Original Article

Tissue inhibitor of metalloproteinase-1 exacerbated renal interstitial fibrosis through enhancing inflammation

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Abstract

Background. Tissue inhibitor of metalloproteinase-1 (TIMP-1) is associated with renal fibrosis. Furthermore, it is a multi-functional protein, and whether it has other roles in renal fibrosis is unknown. As several inflammatory mediators are substrates of matrix metalloproteinases (MMPs), TIMP-1 might affect renal fibrosis via inflammatory pathways.

Methods. Plasmids containing the sense and antisense human TIMP-1 sequence were stably transfected into the human kidney proximal tubular epithelial cell line (HKC), MMP-2 and MMP-9 siRNA were transiently transfected into HKC and the transfected cells were stimulated with phorbol 12-myristate 13-acetate (PMA). In vivo, we established unilateral ureteral obstruction (UUO) models by using homozygote human TIMP-1 transgenic mice. The expression of intercellular adhesion molecule-1 (ICAM-1) in transfected cells and F4/80-positive cells in the renal interstitium were examined by indirect immunofluorescence. Protein levels in the cells and UUO models were examined by western blot, and the activities of the gelatinases and TIMP-1 were examined by gelatin zymography and reverse zymography, respectively.

Results. After stimulation with PMA, the activities of the gelatinases were decreased, ICAM-1 was upregulated, and soluble ICAM-1 in the supernatant was decreased, in HKC transfected with sense TIMP-1, and ICAM-1 was increased in HKC transfected with MMP-9 siRNA. At 14 days after UUO, it was found that compared with wild-type mice, in transgenic mice, with upregulation of TIMP-1, activities of gelatinases were downregulated, ICAM-1, transforming growth factor-β1 (TGF-β1), collagens I and III were upregulated, and the extent of renal fibrosis and infiltration of macrophages was more severe.

Conclusion. Overexpression of TIMP-1 could promote renal interstitial fibrosis through the inflammatory pathway, which might be partly induced by upregulating ICAM-1.

Keywords: inflammation; intercellular adhesion molecule-1; renal fibrosis; tissue inhibitor of metalloproteinase-1; transgene

Introduction

Extracellular matrix (ECM) accumulation is known to be the common final stage of progressive kidney diseases. Matrix metalloproteinases (MMPs) can degrade components of ECM [1]. Activities of MMPs are inhibited by tissue inhibitors of metalloproteinases (TIMPs) through formation of non-covalent 1:1 complexes with MMPs. Four members of the TIMP family have been characterised so far, designated as TIMP-1, TIMP-2, TIMP-3, and TIMP-4. TIMP-1 is capable of inhibiting the activities of most of the MMPs, and plays a key role in maintaining the balance between ECM deposition and degradation [2,3]. With downregulation of MMPs and/or upregulation of TIMPs, degradation of ECM is inhibited. The abundance of TIMP-1 in kidneys was increased significantly in most experimental models and several human renal diseases, and the degree of TIMP-1 increase was associated with the extent of fibrosis [1,4,5]. Although data from experiments with TIMP-1 knock-out mice failed to show the protective effects of TIMP-1 deficiency in the renal fibrosis condition [6,7], it was shown in the CCL4-induced liver fibrosis model of TIMP-1 transgenic mice that TIMP-1 could promote liver fibrosis [8,9]. The reasons why TIMP-1 showed different roles in the above two models were unclear, which might be related to the difference in gene engineering. Furthermore, TIMP-1 was a multi-functional protein, thus, we considered that it might play roles during renal fibrosis via other pathways. It has been found that ECM components are not the only substrates of MMPs. Some inflammatory mediators could also be degraded by MMPs [11,12], especially ICAM-1, which is one of the major molecules involved in promoting the leukocytes firm attachment to the endothelium and transmigration through its expression on the vascular...
endothelium and the binding to β-2 leukocyte integrins [13]. ICAM-1 has been proved to be upregulated in most experimental models of renal diseases and several human renal diseases, implying that upregulation of ICAM-1 could accelerate the progress of renal lesions [14–16]. Renal injury could be attenuated by the inhibition of ICAM-1 through different approaches [17–19].

Therefore, in the present study, we investigated whether TIMP-1 might aggravate renal fibrosis by upregulating the expression of ICAM-1. Human kidney proximal tubular epithelial cell lines (HKCs) were transfected with vectors containing the sense and antisense human TIMP-1 sequence, and MMP-2, MMP-9 siRNA were transiently transfected into the HKC. Then, the change of ICAM-1 on the HKCs were observed after stimulation with phorbol 12-myristate 13-acetate (PMA). Unilateral ureteral obstruction (UUO) in wild-type and homozygote human TIMP-1 transgenic mice models were used as a model of renal interstitial fibrosis in which to explore the proposed mechanism.

**Methods**

**Design of experiment in vitro**

**Cell culture and establishment of stably transfected cell lines.** The human kidney proximal tubular epithelial cell line (HKC) generated by Racussen et al. [20] was provided by Yang from the University of Pittsburgh School of Medicine. Cells were cultured in DMEM/Ham’s F12 medium (Life Technologies, Gaithersburg, MD, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS; HyClone, Logan, UT, USA).

