Renal Expression of the Ets-1 Proto-oncogene during Progression of Rat Crescentic Glomerulonephritis

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Abstract. The ets-1 proto-oncogene is a member of the transcriptional factor family and was identified by homology to the v-ets oncogene. It was recently demonstrated that Ets-1 protein interacts with the promoter region of the genes coding for proteinases, including matrix metalloproteinase-1 (MMP-1), MMP-3, and urokinase-type plasminogen activator, suggesting that it may play an important role in the regulation of MMP expression. The role of the ets-1 proto-oncogene in advanced glomerular diseases, where extracellular matrix accumulation is observed, remains undefined. In this study, the expression of ets-1 mRNA and protein during the progression of rat crescentic glomerulonephritis was examined using immunohistochemical analysis, reverse transcription-PCR, and in situ hybridization. Passive accelerated anti-glomerular basement membrane-induced nephritis was induced in rats by intravenous injection of nephrotoxic serum. Rats were euthanized on day 7, 14, 21, 28, or 42. Immunohistochemical analysis demonstrated significant upregulation of Ets-1 protein expression in glomeruli and the interstitium in anti-glomerular basement membrane-induced nephritis. The numbers of Ets-1-positive cells were increased 8.8-fold on day 21 in glomeruli (1.2 ± 0.1 cells/glomerular cross-section, P < 0.001) and sixfold on day 28 in the interstitium (21 ± 1.3 cells/mm², P < 0.001), compared with control samples. Ets-1 protein was predominantly localized in glomerular epithelial cells, endothelial cells, and interstitial cells. A small number of vascular endothelial cells, macrophages, and T cells also expressed Ets-1 protein. MMP-3 deposition was upregulated and positive cells in the interstitium often coexpressed Ets-1, whereas only a few glomerular cells were positive for both MMP-3 and Ets-1 protein. The expression of ets-1 mRNA was also markedly increased in diseased kidneys. The distribution of ets-1 mRNA was similar to that of the protein. These results indicate that overexpression of the ets-1 proto-oncogene by phenotypically altered renal cells might be associated with the pathogenesis of rat crescentic glomerulonephritis.

Most forms of advanced glomerular diseases are characterized by accumulation of extracellular matrix (ECM) proteins in glomeruli, and proteinuria is caused by changes in the glomerular basement membrane (GBM) and mesangial matrix. Therefore, it is important to elucidate the regulatory mechanisms of ECM metabolism in glomerular diseases.

The normal glomerular ECM is primarily composed of various types of collagens, laminin, fibronectin, and sulfated proteoglycans. In vivo and in vitro studies have demonstrated that these components are secreted by mesangial and visceral epithelial cells (1,2). It has also been demonstrated that phenotypically altered renal cells are mainly responsible for the increased production of these components, as well as disease-specific components that are not expressed in normal kidneys. Several lines of evidence suggest that an imbalance between synthesis and degradation of these components is closely associated with the accumulation of ECM and the subsequent progression of renal diseases (3).

Matrix metalloproteinase (MMP) and tissue inhibitors of metalloproteinases (TIMP) play an important role in ECM remodeling in various glomerular diseases (4,5). MMP are zinc-dependent proteases that play a critical role in the turnover of ECM components, including collagens, elastin, laminin, proteoglycans, fibronectin, and other glycoproteins. MMP are generally classified into five categories on the basis of their properties, i.e., collagensases (MMP-1, -8, -13, and -18), gelatinases (MMP-2 and -9), stromelysins (MMP-3 and -10), membrane-type MMP (MMP-14, -15, -16, and -17), and others (MMP-7, -11, -12, and -19) (6). Previous studies showed that glomerular resident cells express and secrete several types of MMP, including gelatinase A (MMP-2), stromelysin-1 (MMP-3), and gelatinase B (MMP-9). A variety of MMP are expressed by cultured mesangial cells and/or glomerular epithelial cells (GEC) and are thought to be involved in glomerular injury in animal models (7–10). MMP-9, known as type I collagenase, is expressed in cultured mesangial cells as well as GEC. In studies of the passive Heymann nephritis model, McMillan et al. (10) demonstrated a marked increase in MMP-9 synthesis within GEC. MMP-3 is also present in both...
animal and human kidneys. Altered expression of MMP-3 mRNA has been detected in renal biopsy specimens from patients with IgA nephropathy and diabetic nephropathy (11,12).

