Expression of matrix metalloproteinase-9 associated with ets-1 proto-oncogene in rat tubulointerstitial cells

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Abstract

Background. Ets-1 proto-oncogene exhibits multiple activities in the transcriptional regulation of numerous genes including metalloproteinase (MMP)-1, -3 and -9. MMPs play an important role in the remodelling of extracellular matrix in various renal diseases. However, the role of the Ets-1–MMP axis in advanced renal diseases is uncertain. In the present study, we investigated whether Ets-1 is involved in interleukin (IL)-1-mediated expression of MMPs in tubulointerstitial cells.

Methods. Rat renal fibroblasts (NRK-49F) and tubular epithelial cells (NRK-52E) were cultured and allocated to an IL-1β-treated group (10 ng/ml), a platelet-derived growth factor (PDGF)-BB-treated group (25 ng/ml) and a control group. Protein and mRNA were extracted after 1, 6, 12 and 24 h of treatment. Parallel flasks were treated with 2 μM ets-1 antisense oligodeoxynucleotides (ODNs) before exposure to IL-1β. The expression of Ets-1 protein was evaluated by western blotting. The activities of MMPs were evaluated by gelatin zymography. The expression of ets-1 and/or MMP-9 mRNA was evaluated semiquantitatively by real-time reverse transcription–polymerase chain reaction (RT–PCR).

Results. In NRK-49F cells, Ets-1 protein increased significantly by 6.8-fold at 6 h, and MMP-9 activity increased significantly by 9.9-fold at 12 h in the IL-1β-treated group compared with controls. MMP-2 and -3 activities also increased significantly in the IL-1β-treated group. In NRK-52E cells, Ets-1 protein was 3.1 times higher at 1 h, and the latent form of MMP-9 activity increased 3.4-fold at 6 h in the IL-1β group compared with controls. However, MMP-2 or MMP-3 activities were not markedly altered by IL-1β treatment compared with controls. When the cells were treated with ets-1 antisense ODNs before IL-1β treatment, Ets-1 protein expression decreased at least 50%, and MMP-9 activity was clearly inhibited in both cells. We also confirmed that MMP-9 activity was upregulated on days 21 and 28 in renal cortex of rat crescentic glomerulonephritis.

Conclusions. The Ets-1 transcriptional factor may participate in IL-1β-mediated MMP-9 expression in tubulointerstitial cells.

Keywords: ets-1 proto-oncogene; interleukin-1 (IL-1); matrix metalloproteinase-9 (MMP-9); MMP-2; MMP-3

Introduction

Fibrosis and scarring are common pathological features in advanced renal diseases [1], and are characterized by accumulation of extracellular matrix (ECM) and destruction of renal tubules. According to recent studies focusing on phenotypic changes of tubulointerstitial cells, these cells acquire the ability to secrete various forms of inflammatory mediators, including cytokines, chemotactic factors and adhesion molecules, as well as ECM [2]. Interstitial myofibroblasts and tubular epithelial cells (TECs) are known to be mainly responsible for the increased production of ECM components and disease-specific components not expressed in the normal kidney [3,4]. Several lines of evidence suggest that an imbalance between synthesis and degradation of these components is closely associated with accumulation of ECM and subsequent progression of tubulointerstitial fibrosis.

Matrix metalloproteinases (MMPs) play an important role in ECM remodelling in various types of renal diseases [5]. Expression of MMPs is regulated by various stimuli that include mitogens, growth factors,
activators of receptor tyrosine kinases, phorbol esters and proinflammatory cytokines such as interleukin (IL)-1β and tumour necrosis factor-α. MMPs are zinc-dependent proteases that play a critical role in the turnover of ECM components including collagens, elastin, laminin, proteoglycans, fibronectin and other glycoproteins. MMPs are generally classified into five categories based on their specific properties: collagenases (MMP-1, -8, -13 and -18), gelatinases (MMP-2 and -9), stromelysins (MMP-3 and -10), membrane-type MMPs (MMP-14, -15, -16 and -17) and others (MMP-7, -11, -12 and -19). It has been shown that glomerular resident cells express and secrete several types of MMPs including gelatinase A (MMP-2), stromelysin-1 (MMP-3) and gelatinase B (MMP-9), and that interstitial fibroblasts and TECs also secrete gelatinases (MMP-2 and -9) [6]. A previous study demonstrated that both MMP-2 and -9 cleave collagen types IV, V, VII and X, as well as elastin and all forms of degenerate collagen, suggesting that these MMPs play an important role in the destruction of several types of basement membrane [7]. However, it is still unclear whether overexpression of MMPs causes membrane damage that triggers epithelial–mesenchymal transition (EMT) to result ultimately in renal scarring [8].