HKC cells were, respectively, transfected with the empty pcDNA3.1 control vector, TIMP-1S-pcDNA3.1 and TIMP-1AS-pcDNA3.1, constructed by Lin H et al. [21], using Lipofectamine 2000 (Invitrogen Corp., Carlsbad, CA, USA) according to the instructions specified by the manufacturer. Twenty-four hours after transfection and every 3–4 days thereafter, the cells were refed with fresh selective medium containing G418 (geneticin; Invitrogen Corp., Carlsbad, CA, USA) at a final concentration of 800 µg/ml. Neomycin-resistant cells were first visible after 7 days. Passage was performed if the confluent extent of the cells was about 80%. The cells were continuously cultured in selective medium for about 30 days.

Genomic integration of vectors in HKC was identified by polymerase chain reaction (PCR). The DNA primers for identification were designed as follows: neo, upper: 5′-CAG TAA GCA TTT TAA GGT CGT-3′; lower: 5′-CTT CTT AGA AAG AAC CGG ATG TC-3′; sense TIMP-1, upper: 5′-GGT TGC GAG GGG TGG TGG TTA CTC AC-3′; lower: 5′-GTT TAG ACG AAC CGG ATG TC-3′; antisense TIMP-1, upper: 5′-GGT TGC GAG GGG TGG TGG TTA CTC AC-3′; lower: 5′-GGT TGC GAG GGG TGG TGG TTA CTC AC-3′.

HKC cells were, respectively, transfected with the empty pcDNA3.1 control vector, TIMP-1S-pcDNA3.1 and TIMP-1AS-pcDNA3.1, constructed by Lin H et al. [21], using Lipofectamine 2000 (Invitrogen Corp., Carlsbad, CA, USA) according to the instructions specified by the manufacturer. Twenty-four hours after transfection and every 3–4 days thereafter, the cells were refed with fresh selective medium containing G418 (geneticin; Invitrogen Corp., Carlsbad, CA, USA) at a final concentration of 800 µg/ml. Neomycin-resistant cells were first visible after 7 days. Passage was performed if the confluent extent of the cells was about 80%. The cells were continuously cultured in selective medium for about 30 days.

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The products amplified with PCR were 790, 652, and 883 bp, respectively, which indicated that vectors were stably integrated into the genome of HKC (data not shown).

**Confluent parental/HKC, vector/HKC (HKC transfected with empty pcDNA3.1 vector), TIMP-1S/HKC (HKC transfected with TIMP-1S-pcDNA3.1) and TIMP-1AS/HKC (HKC transfected with TIMP-1AS-pcDNA3.1) were incubated in serum-free DMEM/Ham’s F12 medium for 24 h, then treated with phorbol 12-myristate 13-acetate (PMA, Sigma Aldrich Co Ltd., St Louis, MO, USA) 100 nM in serum-free medium for 18 h. Vehicle controls were treated with 1× phosphate-buffered saline (PBS). The selection of the concentration and time for PMA stimulation was based on pre-experimental results (the expression of ICAM-1 in parental/HKC achieved peak after stimulation with PMA for 18 h and 100 nM was the optimal concentration of PMA for stimulation, data not shown).

**Confocal laser scanning microscopy.** Membrane expression of ICAM-1 protein of the above four kinds of cells after treatment with PMA or vehicle was analysed with a Leica confocal laser scanning microscope using a monoclonal ICAM-1 antibody. Briefly, cells were washed with 1× PBS and fixed with 4% paraformaldehyde. Cells were rinsed with 1× PBS and blocked for 30 min with goat serum, with 1× PBS. Then incubated with a 1:50 dilution of rat anti-mouse ICAM-1 monoclonal antibody (Research Diagnostics Inc., Flanders, NJ, USA) for 2 h at room temperature. Cells were washed and incubated with a secondary FITC-conjugated goat anti-rat IgG for 30 min, and rinsed and mounted. Negative control materials used were normal rat IgG (Sigma-Aldrich, St Louis, MO, USA) in place of the primary antibody. Confocal laser scanning microscopy (CLSM) quantitation was determined by analysis of fluorescence intensity. For quantitation of fluorescence intensity, the background laser intensity was set to untreated control conditions, and all subsequent samples were scanned under these conditions so that increases in ICAM-1 signal intensity relative to controls could be determined. Fluorescence intensity was measured in 12–20 representative cells from different slides for each condition by a blinded operator and statistically analysed as described below. Each experiment was repeated three times at least.

**Detection of MMPs/TIMPs and ICAM-1 protein in HKCs with and without stimulation of PMA.** The protein of the above four kinds of cells after treatment with PMA or vehicle was extracted and analysed for the protein expression of MMPs/TIMPs and ICAM-1 by using western blot as described later.