The ets-1 proto-oncogene is a member of the transcription factor family that demonstrates homology to the v-ets oncogene (13,14). Ets-1 protein binds to DNA sequences containing a central GGA (A/T) core sequence (PEA3) via a DNA-binding domain and cooperates with the c-Fos/c-Jun complex at the AP-1 site to activate the expression of certain promoters (15). This motif has been observed in the promoter regions of numerous genes, including MMP-1, -3, and -9, urokinase-type plasminogen activator (u-PA), and TIMP-1 (16–21). Furthermore, Ets-1 protein enhances the promoter activity of MMP-1, MMP-3, and u-PA genes, indicating that the ets-1 proto-oncogene plays a pivotal role in the regulation of matrix proteinase expression. Therefore, it seems that regulation of the activity of several proteinases is one of the most important functions of Ets-1 protein, although it exhibits broad biologic functions.

Although many studies have been performed to elucidate the altered expression of several proteinases in animal and human glomerular diseases, the expression of the ets-1 proto-oncogene in the kidney has not yet been determined.

In this study, we investigated whether the expression of the ets-1 proto-oncogene is involved in the progression of renal injury in a rat model of accelerated anti-GBM-induced glomerulonephritis, using immunohistochemical analysis, Western blotting, reverse transcription (RT)-PCR, and in situ hybridization. This model is characterized by crescentic severe proliferative glomerulonephritis, significant glomerular accumulation of macrophages and T lymphocytes, and severe interstitial inflammation (22–25). These changes ultimately lead to glomerular sclerosis and interstitial fibrosis. Therefore, the model seems to be useful for investigating the imbalance between ECM production and degradation in advanced glomerulonephritis. We also examined whether upregulation of Ets-1 protein in the nephritis model corresponded with renal MMP-3 expression, by correlating Ets-1-positive cells with MMP-3-positive cells in this model.

**Materials and Methods**

**Induction of Rat Crescentic Glomerulonephritis**

Nephrotic serum (NTS) was produced by immunizing male rabbits with a particulate fraction of rat GBM, as described previously (26). Accelerated anti-GBM-induced glomerulonephritis was induced in inbred male Sprague-Dawley rats. Briefly, 40 rats weighing 150 to 200 g were preimmunized by subcutaneous injection of 5 mg of normal rabbit IgG in Freund’s complete adjuvant and were given intravenous injections of 300 µl of rabbit anti-rat GBM serum (NTS) 5 d later (day 0). Groups of eight rats were euthanized on day 7, 14, 21, 28, or 42. In addition, six normal rats were used as control animals. The experimental protocol was approved by the Ethics Review Committee for Animal Experimentation of the Nagasaki University School of Medicine.

**Renal Function and Proteinuria**

For assessment of renal function, each rat was kept in a single-animal metabolism cage and 24-h urine samples were collected at the time of euthanasia. Urinary protein excretion was measured by the sulfosalicylic acid method (SRL, Nagasaki, Japan). Blood samples were obtained from the tail artery at the end of each experimental period, before euthanization, and plasma urea levels were measured by the urease ultraviolet assay.

**Morphologic Analysis**

Renal tissues were fixed overnight in 4% paraformaldehyde/phosphate-buffered saline (PBS) (pH 7.4), embedded in paraffin, sectioned at 4 µm, and stained with periodic acid-Schiff (PAS) stain and Masson trichrome reagent. For each animal, glomerular and tubulo-interstitial damage was evaluated as follows: (1) percentage of glomerular crescents, (2) percentage of global sclerosis, (3) scoring of glomerular hypercellularity, and (4) scoring of tubulointerstitial damage. A minimum of 50 glomeruli/animal was assessed to determine the glomerular score (1 to 3) for each animal. Glomerular hypercellularity was evaluated as total glomerular cell counts/glomerular cross-section (gcs) in PAS-stained sections, as described previously (27), as follows: 0, normal (<50 cells/gcs); 1, mild (50 to 80 cells/gcs); 2, moderate (80 to 120 cells/gcs); 3, severe (>120 cells/gcs).