The ets-1 proto-oncogene is a member of the transcription factor family that shows homology to the v-ets oncogene. Ets-1 protein binds to DNA sequences containing a central GGA(A/T) core sequence (PEA3) via the DNA-binding domain, and cooperates with the c-Fos/c-Jun complex at the AP-1 site to activate the expression of certain promoters [9]. This motif has been found in the promoter region of numerous genes, including those of MMP-1, -3 and -9, as well as urokinase-type plasminogen activator (u-PA). Ets-1 protein enhances the promoter activity of MMPs and the u-PA gene, indicating that the ets-1 proto-oncogene plays a pivotal role in the regulation of matrix proteinase expression.

However, few studies have examined the functions of ets-1 in renal cells [10,11]. A recent experiment demonstrated that not only AP-1 and NF-κB but also Ets-1 transcriptional factors are critically involved in the IL-1β-mediated transactivation of MMP-9 in rat glomerular mesangial cells [11]. As described above, ets-1 may be involved in the pathogenesis of progressive renal diseases through the expression of several proteinases. In the present study, we examined the role of ets-1 in regulating cytokine-mediated MMP expression in tubulointerstitial cells.

Subjects and methods

Cells and culture conditions

Cell lines of rat renal fibroblasts NRK-49F and renal epithelial cells NRK-52E were purchased from the American Type Culture Collection (Manassas, VA). NRK-52E cells are believed to be of proximal tubular origin based on the pattern of collagen secretion and the presence of epidermal growth factor (EGF) receptors. Both cells were grown and maintained in Dulbecco’s modified Eagle’s medium (DMEM) containing 4.5 g/l glucose supplemented with 10% fetal calf serum (FCS) and passaged twice a week. For experimentation, 1 × 10⁶ cells were incubated in 1% FCS/DMEM for 48 h, and exposed to 10 or 20 ng/ml of rat IL-1β (R&D systems, Inc., MN), or 15 or 25 ng/ml of rat platelet-derived growth factor (PDGF)-BB (R&D systems). Protein and mRNA were extracted after 1, 6, 12 and 24 h of treatment.

Effect of ets-1 antisense oligodeoxynucleotides

Ets-1 antisense oligodeoxynucleotides (ODNs) and the mutant control used in this study were single strand phosphorothioate DNA oligonucleotides. Antisense oligonucleotides and controls directed to rat ets-1 were designed and manufactured by Biognostik (Göttingen, Germany). The ets-1 antisense and control ODN sequences were designed as follows: ets-1, GCATGCTCGATACC; control, GAACCAAAGACACC. The binding of oligonucleotides to target mRNA causes a steric or conformational obstacle for protein translation. Both NRK-49F and NRK-52E cells were grown and maintained in DMEM with 10% FCS. For experimentation, 1 × 10⁶ cells were incubated in 1% FCS/DMEM for 48 h with the addition of 2 μM ets-1 antisense ODN or control ODN once daily, and were then exposed to 10 ng/ml of rat IL-1β. Protein and mRNA were similarly extracted at 1, 6 and 12 h. Furthermore, the uptake pattern was monitored using fluorescein isothiocyanate (FITC)-labelled control ODNs that could be visualized directly under a fluorescence microscope to confirm cellular permeabilization to ODNs.

Induction of rat crescentic glomerulonephritis

Nephrotoxic serum (NTS) was produced by immunizing male rabbits with a particulate fraction of rat glomerular basement membrane (GBM), as previously described [12]. Accelerated anti-GBM glomerulonephritis was induced in inbred male Sprague–Dawley rats. Briefly, rats weighing 150–200 g were pre-immunized by subcutaneous injection of 5 mg of normal rabbit IgG in Freund’s complete adjuvant. Five days later (day 0), the animals were given intravenous injections of 300 μl of rabbit anti-rat GBM serum (NTS). Groups of four rats were euthanized on days 14, 21 and 28. In addition, four normal rats were used as controls.