**Co-immunoprecipitation.** Confluent parental/HKC cultured in serum-free medium with PMA or vehicle was lysed with NP-40 1% in lysis buffer as described later. The samples were precleared using a rat IgG1 (Sigma-Aldrich, St Louis, MO, USA) and G protein agarose beads (Amersham, Buckinghamshire, UK), then incubated with rat anti-mouse ICAM-1 monoclonal antibody (Research Diagnostics Inc., Flanders, NJ, USA), and control rat IgG1 (5 µg/mg of protein), respectively, for 18 h and with G protein beads (50 µl/sample) for 2 h. Then, they were washed five times with the lysis buffer. All the procedures were performed at 4°C. The samples were analysed by western blot using the antibody for MMP-2 and MMP-9.
Soluble ICAM-1 detection. Twenty millilitres of serum-free supernatants of parental/HKC, vector/HKC, TIMP-1S/HKC and TIMP-1AS/HKC after treatment with vehicle or PMA was collected and spun (1000 × g for 3 min at 4°C) to remove cell debris, then concentrated 100-fold by Amicon Ultra (Millipore, MA, USA), and protein concentration was measured using the BCA Protein Assay Kit (Pierce Biotechnology, Inc., Milwaukee, WI, USA). The samples were resuspended in non-reducing Laemmli sample buffer, resolved by 8% SDS-PAGE and transferred to a nitrocellulose membrane (Schleicher & Schuell, Dassel, Germany). The membranes were blotted with rat anti-mouse ICAM-1 monoclonal antibody. After incubation with ICAM-1 antibody blots were processed as described for western blot.

MMP-2 and MMP-9 siRNA transfection. To explore whether MMP-2 or MMP-9 could affect ICAM-1, we designed an siRNA experiment. HKCs were cultured in DMEM supplemented with 10% heat-inactivated FBS. Then, just before transfection, the cells were washed twice with PBS and plated in six-well plates at 1 × 10^6 cells per well in 2 ml of serum free DMEM medium. MMP-2, MMP-9 and irrelevant siRNA target sequence were 5'-CAT CAC CTA TTG GAT CCA A-3', 5'-GTT GGC AGT GCA ATA CCT GA-3' and 5'-GAA GAA GTA GTG CTG CTG TTT C-3', respectively. Transfection of siRNA used the Lipofectamine 2000 reagent (Invitrogen Corp., Carlsbad, CA, USA), following the protocol set by the manufacturer. Total RNA and protein of the transfected cells were isolated, then the efficiency of transfection was identified using quantitative real-time PCR and western blot as described later. The membrane-associated ICAM-1 in transfected cells after treatment with PMA or vehicle was examined by indirect immunofluorescence as described above.

Quantitative real-time PCR. Total RNA was isolated from HKCs using an RNeasy Mini Kit (Qiagen, Santa Clarita, CA, USA). First-strand cDNA was synthesised with an iScript cDNA Synthesis Kit (Bio-Rad Laboratories, Hercules, CA, USA). Primers were designed with Beacon Designer software (PREMIER Biosoft International, Palo Alto, CA, USA): MMP-2: Upper: 5'-TGA CCT TGA CCA GAA CAC-3', Lower: 5'-TGT ACG CAA TGG TCC TGT ATG-3'; MMP-9: Upper: 5'-GTG GAC TAC GAT AAG GAC GG-3', Lower: 5'-CTC AAA GAT GAA CCG GAA CAC-3'; GAPDH: Upper: 5'-GAG TTT GGC TAC AGC AAC AGG GT-3', Lower: 5'-TCT CTT CCT CTT GTG CTC TTG CTG-3'. Real-time PCR was performed in duplicates using 25 µl of the reaction mixture that contained 1.5 µl of RT mixture, 0.2 µM of both primers and 12.5 µl of iQ SYBR Green Supermix (Bio-Rad Laboratories, Hercules, CA, USA). The real-time PCR reaction was performed using the iCycler Real-Time Detection System (Bio-Rad Laboratories, Hercules, CA, USA) with a two-step method. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was amplified as control. Data were analysed with the iCycler iQ software. The average Ct (threshold cycle) of fluorescence units was used for analysis. Quantification was calculated using the Ct of the target signal relative to the GAPDH signal in the same RNA sample.

Design of experiment in vivo

Construction of UUO model. All animal procedures were performed according to approved protocols and in accordance with recommendations for the proper care and use of laboratory animals. We used human TIMP-1 transgenic mice that were generated with a National Institutes of Health (NIH) Swiss mouse strain (Harlan, Indianapolis, IN, USA), as previously described [22]. The transgenic mice were established through standard pronuclear microinjection technology. Because hybrid mice with the same genetic background have been used widely as control mice to examine the effect of transgene, the transgenic mice were then used to backcross onto the NIH Swiss mice, and the fifth-generation mice without transgene were used as the control mice (wild-type mice) in this study.

Study mice were bred in our animal facility with standard 12-h light/dark cycles and allowed to grow to a minimum weight of 20 g before the study began. Four groups of weight-matched male TIMP-1 transgenic and wild-type mice were studied: 3 days after UUO operation (n = 8 each), 14 days after UUO operation (n = 8 each), and both 3 and 14 days after sham operation (n = 8 each). UUO and sham operation were performed under general anesthesia with 10% chloral hydrate (0.40 ml/100 g, intraperitoneally). The left ureter was ligated with 4.0 silk at two separate locations in the UUO group, but it was only separated without ligation in the sham group. All mice were killed by exsanguination under general anesthesia. After exsanguination, the left kidney was harvested and the capsule was removed. The kidney was immediately equally divided into five parts: one for paraffin section; four parts of the remaining kidney were snap frozen in liquid nitrogen and stored at −80°C for the frozen section, zymography, reverse zymography and protein extraction. The piece to be used for paraffin sections was fixed in 10% buffered formalin.