Cortical tubulointerstitial injury was characterized by tubular atrophy and dilation, cast formation, interstitial fibrosis, and inflammatory cell infiltration into the interstitium. It was semiquantitatively analyzed in Masson trichrome-stained sections and divided into four grades, as follows: 0, no abnormal findings; 1+, mild (<30% of the cortex); 2+, moderate (30 to 60% of the cortex); 3+, severe (>60% of the cortex).

**Immunohistochemical Analysis**

Immunohistochemical analysis was performed on paraffin-embedded sections, as described previously (28). Briefly, paraffin-embedded sections (4 µm) that had been fixed with 4% paraformaldehyde in PBS (pH 7.4) were deparaffinized with xylene. Sections were blocked for 1 h with either 10% goat serum or 10% rabbit serum, with 5% nonfat dry milk, and were then incubated overnight at 4°C with primary antibodies to the following: Ets-1 (C-20, rabbit polyclonal antibody against the carboxyl-terminal domain of the Ets-1 protein; Santa Cruz Biotechnology, Santa Cruz, CA) (29), MMP-3 (55-2A4, mouse monoclonal antibody against human MMP-3; Fuji Chemical Industries, Japan) (30), α-smooth muscle actin (Dako, Denmark), vimentin (Dako), desmin (Dako), ED1 (monocytes/macrophages and some dendritic cells; Serotec, UK), CD8 (T cell cytotoxic/suppressor subset; Antigenix America), CD4 (T cell helper/inducer subset; Antigenix America), and Ki-B1R (B cells and plasma cells; Biomedicals AG). After washing with PBS, sections were processed further using the Histostain streptavidin-alkaline phosphatase kit (Zymed Laboratories, San Francisco, CA), and reaction products were developed with a mixture of 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium chloride. Preabsorption of the primary antibody with excess recombinant Ets-1 peptide (Santa Cruz Biotechnology) was performed for negative control samples. Spleen tissue served as the internal positive control for Ets-1 protein immunostaining, and Ets-1 expression was confirmed by Western blot analysis. As negative controls for the other antibodies, sections were reacted with 10% normal rabbit serum with 5% nonfat dry milk or PBS, instead of specific antibodies. In addition, sections were also reacted with polyclonal antibodies to the following: laminin (rabbit polyclonal antibody; Chemicon International), collagen III (rabbit polyclonal antibody; Sanbio Co., The Netherlands), and collagen IV (rabbit polyclonal antibody; LSL Co., Japan). Briefly, paraffin-embedded or frozen tissue samples were cut into 4-µm sections and treated for 30 min with 0.3% H₂O₂ in methanol at room temperature, to inactivate
endogenous peroxidase. Sections were then incubated overnight at 4°C with the primary antibody and stained using the Histofine streptavidin-horseradish peroxidase (HRP) kit (Nichirei Laboratories, Japan). The reaction products were observed with 3,3'-diaminobenzidine-4HCl and H₂O₂. As negative controls, normal goat or rabbit serum, with 5% nonfat dry milk, was used at the same dilution. The percentages of Ets-1- or MMP-3-positive cells, CD4- or CD8-positive T cells, B cells, and ED1-positive macrophages were determined in at least 40 gcs, as well as in 40 high-power fields of cortical tubulointerstitial area selected at random in 1 mm², through the eyepiece of the microscope.

Double-Immunostaining

Double-immunostaining was performed to localize Ets-1-expressing cells, as described previously (31). Briefly, sections treated with monoclonal antibodies to MMP-3, α-smooth muscle actin, vimentin, desmin, ED1, CD4, CD8, or Ki-B1R were initially stained using the alkaline phosphatase method and then treated with 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium chloride, which produced dark purple staining. Sections were then pretreated with H₂O₂ to quench endogenous peroxidase activity, further stained for Ets-1 using the peroxidase method, and treated with H₂O₂ and aminoethylcarbazole, which produced intense red staining (Histostain double-staining kit; Zymed). In some cases, sections were initially stained for Ets-1 and then counterstained with the other antibodies, as described above.

