protein expression showed a 4-fold
increase at 4 weeks in diabetic kidneys vs controls.
In proximal and distal tubules, TIMP-2 expression also
showed more than a 2-fold increase in expression
at 4 weeks in diabetic kidneys vs controls (Figure 5B).
Regarding the relationship between urinary protein
excretion and renal MMP-2 and TIMP-2 levels,
urinary protein excretion showed a significant positive
correlation with glomerular and tubular TIMP-2
protein expression, but negative correlations with
these MMP-2 expressions (Table 3). Increased collagen

![Fig. 4. Representative histological and immunostaining findings for MMP-2, TIMP-2 and type IV collagen in experimental animals. Periodic acid-Schiff (PAS) stain (A–C), immunostaining for MMP-2 (D–F), TIMP-2 (G–I), and type IV collagen (J–L). Control rats (A, D, G, J); Diabetic rats 2 days post induction (B, E, H, K); Diabetic rats 4 weeks post induction (C, F, I, L). Original magnification 200× (PAS stain), 400× (immunostaining for MMP-2, TIMP-2, type IV collagen).](image-url)
expression in diabetic rats was mainly observed in the peritubular basement membrane. However, the amount of collagen immunostaining in glomeruli was negligible in all the three groups (Figure 4J–L). Semi-quantitative analysis of type IV collagen in proximal and distal tubules showed significant elevations at 4 weeks post-induction in diabetic kidneys vs controls (Figure 5C).

Discussion

In this study, MMP-2 mRNA and protein expression were suppressed by high glucose and by Ang-II. However, the expressions of TIMP-2 mRNA and protein were significantly increased in response to Ang-II or high glucose. In diabetic rat kidneys, TIMP-2 mRNA and protein levels were significantly higher than in those of the control rats, but MMP-2 levels were similar. Moreover, urinary protein excretion was found to be positively correlated with glomerular and tubular TIMP-2 protein expression, and negatively correlated with MMP-2 expression (both \( P < 0.05 \)).

MMP types are classified on the basis of substrate specificity, e.g. collagenase which cleaves fibrillar interstitial collagen (MMP-1,8,13,18), stromelysins which cleave fibronectin and laminin (MMP-3,10,11), matrilysin which cleaves fibronectin, laminin and vitronectin (MMP-7,26) and gelatinase which cleaves non-fibrillar collagen (type IV collagen) (MMP-2,9), and the membrane-bound form of MMP which activates MMP-2. Four types of Timp have been found to inhibit these MMPs, by binding to an active site of MMPs [1]. Although all MMPs are differentially regulated, their interactions during tissue fibrosis are complex, which makes it difficult to conclude on the specific roles of MMPs in diseases characterized by matrix accumulation. In the present study, we focused on MMP-2 and TIMP-2, because they are the major causes of alterations in ECM proteins in diabetic nephropathy.

Tubular cells may contribute to tissue fibrosis by creating an imbalance between MMP and TIMP, i.e. by increasing the level of TIMP vs MMP, in the tubulointerstitial fibrosis of diabetic nephropathy. Karamessinis et al. [11] reported that an elevated glucose concentration reduced MMP-2 and TIMP-2 expressions, and related these changes to tubular basement thickening in diabetic nephropathy. Although previous reports suggested the role of Ang-II in MMP2 and TIMP2 regulation in other cell lines, including human umbilical vein endothelial cells, this contrasts with our data. However, this difference may be due to the different cell types used [12]. A growing body of evidence suggests that different MMP family members have anti-fibrotic activity in addition to proinflammatory activity [1–3,5,12,13]. The present study shows for the first time that Ang-II is able to modulate the MMP and TIMP system in proximal tubule cells.

Table 3. Correlation analysis between renal MMP-2/TIMP-2 expression and proteinuria in experimental animals

<table>
<thead>
<tr>
<th>Variables</th>
<th>R value</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glomerular MMP-2 protein</td>
<td>−4.10</td>
<td>0.04</td>
</tr>
<tr>
<td>Distal tubule MMP-2 protein</td>
<td>−0.512</td>
<td>0.015</td>
</tr>
<tr>
<td>Glomerular TIMP-2 protein</td>
<td>0.45</td>
<td>0.036</td>
</tr>
<tr>
<td>Proximal tubule TIMP-2 protein</td>
<td>0.424</td>
<td>0.049</td>
</tr>
<tr>
<td>Distal tubule TIMP-2 protein</td>
<td>0.486</td>
<td>0.022</td>
</tr>
</tbody>
</table>