Immunofluorescence. Frozen sections (4 µm thick) of kidney were used to detect the presence of F4/80. Endogenous peroxidase was quenched with 4% hydrogen peroxide in methanol. The non-specific binding was blocked by using 10% goat serum before incubation with rat anti-mouse F4/80 monoclonal antibody (Serotec Ltd., Oxford, UK) diluted in PBS (1:200) overnight at 4°C. Negative control materials used were normal rat IgG (Sigma-Aldrich, St Louis, MO, USA) in place of the primary antibody. All sections were incubated with 1:300 diluted FITC-conjugated goat anti-rat IgG for 60 min at room temperature (Santa Cruz Biotechnology, Santa Cruz, CA, USA), then observed by confocal laser scanning microscope.

Quantitative analysis of renal pathological changes in the obstructed kidney. The 3 µm paraffin sections were stained with Masson’s Trichrome. The relative area of interstitial fibrosis was evaluated by a computer image system with software of VIOP-2M Image/Graphics/Vision Video View series (CAS-DH Co., HZ Image & Vision Inc., Beijing, China). Infiltrating inflammatory cells were counted at a magnification of ×400 and were quantitated as cells per field. Data were expressed as the mean values of 10 fields in each section.
Western blot. Cells were washed three times with PBS and incubated in lysis buffer as described above for 30 min. Each kidney piece was homogenised in 1 ml of lysis buffer (20 mM HEPES–KOH, 250 mM sucrose, 10 mM KCl, 1.5 mM MgCl₂, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 500 μM digoxigenin, 0.1 mM PMSF and 2 mg/ml aprotinin, 10 mg/ml leupeptin, 5 mg/ml pepstatin) with a Tenbroeck Tissue Grinder (Wheaton, Millville, NJ, USA). All samples were spun (10 000 × g for 30 min at 4°C), and the protein concentration in each lysate was determined by BCA Protein Assay Kit (Pierce Biotechnology, Inc., Milwaukee, WI, USA). The extracted proteins were solubilised in SDS loading buffer, denatured by boiling, and then electrophoresed on 10% (for detecting TIMP-1, TIMP-2, TIMP-3, MMP-9 and ICAM-1), 7.5% (for detecting collagens I and III) and 12% (for detecting TGF-β1) polyacrylamide gels (50 μg/lane). The proteins of the gel were transferred to a 0.45-μm pore nitrocellulose membrane (Schleicher & Schuell, Dassel, Germany) by the semidy method (Bio-Rad Laboratories, Richmond, CA, USA). The nitrocellulose membranes were blocked with 10 ml of Tris-buffered saline containing Tween-20 (TBST; 10 mM Tris–HCl, 0.15 M NaCl, 8 mM sodium azide, 0.05% Tween-20) with 3% bovine serum albumin (BSA) overnight at 4°C. The membranes were incubated with the primary antibody, goat polyclonal anti-TIMP-1 (1:200; Santa Cruz Biotechnology, Santa Cruz, CA, USA), rabbit polyclonal anti-TIMP-2 (1:500; US Biological, USA), rabbit polyclonal anti-TIMP-3 (1:300; US Biological, Swampscott, MA, USA), rabbit polyclonal anti-MMP-9 (1:200; Santa Cruz Biotechnology, Santa Cruz, CA, USA), rabbit polyclonal anti-MMP-2 (1:200; Santa Cruz Biotechnology, Santa Cruz, CA, USA), rat monoclonal anti-ICAM-1 antibody (1:200; Research Diagnostics Inc., Flanders, NJ, USA), rabbit polyclonal anti-collagens I and III (1:5000, Rockland Immunochemicals, Inc., Gilbertsville, PA, USA), or rat monoclonal anti-TGF-β1 (1:200, Research Diagnostics, Inc., Flanders, NJ, USA) diluted in the same buffer for 2 h at room temperature. After washing with TBST, the blots were developed with ECL reagents (Santa Cruz Biotechnology, Santa Cruz, CA, USA). After treatment with vehicle, it was shown by western blot that TIMP-1 protein abundance was the highest in TIMP-1S/HKC, and the lowest in TIMP-1AS/HKC (P < 0.05, Figure 1A and B). Little ICAM-1 was expressed on the membrane of any of the four groups of cells after treatment with vehicle, and no difference was found among the four groups (Figure 1A and B). After stimulation with PMA, each group of cells expressed ICAM-1 increasingly. The mean fluorescence intensity of ICAM-1 was the highest in TIMP-1S/HKC, and the lowest in TIMP-1AS/HKC (P < 0.05), while no difference was found between parental/HKC and vector/HKC (P > 0.05, Figure 1A and B).