Western Blot Analysis of Ets-1 Protein Expression

Frozen samples of renal cortex were lysed (lysis buffer was composed of 10 mM phosphate buffer, 1 mM phenylmethylsulfonyl fluoride, 2 μg/ml leupeptin, 2 μg/ml chymostatin, and 0.2% Triton X-100), and the protein concentration was measured with an ultraviolet/visible spectrophotometer (UV-1600; Shimadzu, Japan). Each 100-μg protein sample was subjected to electrophoresis on a 10% sodium dodecyl sulfate-polyacrylamide gel and transferred to polyvinylidene difluoride paper. Then, after blocking for 2 h at room temperature with 20 mg/ml bovine serum albumin dissolved in Tris-buffered saline solution, Ets-1 protein was detected using the polyclonal rabbit antibody (C-20; Santa Cruz Biotechnology). Blots were

**Figure 1.** Serial changes in blood urea nitrogen (BUN) levels (A) and urinary protein excretion (B) in nephritic rats. Each data point represents the mean ± SEM.

**Figure 2.** Histologic findings for rats with nephrotoxic serum (NTS)-induced nephritis. (A) Control rat. (B) Day 21, glomerular crescent formation with narrowing of the capillary lumen. (C) Day 28, interstitial inflammatory cell infiltration, with fibrosis and tubular damage. Original magnification: ×103 for A and C; ×257 for B. Periodic acid-Schiff (PAS) stain.
washed and incubated with HRP-conjugated goat antibody to rabbit IgG (Jackson ImmunoResearch Laboratories, PA). Immunoreactive protein was detected by enhanced chemiluminescence (Amersham, Buckinghamshire, UK).

**In Situ Hybridization**

In situ hybridization was performed to localize ets-1 mRNA-expressing cells by using a T-T-dimerized synthetic oligo-DNA probe, as described previously (32,33). Briefly, rat ets-1 mRNA was detected using an oligo-DNA probe complementary to a fragment of rat mRNA. The sequence of the antisense probe was 5'-GCCAGCT-TCATCACAGAGTCCTATCAGAC-3'. Two TTA repeats were added to both the 5' and 3' ends of that sequence during synthesis (32). After hybridization with ets-1 mRNA was 100% hybridizable RNA in each section by using a competitive study to verify the specificity, the T-T-dimerized sense probe was hybridized with adjacent sections as negative controls. Furthermore, a competitive study was performed by adding a 100-fold excess amount of homologous or unrelated unlabeled oligonucleotides to the hybridization buffer together with the antisense probe. In addition, we evaluated the levels of hybridizable RNA in each section by using in situ hybridization of 28S rRNA, as described previously (32).