Gelatin zymography

Gelatinolytic activity of proteins in cell culture supernatants was assayed as previously described [13]. Briefly, gelatin zymography was performed on a 8% polyacrylamide gel under non-reducing conditions. Heat-denatured FITC-labelled collagen (Japan Institute of Leather Research, Japan) was co-polymerized (final concentration, 0.5 mg/ml gel) in the polyacrylamide gel. A standard stacking gel (5% acrylamide) was used. Aliquots of 10 μl of cell culture supernatants were diluted in sample buffer (50 mM Tris–HCl, pH 6.8, 1% SDS, 0.1% bromophenol blue and 30% glycerol). They were then loaded onto a gel without heat denaturation.
The samples were run at a constant voltage of 120 V for 2 h during cooling in a water bath. After electrophoresis, the gels were incubated overnight at 37°C in digestion buffer containing 50 mM Tris–HCl, 200 mM NaCl, 5 mM CaCl₂ and 0.02% NaN₃ at pH 7.6. After an appropriate period of incubation, the gel was placed directly on the transilluminator. Relative band intensities were assessed using computer densitometry software. Then, we evaluated the activity as the sum of latent and active forms of each MMP. Gelatinolytic activity of the control was arbitrarily set as 1.

Frozen samples of renal cortex from NTS-treated or control rats were lysed by 50 mM Tris–HCl, pH 7.5, and 1% Triton. The protein concentration was measured with an ultraviolet/visible spectrophotometer (UV-1600, Shimadzu, Japan). Samples containing 100 μg of protein were loaded onto a gel without heat denaturation, as described above.

**Western blotting**

Both NRK-49F and 52E cells were lysed by 100 mM Tris–HCl, pH 7.3, 4% SDS and 20 mM EDTA. The protein concentration was measured with an ultraviolet/visible spectrophotometer (UV-1600). Samples containing 100 μg of protein were subjected to electrophoresis on an SDS–10% (w/v) polyacrylamide gel and transferred to polyvinylidene difluoride paper. After blocking for 2 h at room temperature with 20 mg/ml bovine serum albumin (BSA) dissolved in Tris-buffered saline solution, the proteins were detected using anti-Ets-1 (C-20, rabbit polyclonal antibodies against the C-terminal domain of the Ets-1 protein; Santa Cruz Biochemistry, CA), anti-MMP-9 (rabbit polyclonal antibodies against rat; Chemicon, CA), anti-MMP-1 and -3 (mouse monoclonal antibodies; Daiichi Fine Chemical, Japan), anti-MMP-2 (rabbit polyclonal antibodies; Santa Cruz Biochemistry), anti-tissue inhibitor of metalloproteinase (TIMP)-1 and -2 (rabbit polyclonal antibodies; Santa Cruz Biochemistry) and TIMP-3 (goat polyclonal antibodies; Santa Cruz Biochemistry) as primary antibodies. Blots were washed and incubated with the above antibodies in wash buffer containing 5% BSA for 2 h, then washed three times and incubated with horseradish peroxidase-conjugated antibody to rabbit, mouse or goat IgG (Jackson ImmunoResearch Laboratories, PA). Immunoreactive protein was detected by enhanced chemiluminescence (Amersham, Buckinghamshire, UK). Membranes were re-probed with anti-actin (rabbit polyclonal antibody against rat, Sigma, CA) as a loading control. The expression of the target molecules was evaluated semi-quantitatively by Image Analyzer (Image-pro plus version 4.0) as desisometric ratios relative to actin.

Frozen sample of renal cortex from NTS-treated or control rats were lysed by 100 mM Tris–HCl, pH 7.3, 4% SDS and 20 mM EDTA. Protein concentration was measured with an ultraviolet/visible spectrophotometer (UV-1600). Samples containing 100 μg of protein were subjected to electrophoresis on an SDS–10% (w/v) polyacrylamide gel and transferred to polyvinylidene difluoride paper. The proteins were detected using anti-Ets-1 (C-20; Santa Cruz Biochemistry) and anti-MMP-9 (Chemicon). Immunoreactive protein was detected by enhanced chemiluminescence (Amersham). Membranes were re-probed with anti-actin (Sigma) as a loading control as described above.