Protein expression of MMPs/TIMPs and ICAM-1 in the four groups of cells

After treatment with vehicle, it was shown by western blot that TIMP-1 protein abundance was the highest in TIMP-1S/HKC, and the lowest in TIMP-1AS/HKC (P < 0.05), while no difference was found between parental/HKC and vector/HKC (P > 0.05, Figure 2A and B). No differences were observed in TIMP-2, MMP-2, MMP-9 and ICAM-1 protein expression among the four groups of cells (P > 0.05, Figure 2A, C–F).

Reverse zymography. Briefly, samples prepared as described above were electrophoresed on non-denaturing 10% SDS, 12% polyacrylamide gels containing 1 mg/ml gelatin and conditioned media from MDA-MB-468 cell line (human breast cancer cell line), which was added to 8% (vol/vol) as a source of gelatin-degrading enzyme. Electrophoresis was carried out at 4°C after which the gel was washed at room temperature in buffer containing 2.5% Triton X-100, 50 mM Tris–Cl (pH 7.5), and 5 mM CaCl₂ once for 15 min, then again overnight. The next day, the gel was rinsed once in water and incubated in 50 mM Tris–Cl (pH 7.5) and 5 mM CaCl₂ for 24 h at 37°C and stained with Coomassie blue. The optical value of each band was measured using the AlphaImager 2200 analysis program.

Statistical analysis

All values were presented as means ± SD. Kruskal–Wallis test followed by Mann–Whitney U-test was used for statistical analysis. P < 0.05 was considered to be significant.
TIMP-1 and renal fibrosis

Fig. 1. Membrane expression of ICAM-1 in parental/HKC, vector/HKC, TIMP-1S/HKC and TIMP-1AS/HKC. (A) CLSM analysis of ICAM-1 expression in parental/HKC, vector/HKC, TIMP-1S/HKC and TIMP-1AS/HKC after treatment with vehicle or PMA was shown. (B) The mean fluorescence intensity expressed as mean ± SD was illustrated (original magnification ×600). *P < 0.05, compared with each corresponding group cell after treatment with vehicle. # P < 0.05, compared with parental/HKC and vector/HKC after treatment with PMA.

protein was the highest in TIMP-1S/HKC and the lowest in TIMP-1AS/HKC (P < 0.05, Figure 2A and B). ICAM-1 protein was the highest in TIMP-1S/HKC and the lowest in TIMP-1AS/HKC (P < 0.05, Figure 2A and F). There was no difference in TIMP-2, MMP-2 and MMP-9 protein expression among the four groups of cells (P > 0.05, Figure 2A, C, D and E).

Activities of gelatinases and TIMP-1 in the four groups of cells

After treatment with vehicle, among the culture supernatants of the four groups of cells, activities of gelatinases were the highest in TIMP-1AS/HKC and the lowest in TIMP-1S/HKC (P < 0.05, Figure 3A–C), and TIMP-1 activity was the highest in TIMP-1S/HKC and the lowest in TIMP-1AS/HKC (P < 0.05, Figure 3D and E). After stimulation with PMA, the activities of gelatinases and TIMP-1 were upregulated, while the activities of gelatinases were lower in TIMP-1S/HKC and higher in TIMP-1AS/HKC than those in parental/HKC or vector/HKC (P < 0.05, Figure 3A–C); TIMP-1 activity was higher in TIMP-1S/HKC and lower in TIMP-1AS/HKC than that in parental/HKC or vector/HKC (P < 0.05, Figure 3D and E).

Co-immunoprecipitation and sICAM-1 detected in culture supernatant of the four groups of cells

Western blot analysis of anti-ICAM-1 antibody immunoprecipitates from lysates of parental/HKC stimulated with PMA revealed the presence of a dominant 92 kDa species corresponding to pro-MMP-9, with no positive species detected when immunblotted with a control rat IgG1, suggesting that ICAM-1 and MMP-9 could interact with each other (Figure 4A). No interaction was found between ICAM-1 and MMP-2 (data not shown).

Soluble ICAM-1 in the supernatant was mainly derived from the shedding of the extracellular part of ICAM-1 [10]. After treatment with vehicle, sICAM-1 in the supernatant showed no difference among the four groups of cells. After stimulation with PMA, the level of sICAM-1 was the lowest in the culture supernatant of TIMP-1S/HKC, and was the highest in that of TIMP-1AS/HKC (P < 0.05, Figure 4B, C).

Membrane expression of ICAM-1 in HKCs transiently transfected with MMP-2 and MMP-9 siRNA

It was shown by real-time PCR and western blot that the mRNA and protein levels of MMP-2 or MMP-9 in HKCs were downregulated, respectively, after being transfected with MMP-2 or MMP-9 siRNA, whether after treatment
Fig. 2. Protein expression of MMPs/TIMPs and ICAM-1 in parental/HKC, vector/HKC, TIMP-1S/HKC and TIMP-1AS/HKC. (A) Protein expression of TIMP-1, TIMP-2, MMP-2, MMP-9 and ICAM-1 in parental/HKC, vector/HKC, TIMP-1S/HKC and TIMP-1AS/HKC after treatment with PMA or vehicle was shown. (B–F). Densitometric quantifications of relative band intensities were shown. The relative band intensity calculated by the intensity ratio of each band to β-actin was expressed as mean ± SD. *P < 0.05, compared with corresponding each group cell after treatment with PMA. #P < 0.05, compared with parental and vector/HKC after stimulation with PMA. ▲P < 0.05, compared with parental and vector/HKC after treatment with vehicle.
TIMP-1 and renal fibrosis