**RT-PCR**

RT-PCR was used to detect ets-1 mRNA expression, as described previously (34). Briefly, total RNA was extracted from the renal cortex by using the acid guanidinium phenol method. For this purpose, 3 μg of total RNA template was incubated with 50 ng of random hexamers at 70°C for 10 min and then quickly chilled on ice, followed by incubation for 10 min at room temperature. The sample was then treated for 50 min at 42°C with 200 U of Superscript RT, 500 μM dNTP mixture, 10 mM dithiothreitol, 20 mM Tris-HCl (pH 8.4), 5 mM KCl, and 2.5 mM MgCl<sub>2</sub> in a final volume of 20 μl, using a single-stranded cDNA synthesis kit (Superscript Preamplification...
System, Life Technologies-BRL). The reaction was terminated by heating to 90°C for 5 min. The primers for rat ets-1 were 5'-GC-CCAGCTTCATCACAGAGT-3' (upper) and 5'-TGTTGAAAGAT-GACTGGCTG-3' (lower), which were synthesized by BEX Co. The PCR product from cDNA amplification using the aforementioned primers was 296 bp. PCR was performed by using the GeneAmp PCR reagent kit (Perkin Elmer Cetus, CT). Reactions were performed in a total volume of 25 μl, containing 5 μl of the cDNA preparation, 10 mM Tris-HCl, 50 mM KCl, 1.5 mM MgCl₂, 0.001% gelatin, 200 μM levels of each dNTP, 2.5 U of Taq DNA polymerase, and 0.25 μM levels of each primer. Samples were subjected to 29 cycles of PCR amplification using a thermocycler (PC701; Astec Co., Japan). The PCR conditions for ets-1 were denaturation at 94°C for 1 min, annealing at 58°C for 1 min, and extension at 72°C for 1.5 min. As a positive internal control for RT-PCR efficacy, primers were derived for the constitutively expressed housekeeping gene β-actin. The primers for rat β-actin were 5'-CTGATCCACATCTGCTGGAG-GTG-3' (upper) and 5'-ACCCTCAACACCGCCATGTACG-3' (lower). The PCR conditions were the same as described above, and the product from cDNA amplification was 703 bp. The reaction products were subjected to electrophoresis on a 2% agarose gel and stained with ethidium bromide. Semiquantitative analysis was performed using an image analyzer (Chemi Imager; Astec), and results were expressed as the Ets-1/β-actin signal intensity ratio. Sequential analysis of the PCR products was performed with an autosequencer, to confirm specific amplification of ets-1 mRNA. Briefly, ets-1 primers were labeled with fluorescein at the 5'-end by 5'-oligolabeling for fluorescein (Amersham). DNA sequencing was performed using Thermo-Sequenase (Amersham) and the fluorescein-labeled primer in a DNA sequencer (DSQ-1000L; Shimadzu, Japan). The reactions were performed according to the instructions provided by the manufacturer.

### Statistical Analyses

Data were expressed as mean ± SEM. Differences between groups were examined for statistical significance using the Mann-Whitney U test or one-way ANOVA. Correlations were examined by Pearson’s correlation analysis. A P value of <0.05 indicated a statistically significant difference.

### Results

#### Renal Function and Proteinuria

Injection of NTS resulted in a rapid increase in blood urea nitrogen levels, reaching a plateau level on day 7 (Figure 1A). Injection of NTS also resulted in proteinuria, reaching a maximal level on day 28 (NTS, 5119 ± 1308 mg/dl; control, 141 ± 18.5 mg/dl; P < 0.001) (Figure 1B).
Histologic Findings

Injection of NTS resulted in the development of crescentic necrotizing glomerulonephritis in all rats (Figure 2; Table 1). On day 21, treated rats exhibited moderate to severe hypercellularity, segmental lesions, and marked cellular or fibrocellular crescents. Furthermore, marked tubulointerstitial damage, such as tubular atrophy and degeneration, interstitial fibrosis, and infiltration into the interstitium, was noted. Although glomerular hypercellularity diminished on day 42, severe glomerular sclerosis, including global sclerosis, was most pronounced. Furthermore, extensive tubular damage and interstitial fibrosis were noted.
Immunohistochemical Assessment of Infiltrating Cells
Infiltrating ED1-positive macrophages, CD4-positive T cells, CD8-positive T cells, and Ki-B1R-positive B cells were identified by immunohistochemical analysis at different times during the progression of glomerulonephritis (Table 2). A few macrophages were detected in the glomeruli and interstitium in normal rat kidney. In contrast, numerous ED1-positive macrophages were observed in both the glomeruli and interstitium on day 7, and numbers further increased between day 14 and day 42. The maximal numbers of macrophages were observed on day 21 in the glomeruli and on day 28 in the interstitium. The densities of CD4-positive and CD8-positive T cells were also significantly increased in glomeruli on day 7; however, these cells were more frequently observed in the interstitium and reached peak densities on day 28. Furthermore, there were significant increases in B cell counts in both the glomeruli and interstitium, although their infiltration was less than that of other cell types.