**Real-time RT–PCR**

Real-time reverse transcription–polymerase chain reaction (RT–PCR) was used to evaluate ets-1 and MMP-9 mRNA expression. Briefly, total RNA was extracted from NRK-49F and NRK-52E cells using the acid–guanidinium–phenol method. Each 100 ng RNA sample was analysed by quantitative real-time RT–PCR using an ABI Prism 7700 Sequence Detection System (PE Applied Biosystems, Foster City, CA). This system is based on the ability of the 5'-nucleotide activity of Taq polymerase to cleave a non-extendable dual-labelled fluorogenic hybridization probe (TaqMan probe) during the extension phase of PCR. The increase in fluorescence signal is detected only if the target sequence is complementary to the probe, and is amplified during PCR. Relative quantities of ets-1, MMP-9 and glyceraldehyde phosphate dehydrogenase (GAPDH) mRNA were calculated using the comparative threshold cycle number (Cₚ) for each sample fitted to a five-point standard curve (ABI Prism 7700 User Bulletin #2, PE Applied Biosystems). Expression levels of ets-1 and MMP-9 mRNA were normalized to GAPDH. The following sequence-specific primers for rat ets-1, MMP-9 and GAPDH were designed using Primer Express Software 1.0 (PE Applied Biosystems): ets-1 forward primer, 5'-GGCCCTTCGCTATTACGACAA-3', reverse primer, 5'-TGCCGACAAACACGGCTAC-3' and probe, 5'-FAM-ATCATCCAAAGACGGCGGCA-TAMRA-3'; MMP-9 forward primer, 5'-TAAACCTGGTGATCCCCGACTTC-3', reverse primer, 5'-ATACGGTTCCCCGGCTGATCAG-3', and probe, 5'-FAM-CGCGGT CGTGAGGAGCGG- TAMRA-3'. Primers were used at a concentration of 300 nM, and probes at 100 nM in each reaction. Multiscribe reverse transcriptase and AmpliTaq Gold polymerase (PE Applied Biosystems, Foster City, CA) were used in RT–PCRs. The RT–PCR protocol was as follows: 48°C for 30 min (reverse transcription); 95°C for 10 min (AmpliTaq Gold activation); and 40 cycles at 95°C for 15 s (denaturation) and 60°C for 1 min (annealing/extension). Expression levels were normalized to GAPDH mRNA and related to relevant controls.

**Immunohistochemical analysis**

Immunohistochemical analysis was performed on frozen sections of both control and NTS-treated rats. Briefly, frozen tissue samples were cut into 4 μm sections and treated for 30 min with 0.3% H₂O₂ in methanol at room temperature in order to inactive endogenous peroxidase. Sections were blocked for 1 h with 5% non-fat dry milk, and were then incubated overnight at 4°C with rabbit polyclonal antibody against rat MMP-9 (Chemicon). After washing with phosphate-buffered saline, sections were stained with tetramethylrhodamine isomer R (TRITC)-conjugated swine anti-rabbit immunoglobulin (DAKO, Denmark). As negative control, normal rabbit serum with 5% non-fat dry milk was used at the same dilution.

**Statistical analysis**

In all cell culture experiments, three experiments were performed at different times in duplicate. In animal studies, four rats were used in each group. Data are expressed as means ± SEM. Differences between groups were examined...
for statistical significance using the Mann–Whitney U test or one-way analysis of variance (ANOVA). P-values < 0.05 were considered to indicate statistically significant differences.

**Results**

*Expression of Ets-1 protein and mRNA*

In the renal fibroblast cell line NRK-49F, western blot analysis demonstrated that the peak Ets-1 protein expression was increased 6.8-fold after 6 h of stimulation with 10 ng/ml of IL-1β compared with the control (Ets/actin ratio: 0.85 ± 0.04 vs 0.13 ± 0.01, *P* < 0.01) (Figure 1A). A greater increase was observed with 20 ng/ml of IL-1β (0.96 ± 0.02). The peak expression of ets-1 mRNA was also significantly increased 3.3-fold compared with control after 6 h of IL-1β (10 ng/ml) stimulation (3.3 ± 0.7 vs 1.0 ± 0.0, *P* < 0.05) (Figure 1B).

In the proximal tubular cell line NRK-52E, three distinct bands with molecular sizes of 54, 50 and 45 kDa were detected with anti-Ets-1 antibody C-20.
Therefore, we evaluated the signal intensity of the most predominant band at 54 kDa. The Ets-1 protein expression increased markedly to almost peak levels after 1 h of IL-1β stimulation, and was 3.1 times higher than the control at 10 ng/ml of IL-1β (Ets/actin ratio: 0.63 ± 0.02 vs 0.21 ± 0.01, P < 0.01) (Figure 1C). A greater increase was observed with 20 ng/ml of IL-1β (0.78 ± 0.10). The peak expression of ets-1 mRNA was also significantly increased compared with control (P < 0.05) after 1 h of IL-1β (10 ng/ml) stimulation (Figure 1D). Peak expression occurred earlier in NRK-52E than in NRK-49F cells.