Fig. 3. Activities of gelatinases and TIMP-1 in parental/HKC, vector/HKC, TIMP-1S/HKC and TIMP-1AS/HKC. Activities of MMP-2 and MMP-9 in parental/HKC, vector/HKC, TIMP-1S/HKC and TIMP-1AS/HKC after treatment with vehicle or PMA detected by gelatinzymography were shown in A (Markers were MMP-2 and -9 standards). TIMP-1 activity in parental/HKC, vector/HKC, TIMP-1S/HKC and TIMP-1AS/HKC after treatment with vehicle or PMA detected by reverse zymography was shown in D. Densitometric quantifications of optical value expressed as mean ± SD were shown in (B), (C) and (E). *P < 0.05, compared with each corresponding group cell after treatment with vehicle. # P < 0.05, compared with parental/HKC and vector/HKC after treatment with vehicle.
Fig. 4. Co-immunoprecipitation and sICAM-1 detected in culture supernatant of parental/HKC, vector/HKC, TIMP-1S/HKC and TIMP-1AS/HKC. (A) Lysates of PMA-treated (lanes 1, 2) and vehicle-treated (lanes 3, 4) parental/HKCs were incubated with a control rat IgG1 (lanes 1, 3) or with anti-ICAM-1 antibody (lanes 2, 4) and the immunoprecipitates were subjected to western blot analysis using an anti-MMP-9 antibody. (B) Soluble ICAM-1 in supernatants of parental/HKC, vector/HKC, TIMP-1S/HKC and TIMP-1AS/HKC after treatment with vehicle or PMA was examined by western blot. (C) Histogram indicated the change of sICAM-1 among the four kinds of cells after treatment with vehicle or PMA. The mean optical value was expressed as mean ± SD. ∗P < 0.05, compared with each corresponding group cells after treatment with vehicle. †P < 0.05, compared with parental/HKC and vector/HKC after treatment with PMA.

with vehicle or PMA (P < 0.05, Figure 5A and B). After stimulation with PMA, the expression of ICAM-1 was the highest in HKC transfected with MMP-9 siRNA, while no difference was found in parental/HKC, HKC transfected with irrelevant siRNA or MMP-2 siRNA (P < 0.05, Figure 5C and D).

Fibrosis and infiltration of macrophages in renal tubulointerstitium of transgenic and wild-type mice after UUO operation

At 3 and 14 days after UUO, it was found that the relative areas of interstitial fibrosis of two genotypes were larger than those of their sham groups, respectively, while they were larger at 14 days than at 3 days in the same genotype (P < 0.05), and more serious in transgenic than in wild-type at 14 days (P < 0.05, Table 1).

At 14 days after UUO, the number of macrophages infiltrated in tubulointerstitium of the transgenic group was more than that of the wild-type group (P < 0.05, Figure 6; Table 1).

Protein expression of MMPs/TIMPs, ICAM-1, TGF-β1, collagens I and III in two genotypes after UUO. After sham operation, compared with wild-type, TIMP-1 expression in transgenic was much higher, while MMP-2 and TIMP-2 expression was lower (P < 0.05, Figure 7A, C and E). No differences in TIMP-3, MMP-9, ICAM-1, TGF-β1, collagens I and III protein were found between the two genotypes (P > 0.05, Figures 7A, D, F and 8A–E).
Fig. 5. Membrane expression of ICAM-1 in HKCs transiently transfected with MMP-2 and MMP-9 siRNA. (A) and (B) After treatment with vehicle or PMA, the mRNA and protein levels of MMP-2 or MMP-9 in HKCs transfected with MMP-2 or MMP-9 siRNA were detected by real-time PCR and western blot, respectively (parental/HKC (lanes 1, 5), irrelevant siRNA/HKC (lanes 2, 6), MMP-9 siRNA/HKC (lanes 3, 7), and MMP-2 siRNA/HKC (lanes 4, 8) stimulated with without PMA (lanes 1–4) and with PMA (lanes 5–8)). (C) CLSM analysis of ICAM-1 expression in parental/HKC, irrelevant siRNA/HKC, MMP-9 siRNA/HKC and MMP-2 siRNA/HKC after treatment with vehicle or PMA was shown (original magnification ×600). (D) Histogram indicated the mean fluorescence intensity expressed as mean ± SD. *P < 0.05, compared with each corresponding group cell after treatment with vehicle. #P < 0.05, compared with the other three groups after treatment with PMA.
Table 1. Quantitative analysis of renal pathological changes in UUO model