Expression of Ets-1 and MMP-3 in Anti-GBM Disease
We also investigated the expression of Ets-1 protein in the renal cortex by immunohistochemical analysis and Western blotting (Figures 3, 4, and 5). Ets-1-positive cells were only occasionally detected in the glomeruli and tubulointerstitium in control rat kidneys. In contrast, numerous Ets-1-positive glomerular intrinsic cells and interstitial cells and some tubular epithelial cells were noted in NTS-treated rat kidneys. Staining in immunopositive cells was limited to the nuclei, as reported previously (29). The number of Ets-1-positive cells increased approximately 8.5- to 8.8-fold on days 14 to 21 in glomeruli in this model, compared with control kidneys, and increased approximately 5.7- to 6.0-fold on days 21 to 28 in the interstitium. These results
indicated that the peak expression of Ets-1 occurred earlier in the glomeruli (days 14 to 21) than in the interstitium (days 21 to 28) (Figure 4). Moreover, we demonstrated, by Western blotting, an Ets-1 band (54 kD) in the renal cortex of diseased rats (days 14 and 21) (Figure 5).

MMP-3 expression was also significantly upregulated in the glomeruli and interstitium during the progression of glomerulonephritis, compared with control kidneys (Figures 6 and 7). In particular, MMP-3-immunopositive cells were similar to Ets-1-expressing cells in the interstitium of diseased rats (days 14 and 21) (Figure 7).

Expression of ECM in Anti-GBM Disease

To investigate the expression of ECM known to be degraded by MMP-3, we performed additional immunostaining, including staining for type III and IV collagen and laminin. Positive immunostaining for type IV collagen and laminin was present in the mesangium and GBM in the glomeruli of control rats, but no intraglomerular immunostaining for type III collagen was observed. In the tubulointerstitium of control rats, focal deposition of type III collagen was observed in the interstitium, whereas type IV collagen and laminin were observed mainly in the tubular basement membrane. As we previously described for other types of human and/or animal nephritis (28,31), type III collagen was strongly present in interstitial fibrosis, fibrous crescentic lesions, and the sclerotic matrix of glomeruli in NTS-treated rats. The level of expression was proportional to the progression of glomerular sclerosis and interstitial fibrosis (Figure 8). Type IV collagen was also identified in the sclerotic lesions of glomeruli and in the thickened tubular basement membrane, compared with the lesser accumulation of laminin (data not shown).

Characterization of Ets-1-Positive Cells by Double-Immunostaining

To identify cells expressing Ets-1 protein, we performed double-staining for Ets-1 and various marker molecules, such
as α-smooth muscle actin, desmin, and vimentin, as noted above (Figures 9 and 10). α-Smooth muscle actin and desmin were used as references for phenotypic changes of mesangial cells and visceral epithelial cells in glomeruli, whereas vimentin and α-smooth muscle actin were used for identification of injured tubular epithelial cells and myofibroblasts in the tubulointerstitium, respectively (35–37). To examine the relationship between Ets-1 and infiltrating cells, double-immunostaining was performed for CD4, CD8, ED1, and Ki-B1R. In addition, we also double-stained the tissues for Ets-1 and MMP-3. In glomeruli of NTS-treated rats, Ets-1-positive cells were identified as predominantly desmin-positive GEC (including crescentic cells) and glomerular endothelial cells, whereas only a few ED1-positive macrophages and CD4-positive T cells were positive for Ets-1 protein. Furthermore, only a few cells were positive for both Ets-1 and MMP-3 in glomeruli (Figure 9). In the tubulointerstitial, Ets-1-positive cells were predominantly observed in α-smooth muscle actin-positive myofibroblasts and vascular smooth muscle cells and in a small number of vascular endothelial cells. In addition, ED1-positive infiltrative macrophages and CD4- and/or CD8-positive T cells demonstrated modest Ets-1 expression. In contrast, the double-staining procedure demonstrated that MMP-3-positive interstitial cells were more often colocalized with Ets-1 expression than were those in the glomeruli (Figure 10).