Because previous studies demonstrated that PDGF-BB induced a dose-dependent expression of ets-1 mRNA in vascular smooth muscle cells (VSMCs) [14], we also investigated whether Ets-1 protein was increased by PDGF-BB. PDGF-BB at 25 ng/ml increased Ets-1 protein expression 1.3 times compared with the control at 6 h in NRK-49F cells (0.17 ± 0.01 vs 0.13 ± 0.01, P < 0.05) (Figure 1A), and 1.4 times compared with the control at 12 h in NRK-52E cells (0.40 ± 0.01 vs 0.28 ± 0.03, P < 0.05) (Figure 1C).

**Evaluation of MMP-9 activity and mRNA**

We performed gelatin zymography on the cell culture supernatant because findings from this method reveal the intrinsic enzyme activity of MMPs. In NRK-49F, the intensity of the lysis band of MMP-9 was significantly augmented from 6 h onward in IL-1β (10 ng/ml)-stimulated cells compared with control (Figure 2A). In particular, both latent (92 kDa) and active (84 kDa)
forms of the MMP-9 were clearly increased from 6 h. The peak intensity of these bands increased 9.9 times compared with the control at 12 h (10 ng/ml: 9.9±0.9 vs control: 1 ±0.0, P <0.01). A greater increase was observed with 20 ng/ml (11.1±0.3). MMP-9 mRNA was significantly increased to 11.1-fold of control after 12 h stimulation by IL-1β at 10 ng/ml (11.1±1.6 vs 1±0.0, P <0.01) (Figure 2B). In NRK-52E, the active form of MMP-9 was not increased throughout the course of study in these cells. However, the intensity of the latent form of MMP-9 was upregulated from 1 h onward, and increased to a peak that was 3.4 times
higher than controls at 12 h at an IL-1β concentration of 10 ng/ml (3.4 ± 0.5 vs control: 1 ± 0.0, \( P < 0.01 \)) (Figure 2C), with a greater increase at 20 ng/ml (5.2 ± 0.3). MMP-9 mRNA levels were also increased 8.6-fold above controls after 12 h of IL-1β stimulation (8.6 ± 0.4 vs 1 ± 0.0, \( P < 0.01 \)) (Figure 2D). In summary, induction of both Ets-1 protein and MMP-9 activity by IL-1β was faster in NRK-52E cells than in NRK-49F cells. The IL-1β-induced augmentation was more marked at a concentration of 20 ng/ml.

However, the activity of MMP-9 did not change significantly when treated with 25 ng/ml of PDGF-BB as compared with controls in both cell types (Figure 2A and C).

Sequential changes in activity of other MMPs
We evaluated the activities of other MMPs such as MMP-1, -2 and -3 by gelatin zymography. As shown in Figure 3, different patterns were observed in NRK-49F and NRK-52E cell types. In NRK-49F cells, the intensities of both latent (72 kDa) and active (62 kDa) forms of MMP-2 were significantly increased from 6 h onward in IL-1β-stimulated cells compared with controls, with peak levels ~2 times higher than controls at 12 h (10 ng/ml, 1.8 ± 0.1; 20 ng/ml, 2.3 ± 0.5 vs control, 1 ± 0.0, \( P < 0.01 \)) (Figure 3A). Although the active form (43 kDa) of MMP-3 was not observed in the cell lines, the latent form (52 kDa) of MMP-3 activity was
increased significantly from 12 to 24 h in IL-1β-stimulated cells compared with controls (at 12 h, 10 ng/ml, 2.8±0.1, 20 ng/ml, 4.3±0.6 vs control, 1±0.0, *P < 0.01) (Figure 4A). In NRK-52E cells, however, the activity of both MMP-2 and -3 showed no perceptible change after IL-1β treatment, even at 20 ng/ml of IL-1β (Figures 3B and 4B). In particular, the gelatinolytic band of MMP-3 was scarcely observed in NRK-52E cells. On the other hand, PDGF (15 or 25 ng/ml) did not stimulate the activity of MMP-2 or -3 in either cell line (Figures 3 and 4).

We performed western blot analysis to demonstrate the expression of MMP-1, -2, -3 and -9 in both cell types (data not shown). It was important to confirm the expression of MMP-3 protein by western blotting, since MMP-3 cannot be distinguished from MMP-1 in gelatin zymography due to their similar molecular weights. MMP-1 protein was not detected in either cell
type by western blot analysis although the results for MMP-2, -3 and -9 were equivocal.