<table>
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<tr>
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<th>Relative area of interstitial fibrosis (%)</th>
<th>Number of F4/80-positive cells (cells/×400 field)</th>
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<tr>
<td>Wild-type 3 d</td>
<td>8</td>
<td>2.35 ± 0.34</td>
<td>5.5 ± 1.3</td>
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<tr>
<td>Wild-type 3 d</td>
<td>8</td>
<td>9.57 ± 0.87#</td>
<td>14.6 ± 4.5 #</td>
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<tr>
<td>Transgenic 3 days</td>
<td>8</td>
<td>3.01 ± 0.48</td>
<td>6.1 ± 1.6</td>
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<tr>
<td>Wild-type 14 days</td>
<td>8</td>
<td>10.42 ± 1.57 #</td>
<td>15.8 ± 5.4 #</td>
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<tr>
<td>Wild-type 14 days</td>
<td>8</td>
<td>36.33 ± 5.12 ▲</td>
<td>52.4 ± 13.3 ▲</td>
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<tr>
<td>Transgenic 14 days</td>
<td>8</td>
<td>2.84 ± 0.42</td>
<td>6.2 ± 1.5</td>
</tr>
<tr>
<td>Transgenic 14 days</td>
<td>8</td>
<td>46.24 ± 6.58 #</td>
<td>68.9 ± 15.6 #</td>
</tr>
</tbody>
</table>

*P < 0.05, compared with wild-type at the same time point under the same treatment. #P < 0.05, compared with the sham group of the same genotype at the same time point. ▲P < 0.05, compared with 3 days after UUO of the same genotype.

At 3 and 14 days after UUO, in the same genotype, TIMP-1 was significantly increased, compared with sham group, and its expression in transgenic was higher than in wild-type at each time point (P < 0.05, Figure 7A, B). The protein expression of TIMP-2 and TIMP-3 was also increased but with no difference between two genotypes at each time point (P > 0.05, Figure 7A, C and D). MMP-2 and MMP-9 were decreased in two genotypes after UUO (P < 0.05), at 14 days after UUO, it was found that the protein expression of MMP-2 and MMP-9 in transgenic type was lower than that in the wild-type (P < 0.05, Figure 7A, E and F). The protein expression of ICAM-1, TGF-β1, collagens I and III was increased at each time point after UUO in the same genotype and at 14 days after UUO it was higher in transgenic than in wild-type (P < 0.05, Figure 8A–D).

Activities of gelatinases and TIMP-1 in two genotypes after UUO

In transgenic mice after sham operation, the activity of MMP-2 was lower, while the activity of MMP-9 was higher than that of the wild-type (P < 0.05). At 3 and 14 days after UUO, the activities of gelatinases were not only lower than those of the sham group in the same genotype, but also lower in transgenic than in wild-type at each time point (P < 0.05, Figure 9A–C). After UUO, TIMP-1 activity was not only upregulated in the same genotypes, but also significantly higher in transgenic than in wild-type at each time point (P < 0.05, Figure 9D and E).

Discussion

Inflammation could accelerate progression of renal diseases, in which lots of inflammatory mediators and their receptors played important roles [23–25]. In addition, MMPs could act as inflammatory mediators by destroying tissue structure and promoting inflammatory cell infiltration during the acute phase of glomerulonephritis [26] and modulators of inflammation and innate immunity [27], TIMPs, also played important roles in the inflammatory reaction [28,29]. Also, some inflammatory mediators were substrates of MMPs [10–12], suggesting that MMPs/TIMPs imbalance has a relationship with the inflammatory reaction during renal fibrosis.

As described above, ICAM-1 is an inflammatory mediator which plays important roles in the progression of renal diseases. It has been found that MMP-9 could proteolytically cleave to the extracellular part of ICAM-1, increasing the tumour cell resistance to natural killer cell-mediated cytotoxicity [10]. Also, the membrane proximal domain of ICAM-1 contained the P-G-N-W-T motif that was consistent with an identified consensus sequence for MMP-9.

Fig. 6. Photomicrographs of interstitial monocytes/macrophages detected by indirect immunofluorescence staining for the F4/80 antigen. The number of positive cells per ×400 field examined by computer image analysis system was shown in Table 1.
Fig. 7. Protein expression of MMPs/TIMPs in transgenic and wild-type mice after UUO. (A) Protein expression of TIMP-1, TIMP-2, TIMP-3, MMP-2 and MMP-9 at 3 days and 14 days after sham and UUO operation in wild-type and transgenic mice was detected by western blot. (B–F) Densitometric quantifications of relative band intensities were shown. The relative band intensity calculated by the intensity ratio of each band to β-actin was expressed as mean ± SD. *P < 0.05, compared with wild-type at the same time point under the same treatment. #P < 0.05, compared with the sham group of the same genotype at the same time point. ▲P < 0.05, compared with 3 days after UUO of the same genotype.
substrates. The mutation of a single residue of this sequence, as well as its entire deletion, resulted in the abrogation of MMP-9-mediated cleavage [30]. In our study, the results of co-immunoprecipitation and shRNA interference experiments showed that it was not MMP-2 but MMP-9 that had an interaction with ICAM-1, further supporting the view that ICAM-1 is an MMP-9 substrate.