**Expression of ets-1 mRNA in Anti-GBM Disease**

The expression of ets-1 mRNA in the renal cortex was determined by RT-PCR analysis of RNA isolated from rats with anti-GBM-induced nephritis (Figure 11). Expression of ets-1 mRNA was present in all samples, including those from control rats, and sequential analysis confirmed that the amplification product of 296 bp exhibited 100% homology with the published rat ets-1 cDNA sequence (data not shown). Semi-quantitative analysis demonstrated that ets-1 mRNA levels, relative to β-actin levels, were 3.7 to 6.5 times higher in rats with anti-GBM-induced nephritis, compared with control rats. We also examined the distribution of ets-1 mRNA expression in control and nephritic rats on days 14 and 21 by in situ
hybridization (Figure 12). A few glomerular and interstitial cells that were weakly positive for ets-1 mRNA expression were observed in control kidneys. In contrast, in situ hybridization revealed a significant increase in the number of ets-1 mRNA-positive glomerular and interstitial cells in the renal tissue of NTS-treated rats. Glomerular cells expressing ets-1 mRNA were identified as GEC or endothelial cells. A small number of infiltrating cells also expressed ets-1 mRNA. However, there was little or no expression in glomerular mesangial cells. In the tubulointerstitium, signals for ets-1 mRNA were observed in some interstitial cells, as well as a few vascular endothelial cells and infiltrating cells. These results for the glomeruli and interstitium were similar to those for Ets-1 protein expression, as detected by immunohistochemical analysis.

Discussion

To clarify the involvement of the ets-1 proto-oncogene in the development and progression of glomerular sclerosis and interstitial fibrosis in glomerulonephritis, in this study we investigated the expression of ets-1 mRNA and protein in a rat model of accelerated anti-GBM-induced glomerulonephritis, using immunohistochemical analysis, Western blotting, RT-PCR, and in situ hybridization. Our results indicated induced expression of the ets-1 proto-oncogene in GEC, glomerular endothelial cells, interstitial myofibroblasts, and a small number of vascular endothelial cells and infiltrating cells in the model kidneys. These results demonstrate, for the first time, that the expression of ets-1 mRNA and protein is upregulated and that such expression is closely related to the progression of glomerular and tubulointerstitial damage.

The ets-1 proto-oncogene is a transcription factor that was originally identified by sequence homology with the viral v-ets oncogene of the E26 avian leukemia retrovirus (13). Expression of the ets-1 proto-oncogene has been detected in various cells, and the role of the ets-1 gene expressed in mesodermal lineage cells, such as fibroblasts and endothelial cells, has drawn wide attention in the fields of embryogenesis and angiogenesis (29,38 – 40). Recent studies have also demonstrated that Ets-1 protein exhibits multiple activities in the transcriptional regulation of numerous genes. Among these activities, one of the most important functions seems to be activation of the transcription of matrix proteinases such as MMP-3 and u-PA (16 –19,21). This study demonstrated that not only serial changes in expression but also the cellular distribution of MMP-3 was similar to that of Ets-1 protein. Moreover, MMP-3-positive interstitial cells in diseased kidneys often expressed Ets-1 protein, as demonstrated by double-immunostaining, thus confirming the close correlation between Ets-1 and MMP-3 expression. The upregulated expression of MMP-3 induced by Ets-1 might participate in matrix remodeling in injured renal tissues. However, only a few cells in the glomerular interstitium expressed ets-1 mRNA, as observed by reverse transcription-PCR analysis (Figure 11). In contrast, in situ hybridization revealed a significant increase in the number of ets-1 mRNA-positive glomerular and interstitial cells in the renal tissue of NTS-treated rats. Glomerular cells expressing ets-1 mRNA were identified as GEC or endothelial cells. A small number of infiltrating cells also expressed ets-1 mRNA. However, there was little or no expression in glomerular mesangial cells. In the tubulointerstitium, signals for ets-1 mRNA were observed in some interstitial cells, as well as a few vascular endothelial cells and infiltrating cells. These results for the glomeruli and interstitium were similar to those for Ets-1 protein expression, as detected by immunohistochemical analysis.

Figure 11. Reverse transcription-PCR analysis of ets-1 mRNA expression in control rats and rats with NTS-induced nephritis. (A) Renal cortex from control rats and from diseased rats at days 7 to 28, demonstrating the expression of 296-bp ets-1 mRNA. (B) Expression of β-actin, as an internal control. Numbers at the bottom indicate the normalized ets-1/β-actin mRNA ratios, as determined using an image analyzer (Chemi Imager; Astec).