Glomerular and tubular protein expressions based on the immunostaining scores for MMP-2 and TIMP-2 in renal tissues. Correlation analysis was performed using Spearman rank correlations.
Recent evidence indicates that the MMP/TIMP system has an important function in the pathogenesis of diabetic nephropathy [14]. Moreover, the exposure of proximal tubule cells to high glucose levels induces type IV collagen and fibronectin accumulation by increasing TIMP protein synthesis, which reduces the degradation of matrix proteins [15]. In support of the role of the MMP/TIMP system in diabetic nephropathy, it has been reported that reduced MMP activity and increased TIMP-1 transcription associated with the disease, are attenuated by angiotensin converting enzyme (ACE) inhibitor in type I diabetic rats [16]. Consistent with the results of a previous study [11], our results indicate that MMP-2 levels are markedly suppressed by high glucose levels. Interestingly, Ang-II exhibited more potent suppressive effects on MMP-2 gene transcription and enzymatic activity and more potent increases in TIMP-2 synthesis in proximal tubule cells. It is of note that the AT1 receptor is involved in the Ang-II-mediated regulations of MMP-2 and TIMP-2. This finding concurs with a recently reported study, which showed that angiotensin II increases the expression of TIMP-1 and collagen synthesis through AT1 receptor in skin fibroblasts [17].

TIMP-2 is an MMP-2-specific inhibitor, and is considered to be an important regulator of tissue remodelling. TIMP-2 levels were found to be up-regulated in high glucose media in cultured podocytes, which may have contributed to ECM accumulation [18]. Advanced glycation end products were found to increase the expression of TIMP-2 [19]. In addition, high levels of glucose and exogenous TGF-β1 have been shown to increase the levels of TIMP-2 in cultured mesangial cells [20]. One interesting finding, in the present study, concerned Ang-II, which reduced MMP-2 activity but concurrently increased TIMP-2 levels.

Another observation made during the present study was that Ang-II reduced MMP-2 expression and activity and increased TIMP-2 production through a TGF-β1-dependent pathway. Moreover, we observed a significant increase in TGF-β1 gene expression and protein synthesis in response to Ang-II stimulation. Furthermore, the finding that TGF-β1 inhibition by TGF-β1 antibody markedly prevented angiotensin II-induced MMP-2 and TIMP-2 dysregulation suggests that Ang-II induced MMP-2 and TIMP-2 dysregulation via a TGF-β1-dependent mechanism. In comparison, high glucose stimuli also significantly increased TGF-β1 gene expression and protein synthesis. However, the inhibition of TGF-β1 with TGF-β1 antibody did not affect high glucose-induced MMP-2 and TIMP-2 synthesis, which suggests that glucose-induced MMP-2/TIMP-2 dysregulation occurs via a TGF-β1-independent pathway. These results contrast with those of some previous studies, which suggested the presence of a TGF-β1-dependent pathway for the down-regulation of MMP-2 by high glucose [20,21]. However, these studies used mesangial cells, and it is not known whether glucose-induced MMP-2 down-regulation occurs in a TGF-β1-dependent manner in proximal tubule cells.

In the present study, we observed changes in the expressions of MMP-2 and TIMP-2, and in urinary protein excretion and type IV collagen expression during early stage diabetic nephropathy in type I diabetic rats. Four weeks after diabetes induction, when significant proteinuria had developed, TIMP-2 gene expression and protein synthesis were markedly up-regulated, in association with an increase in type IV collagen mRNA and protein production. However, MMP-2 expression in diabetic kidneys was no different from that in control rats. Taken together, relative overactivity of TIMP-2 vs MMP-2 was observed at 4 weeks post-induction in diabetic kidneys. We also noted that urinary protein excretion showed a significant positive correlation with glomerular and tubular TIMP-2 protein expression, and negative correlation with MMP-2 expression.

The major limitation of this study is that we did not demonstrate a direct causal role between MMP-2 and TIMP-2 imbalance and tissue fibrosis. In addition, MMP-2 and TIMP-2 are small components in the matrix degradation pathway, and thus, other matrix metalloproteinases and their inhibitors increase the complexity of the renal fibrosis process. However, our aim was to determine changes in MMP-2 and TIMP-2, which are the known important degraders of type IV collagen in response to high glucose or Ang-II, and to observe changes in these molecules during the early stages of diabetic nephropathy.

In conclusion, our results suggest that a significant up-regulation of renal TIMP-2 vs MMP-2 occurs during the early stage of diabetic kidneys. In addition, urinary protein excretion was found to be significantly positively correlated with glomerular and tubular TIMP-2 protein expression, and significantly negatively correlated with MMP-2 expression. In cultured proximal tubule cells, Ang-II, via AT1 receptor, was found to dysregulate MMP-2 and TIMP-2, by favouring TIMP-2 up-regulation via a TGFβ1-dependent mechanism. These results suggest that an imbalance within the MMP-2 and TIMP-2 system, has a role in the pathogenesis of diabetic nephropathy.

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Conflict of interest statement. None declared.

References


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