TIMP-1 is the natural inhibitor of MMP-9, therefore, it might regulate ICAM-1 through influencing MMP-9. It was shown by *in vitro* study that ICAM-1 protein was the highest in TIMP-1S/HKC and the lowest in TIMP-1AS/HKC, soluble ICAM-1 was the lowest in TIMP-1S/HKC and the highest in TIMP-1AS/HKC, and MMP-9 activity was the lowest in TIMP-1S/HKC and the highest in TIMP-1AS/HKC after stimulation with PMA, indicating that TIMP-1 might upregulate ICAM-1 protein level by affecting the degradation of ICAM-1 through inhibition of MMP-9 activity.
In order to elucidate whether TIMP-1 has effects on renal inflammation and fibrosis through ICAM-1 in vivo, we used homozygote human TIMP-1 transgenic mice to establish a UUO model, in which inflammation is involved in the progress of renal interstitial fibrosis [31].

In the present study, at 14 days after UUO, the extent of renal fibrosis, expressions of collagens I and III in the kidney were much more serious in the transgenic type than in the wild-type, the expression and activity of TIMP-1 in the transgenic type was higher while activities of the gelatinases were lower, than those of corresponding the wild-type,
indicating in vivo that TIMP-1 could promote renal fibrosis in this kind of TIMP-1 transgenic mice. Macrophages infiltrated the tubulointerstitium at 14 days after UUO increased as the expressions of ICAM-1 protein were upregulated, which was much more serious in the transgenic type than in the wild-type, suggesting that TIMP-1 promoted renal inflammation during renal fibrosis, which was associated with an increase of ICAM-1 expression. Combined with the results in vitro, we considered that TIMP-1 upregulated ICAM-1 in vivo, at least partially, through inhibiting the activity of MMP-9. The activity of MMP-2 was also downregulated with a high expression of TIMP-1, which might also play important roles in inflammation during renal fibrosis, because some inflammatory mediators, such as interleukin-1β (IL-1β) and monocyte chemotactic protein-3 (MCP-3) could be degraded by MMP-2 [11,12]. If the activity of MMP-2 had been inhibited by the high expression of TIMP-1, such mediators would be upregulated, then inflammation would be exacerbated.

TGF-β1, a cytokine to promote ECM accumulation, played an important role in many renal diseases [32]. It was found in our study that TGF-β1 protein level was higher in the transgenic type than in wild-type at 14 days after UUO, which might be attributed to the aggravation of inflammation. Monocytes could not only synthesise TGF-β1 by themselves, but also stimulate mesangial cells and tubular epithelial cells to synthesise and secrete TGF-β1 [33,34], so that upregulation of ICAM-1 might accelerate the expression of TGF-β1 by facilitating the adhesion and infiltration of monocytes and macrophages. It was reported that TGF-β1 could upregulate TIMP-1 through smad or the activator protein 1 (AP-1) signal transduction pathway [35], therefore, we considered that the renal fibrosis promoted by overexpression of TIMP-1 might included several mechanisms, such as inhibition of MMP activities, acceleration of inflammation and upregulation of TGF-β1.

Our study and the data from Yoshiji et al. [8,9] indicated that TIMP-1 was a promoter for fibrosis of the kidney and liver in TIMP-1 transgenic mice, however, it was shown in TIMP-1 knock-out that TIMP-1 deficiency had no protective effect on renal fibrosis [6,7]. We considered that these controversial results might be attributed to different means of gene engineering. On the other hand, compensative roles against changes induced by gene engineering in vivo would take place at certain levels. If these changes exceeded the compensative ability, functions of the target gene would be predominant.

The renal expression and activity of TIMP-1 in the transgenic type undergoing sham operation were much higher than those of the corresponding wild-type, while no significant histological changes were found between them, implying that TIMP-1 was not the direct cause of renal fibrosis [6,7]. Further results showed decreased expressions of TIMP-2 and MMP-2. Decreased activity of MMP-2, and increased activity of MMP-9 in the kidney of transgenic mice, indicated that effects of TIMP-1 overexpression in vivo might, to a certain extent, be compensated by the roles of gelatinases and other TIMPs. The reason that TIMP-1 was expressed highly while activity of MMP-9 was upregulated in this kind of transgenic mice was unclear. Besides TIMPs, there were several kinds of MMP inhibitors, for example, procollagen C-terminal proteinase enhancer (PCPE), reversion-inducing cysteine-rich protein with Kazal motifs (RECK), and tissue factor pathway inhibitor-2 (TFPI-2) (3). Since changes of these inhibitors in this kind of transgenic mice were not examined, it was assumed that upregulation of MMP-9 activity might be due to the changes TIMPs and other MMPs inhibitors induced by TIMP-1 transgene.

In summary, based on our research in TIMP-1 transgenic mice, it was considered that besides inhibiting degradation of ECM, overexpression of TIMP-1 could promote renal interstitial fibrosis through the inflammatory pathway, which might be partly induced by upregulating ICAM-1. However, TIMP-1 also has multiple other biological activities, such as regulating steroid hormone synthesis [36], inhibiting angiogenesis [37], and affecting proliferation and apoptosis of many kinds of cells [21,38]. Other roles of TIMP-1 in renal fibrosis are worth exploring further.

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Conflict of interest statement. None declared.

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TIMP-1 and renal fibrosis

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