Figure 12. In situ hybridization study of ets-1 mRNA expression in the renal tissue of nephritic rats. (A) Day 21. Localization of ets-1 mRNA is observed primarily in glomerular epithelial cells and occasionally in glomerular endothelial cells by counterstaining with PAS (arrowhead). (B) Day 14. Increased signal for ets-1 mRNA is observed in spindle-shaped interstitial cells (arrowhead). (C) Day 21, negative control. The section was hybridized with ets-1 sense T-T-dimerized oligo-DNA probe, which yielded no specific signals. Magnifications: ×357 for A and C; ×257 for B.
ulteriorly exhibited coexpression by double-staining. Although the reason for this discrepancy is not fully understood, it is possible that the turnover of Ets-1 protein is too rapid for colocalization with MMP-3 in glomerular resident cells. Alternatively, it is possible that other transcriptional factors, such as c-Jun and c-Fos, are engaged in the induction of MMP-3 expression, in combination with Ets-1 protein (8,41).

MMP-3 is important for the degradation of ECM components, such as type III, IV, and VI collagen, laminin, aggrecan, and fibronectin (6). As previously demonstrated in advanced glomerulonephritis (3), the expression of major matrix substrates for MMP-3, such as type III or IV collagen, increases in advanced glomerulonephritis and further increases with the progression of renal injury, as confirmed by immunohistochemical analysis. Furthermore, overexpression of these matrices in the kidney seems to coincide with the time course of enhanced MMP-3 expression. These results suggest that MMP-3 plays a pivotal role in ECM remodeling in our system.

Previous studies demonstrated signals for MMP-3 mRNA in glomerular resident cells, including mesangial, epithelial, and endothelial cells, and cells of Bowman’s capsule in human diseased kidneys, by in situ hybridization analysis (11,12). However, these findings for NTS-treated rats demonstrated minimal expression of MMP-3 in glomerular mesangial cells. Considering that the major changes in the NTS model occur in the phenotype and mitotic rates of GEC, rather than mesangial cells (as is more common in other types of glomerulonephritis), the discrepancy in the expression of MMP-3 could be explained by the different features of glomerular damage. Further studies using other forms of experimental and human glomerulonephritis are necessary to clarify the relationship between the expression of MMP-3 and glomerulonephritis, including the involvement of MMP inhibitors such as TIMP-1.

High levels of ets-1 gene expression have been demonstrated in T lymphocytes, and there is evidence that such expression is essential for the maintenance of the normal pool of resting T cells (42,43). Moreover, the Ets-1 binding site is present in the T cell receptor gene enhancer and CD4 gene core promoter (44,45), and it was reported that the ets-1 proto-oncogene might play an important role in T cell activation (46). Because the model of nephrotoxic nephritis is known to be closely associated with the accumulation of T cells in the kidney, as described above, we expected to observe the ets-1 gene predominantly expressed in T cells in nephritic kidneys. Unexpectedly, however, only a small number of T cells expressed ets-1 mRNA and protein. In this context, it should be noted that Ets-1 products are involved in maintaining T cells in a quiescent state and ets-1 gene levels are decreased after the activation of T cells (46,47), indicating that most infiltrating T cells might already be activated in the kidneys of NTS-treated rats.

Finally, recent studies demonstrated that the ets-1 gene is essential for the normal development of mammalian kidneys and the maintenance of glomerular integrity and that the Ets-1 protein may act as an upstream regulator of the expression of FREAC-4, a winged helix transcriptional factor that is detected during kidney development (48,49). In fact, kidneys from ets-1-knockout mice exhibited various glomerular abnormalities, including sclerosis, atrophy, and markedly fewer and immature glomeruli (48). These results might indicate a protective reaction against destruction of the normal structure of the kidney, in the presence of Ets-1 protein overexpression. Further studies are needed to determine whether the ets-1 proto-oncogene acts as a progression factor or a protection factor in various forms of glomerulonephritis.

In conclusion, this study demonstrated that the expression of the ets-1 proto-oncogene was upregulated in the renal tissue of a rat model of crescentic glomerulonephritis. Expression of ets-1 mRNA was observed primarily in glomerular resident cells, interstitial myofibroblasts, and some vascular endothelial cells and infiltrating cells. Overexpression of the ets-1 proto-oncogene might be associated with the pathogenesis of glomerulonephritis through the expression of MMP.

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