Expression of TIMP-1, -2 and -3 protein

We also examined the expression of TIMP-1, -2 and -3 proteins by western blot analysis. TIMP-1 protein expression was weakly detected in the three groups in both cell types (Figure 5A). However, TIMP-1 protein was not significantly changed by IL-1β (10 or 20 ng/ml) or PDGF treatment (15 or 25 ng/ml) in both cell types. TIMP-2 expression was not detected in any of the three groups in NRK-49F and NRK-52E cells (Figure 5B). TIMP-3 protein was slightly and equally observed in

Fig. 4. Effects of IL-1β (10 ng/ml) and PDGF-BB (25 ng/ml) on the activity of MMP-3 analysed by gelatin zymography. Cellular supernatant was extracted after 1, 6, 12 and 24 h of each treatment. Migration properties were determined using standard molecular weight markers (the latent form 52 kDa and the active form 43 kDa). Densitometric evaluation of the activity of MMP-3 (the sum of both forms) is presented as relative densitometric units. Gelatinolytic activities determined with controls were arbitrarily set as 1. (A) Enzyme activity of MMP-3 in NRK-49F cells. (B) Enzyme activity of MMP-3 in NRK-52E cells. Data are presented as means±SEM for each group. **P < 0.01, *P < 0.05, compared with controls.
the three groups in NRK-49F, but was not detected in NRK-52E cells (data not shown).

**Effect of ets-1 antisense ODN on MMPs activity**

To confirm further that *ets-1* is involved in IL-1β-mediated MMP expression in rat tubulointerstitial cell lines, we evaluated the effect of *ets-1* antisense ODN on MMP expression in NRK-49F and NRK-52E cells. We first validated whether *ets-1* antisense ODN indeed suppresses IL-1β-induced Ets-1 protein expression. After treatment with *ets-1* antisense ODN, Ets-1 expression decreased by at least 50% in both cell lines, as compared with control ODN (Figure 6A and B). Moreover, we confirmed the cellular uptake of ODN using FITC-labelled control ODN that can be visualized directly using fluorescence microscopy (data not shown).

We next investigated the ability of *ets-1* antisense ODN to inhibit IL-1β-induced MMP activity by suppressing Ets-1 protein expression. The activities of latent and active forms of MMP-9 were significantly reduced by treatment with *ets-1* antisense ODN compared with control ODN in NRK-49F cells.
The latent form of MMP-9 was obviously decreased in NRK-52E cells (12 h: 3.1±0.3 vs 1.3±0.1, \(P<0.01\)) (Figure 6A and B). In NRK-49F cells, the activity of latent MMP-3 was almost completely inhibited by treatment with \(\text{ets-1}\) antisense ODN compared with control ODN (12 h: 2.7±0.5 vs 0.9±0.2, \(P<0.01\)), and the activities of latent and active forms of MMP-2 were significantly reduced (12 h: 1.9±0.3 vs 1.3±0.4, \(P<0.01\)). However, neither MMP-2 nor MMP-3 activity was visibly changed by antisense ODN in NRK-52E cells.

We further investigated the ability of \(\text{ets-1}\) antisense ODN to inhibit the expression of TIMP-1, -2 and -3 proteins by suppressing Ets-1 protein expression.
Western blotting showed no clear changes in these proteins in either cell line (data not shown). Although this result is not conclusive, our present data indirectly demonstrated that Ets-1 transcriptional factor may participate in IL-1β-mediated MMP-9 expression in tubulointerstitial cells.

Expression of Ets-1 and MMP-9 in anti-GBM disease

We previously observed that the number of the Ets-1-positive cells increased ~5.7- to 6.0-fold on days 21 to 28 in the interstitium compared with control kidneys [10]. Western blotting also identified an increase in Ets-1 protein expression in renal cortex on days 14–28 after induction of anti-GBM disease (day 21: 0.86±0.24 vs 0.14±0.05, P<0.01) (Figure 7).

MMP-9 activity in the renal cortex of NTS-treated rats was estimated by gelatin zymography. As shown in Figure 8A, the intensities of the latent and active forms of MMP-9 were significantly increased on days 21–28 (stage of advanced renal scarring) compared with control kidneys (day 21: 3.3±0.3 vs 1.0±0.0, P<0.01). Western blotting confirmed that MMP-9 protein was upregulated on days 14–28 in the renal cortex of NTS-treated rats (Figure 8B). We also investigated MMP-9 protein in the renal cortex by immunohistochemical analysis. MMP-9 reactivity was detected on some TECs and interstitial cells (Figure 8C), and modestly on glomerular intrinsic cells in NTS-treated rat kidney.

