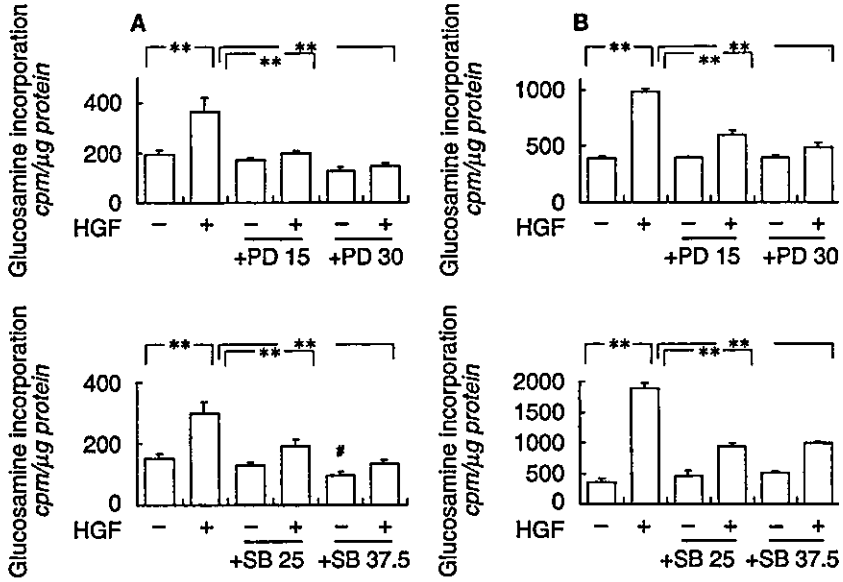

Fig. 5. Effects of MAPK/ERK kinase (MEK) inhibitor and p38 mitogen-activated protein kinase (MAPK) inhibitor on hepatocyte growth factor (HGF)-induced changes in cell-associated and secreted proteoglycan synthesis in NRK-49F cells. Quiescent cells were pretreated with (upper panel) the MEK inhibitor PD98059 (PD 15:15 μmol/L; PD 30:30 μmol/L), or (lower panel) the p38 MAPK inhibitor SB203580 (SB 25:25 μmol/L; SB 37.5:37.5 μmol/L) or vehicle [dimethyl sulfoxide (DMSO)], prior to stimulation with 20 ng/mL HGF for 48 hours, then proteoglycan synthesis in the cell layer (*A*) and the media (*B*) was assayed as described in the **Methods** section. Results shown are the mean ± SEM ($N = 4$ per assay point). $^{**}P < 0.01$ vs. the respective groups; $\#P < 0.05$ vs. control HGF(−) (column 1).

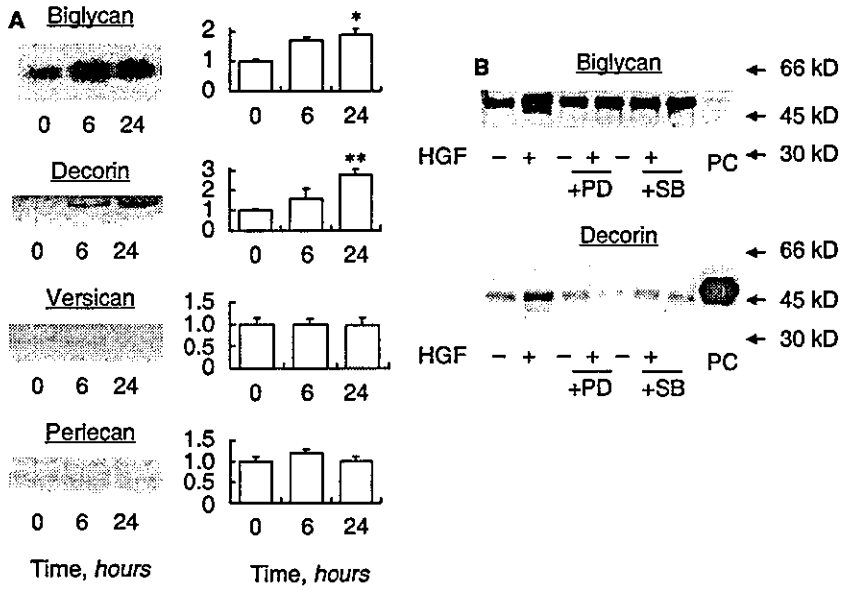

Fig. 6. Effects of hepatocyte growth factor (HGF) on proteoglycan core protein expression. (*A*) NRK-49F cells were treated with HGF (20 ng/mL) for the indicated times, and levels of biglycan, decorin, versican, and perlecan mRNA were assessed by Northern blot analysis (biglycan), or reverse transcription-polymerase chain reaction (RT-PCR) (decorin, versican, perlecan). Representative image of Northern blot or RT-PCR assay (left panels). Results of laser densitometric quantitation (right panels). Results shown are the mean ± SEM ($N = 4$ per assay point). $^{*}P < 0.05$ vs. control; $^{**}P < 0.01$ vs. control. (*B*) Neonatal human fibroblast (NHF) cells were pretreated with the MEK inhibitor PD98059 (PD 30 μmol/L), or the p38 MAPK inhibitor SB20350 (SB 37.5 μmol/L), or vehicle [dimethyl sulfoxide (DMSO)], then incubated with or without HGF (20 ng/mL) for 72 hours, and levels of biglycan and decorin proteins in the media were assessed by Western blot as described in the **Methods** section. PC, positive control; 10 pmol of purified bovine biglycan or decorin were treated with chondroitinase ABC and run as positive controls. Arrows show the position of molecular size markers.