Discussion

The ets-1 proto-oncogene is involved in a diverse range of biological phenomena, including activation of protease gene transcription and promotion of angiogenesis [9]. We have recently demonstrated that ets-1 expression is upregulated in glomeruli and interstitium during disease progression in a rat model of anti-base membrane nephritis [10]. Furthermore, ets-1 has been confirmed to participate in the activation of MMP-9 and MMP-2 transcription in glomerular mesangial cells [11,15]. Among the MMPs, the gelatinases (MMP-9 and MMP-2) have been shown to be involved in remodelling of the ECM of glomeruli and tubulo-interstitium during the development of renal disease. However, their precise roles remain unresolved, and their involvement in glomerulosclerosis and interstitial fibrosis remains to be fully defined.

Only a few studies have examined the ets-1 proto-oncogene and MMPs in the tubulo-interstitium. We therefore examined the role of ets-1 in the expression of MMPs induced by IL-1β in interstitial fibroblasts and TECs in vitro. The present studies demonstrated that the expression of MMP-9 and ets-1 at both protein and mRNA levels was clearly increased by IL-1β stimulation in cultured renal fibroblasts and tubular cells, even though the upregulation of MMP-9 activity was limited to the latent form in tubular cell lines. Although induction of both Ets-1 and MMP-9 was faster in
tubular cells than in fibroblasts, peak expression of ets-1 mRNA and protein was observed >6 h earlier than that of MMP-9 in both cell types. Previous studies have demonstrated that proinflammatory cytokine IL-1β induced the expression of MMP-9 via ets-1 transcriptional factor in VSMCs and mesangial cells [11,16]. We therefore suggest that the Ets-1–MMP-9 axis hypothesis may also be applied to both the TECs and renal fibroblasts that were tested in this study.

We next assessed the effects of ets-1 antisense ODNs on IL-1β-induced MMP-9 expression at the protein and mRNA levels. We confirmed by western blot analysis that ets-1 antisense ODNs blocked Ets-1 protein synthesis at the translational step as compared with control ODNs. Concurrent changes in MMP-9 activity were also examined. In both cell lines, ets-1 antisense ODNs remarkably inhibited MMP-9 activity. This finding provided indirect evidence that the ets-1 proto-oncogene is involved in IL-1β-induced MMP-9 expression in interstitial fibroblasts and TECs.

Of particular interest, our results showed that the expression profile of MMP-2 and -3 was different from that of MMP-9 after IL-1β stimulation in tubulointerstitial cells. Although IL-1β induced upregulation of the latent and/or active forms of MMP-2 and the latent form of MMP-3 in renal fibroblast cell line was similar to MMP-9, the cytokine did not visibly change MMP-2 or -3 activities in the TEC line. Furthermore, neither MMP-2 nor MMP-3 activities were remarkably changed by treatment with ets-1 antisense ODN in
NRK-52E cells, which is in contrast to NRK-49F cells. A previous study demonstrated that IL-1β increased expression and activity of MMP-2 and -3 in mesodermal cell lines, and that ets-1 activated these MMPs at the transcriptional level in several cell lines, including rat mesangial cells [15]. In contrast, another study reported that the addition of IL-1β to glomerular epithelial cells stimulated the secretion of MMP-9 but not MMP-2 [17]. These data support the hypothesis that MMPs are independently regulated by different cytokines and/or transcriptional factors in individual cell types. Our present findings suggest that the expression and activity of MMP-2, -3 and -9 may be individually regulated by different cytokines, and that Ets-1 protein may play only a small role in the transactivation of MMP-2 and -3 in TECs.

We also investigated changes in TIMPs following IL-1β treatment because ECM degradation is due to a balance between MMP levels and TIMP levels. Our data indicated that IL-1β did not induce the expression of TIMP-1, -2 and -3, and that these TIMPs are perhaps not influenced by the ets-1 transcriptional factor in rat tubulointerstitial cells. Although Logan et al. [18] demonstrated that TIMP-1 contains not only the AP-1-binding site but also the Ets-binding site (PEA3) on its promotor in the F9 teratocarcinoma cell line, no studies have shown that Ets-1 protein induces activation of TIMP-1 in other cells. Even though the protein expression of TIMPs showed no change in either cell lines in the present study, we cannot exclude the possibility of ets-1 factor involvement in the regulation of TIMPs.

We additionally examined whether the inflammatory cytokine PDGF-BB is involved in the expression of ets-1 and MMPs in the cell cultures. These experiments were performed because PDGF-BB has recently been reported to induce ets-1 mRNA expression via protein kinase C at 25 ng/ml in VSMCs in vitro [14]. Our results showed that stimulation with 25 ng/ml of PDGF-BB significantly increased Ets-1 protein in both NRK-49F and NRK-52E cells, but did not increase the activity of MMPs or the expression of TIMPs in either cell line. These results suggest either that PDGF alone is incapable of readily upregulating MMP-9, or that 25 ng/ml of PDGF-BB fails to induce an increase in Ets-1 transcriptional factor that is adequate for increasing the activity of MMPs in tubulointerstitial cells. Although some studies have demonstrated that PDGF by itself induces MMPs or TIMPs, further studies will be needed to confirm these findings.

Because a pathogenic role for IL-1 in the development and progression of interstitial fibrosis has been demonstrated by the finding that IL-1 receptor antagonism halts progression of glomerulonephritis...
in anti-GBM involved in glomerulonephritis [12], we examined whether ets-1 and MMP-9 expression is actually increased in this model. As previously described [10], these rats are characterized by severe proliferative crescentic glomerulonephritis, significant glomerular accumulation of macrophages and T lymphocytes after 1 week, and severe interstitial inflammation ultimately leading to glomerular sclerosis and interstitial fibrosis after 3 weeks. We previously showed by immunohistochemistry and western blot analysis that Ets-1 protein expression peaked on day 21 in glomeruli and on day 28 in the interstitium, and demonstrated by RT–PCR and in situ hybridization that ets-1 mRNA expression was clearly increased and localized in glomerular epithelium, endothelial cells and interstitial fibroblasts [10]. To complement our previous studies, we examined MMP-9 activity and protein expression in the renal cortex by gelatin zymography and western blot. Our present results confirmed significant increases in MMP-9 activity (latent and active forms) and protein expression on days 14–28 that were associated with glomerular and interstitial destruction. We also showed that the MMP-9 protein was mainly localized in the cytoplasm of interstitial cells and TECs. Although we could not confirm co-localization of Ets-1 and MMP-9 in the present study, the expression of both proteins was shown to increase almost simultaneously in this model of renal failure. These findings suggest that MMPs together with ets-1 transcriptional factor may be involved in the pathogenesis of renal scarring. Further studies will be necessary to confirm involvement of these factors in renal diseases in humans.

Several studies have demonstrated that a number of mechanisms lead to a common pathway that causes TECs and interstitial fibroblasts to change phenotypically into myofibroblasts. The phenotypic transformation of tubular epithelium was recently termed epithelial–mesenchymal transition or tubular epithelial–myofibroblast transdifferentiation. Cells that transform to myofibroblasts acquire the properties of inflammatory cells, including the release of cytokines [such as transforming growth factor (TGF)-β] and chemotactic factors (such as monocyte chemoattractant protein-1), expression of adhesion molecules (such as intercellular adhesion molecule-1) and synthesis of ECM components (such as type I and type III collagen) [1,2]. These properties promote fibrosis of the tubulointerstitium. The findings from our study suggest that phenotypically transformed interstitial cells may overexpress the ets-1 proto-oncogene that upregulates MMP-9 and other matrix proteinases. Previous studies have shown that TGF-β, EGF and fibroblast growth factor-2, acting alone or synergistically, promote EMT of TECs [8]. Recently, Fan et al. [19] found that stimulation with 10 ng/ml or higher levels of IL-1 induced EMT in NRK-52E cells. This finding supports the hypothesis that IL-1 induces TECs that have been transformed to mesenchymal cells to overexpress ets-1 and induce MMPs. In addition to MMP induction, ets-1 has a number of diverse functions. A recent study demonstrated that Ets-1 regulated angiogenesis through the induction of angiogenic growth factors (VEGF and HGF) [20]. Further studies are needed to determine whether ets-1 acts as a progression factor or a regression factor in tubulointerstitial fibrosis.

In conclusion, findings from the present study suggest that the ets-1 proto-oncogene is involved in IL-1β-induced expression of MMP-9 in renal interstitial fibroblasts and TECs. Future studies should examine in greater detail the roles of matrix proteinases and upstream transcription factors including ets-1 in the development of tubulointerstitial fibrosis.

Acknowledgements. We thank Mr Yoshiyuki Moriguichi, Mr Koichi Kawahara and Dr Masato Higuchi, PhD (Chugai Pharmaceutical Co., Ltd), and Dr Shunji Hattori, PhD (Japan Institute of Leather Research) for excellent technical support.

Conflict of interest statement. None declared.

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Received for publication: 22.5.04
Accepted in revised form: 4.6.05