was assessed using an ELISA for active TGF-β1. No significant changes in either active TGF-β1 (without biglycan, 0.87 ± 0.11 ng/mL; with biglycan, 1.14 ± 0.11 ng/mL; $N = 4$) or total (active + latent) TGF-β1 (without biglycan, 1.27 ± 0.03 ng/mL; with biglycan, 1.31 ± 0.04 ng/mL; $N = 4$) were detectable in the culture media by ELISA.

DISCUSSION

The growth factor HGF is receiving increasing interest as a potential therapeutic candidate for the treatment of renal disease. It has been shown that the actions of HGF are mediated through a specific receptor c-Met [2]. This receptor is a transmembrane protein possessing an intracellular tyrosine kinase domain, and is activated by HGF leading to autophosphorylation of the receptor. Potential targets for HGF action in the kidney include components of the renal glomeruli (endothelial cells, mesangial cells, and epithelial cells), as well as tubular epithelial cells in the tubulointerstitial region [2, 3]. Our results using fibroblasts from two different sources suggested that these cells also express the c-Met mRNA. Moreover, we

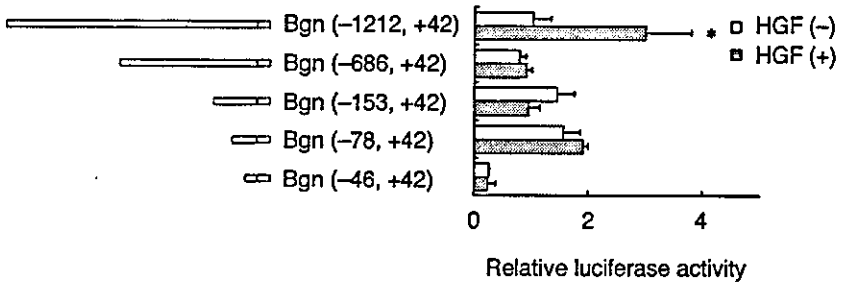

Fig. 7. Effects of hepatocyte growth factor (HGF) on biglycan promoter activity in NRK-49F cells. Cells were transfected with the indicated biglycan promoter-luciferase constructs, then treated with or without HGF (20 ng/mL) prior to assay of luciferase activity as described in the **Methods** section. Results shown are the mean ± SEM ($N = 4$ per assay point). *$P < 0.05$ vs. HGF (–).

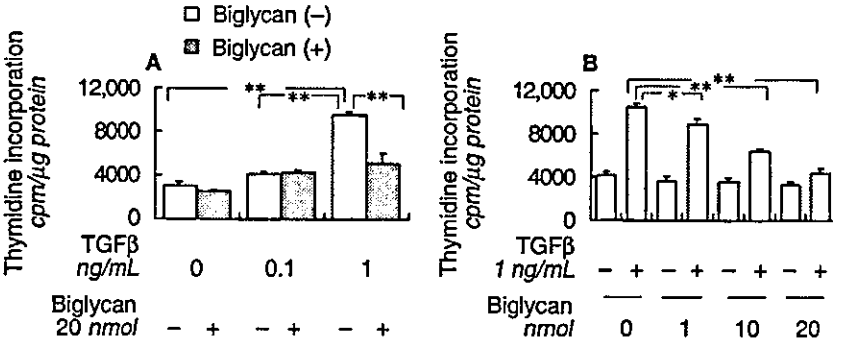

Fig. 8. Inhibition of transforming growth factor-β1 (TGF-β1)–mediated proliferative responses by biglycan in NRK-49F cells. Cells were stimulated with various concentrations of TGF- β1 together with a fixed concentration of biglycan (20 nmol/L) (*A*) or a fixed concentration of TGF- β1 (1 ng/mL) together with various concentrations of biglycan (*B*), then thymidine incorporation was assayed as described in the **Methods** section. *$P < 0.05$ vs. the respective groups; **$P < 0.01$ vs. the respective groups.

found that HGF can interact with the expressed receptors resulting in their phosphorylation and can also activate ERK1/2 and p38 MAPK, suggesting signal pathway mechanisms similar to those found in other cells such as lung adenocarcinoma cells [23]. The extension of potential targets of HGF in the kidney to fibroblasts may be important since these cells play a central role in the processes of interstitial fibrosis.

Previous studies from our and other laboratories have implicated changes in proteoglycan synthesis as an important factor in the actions of vasoactive peptides, hormones, and their antagonists [15, 16, 24]. These glycoproteins consist of a core protein covalently bound to one or more glycosaminoglycan (GAG) side chains [24]. The proteoglycans found in the kidney can be classified according to the composition of the GAG side chains into CSPG, DSPG, and HSPG. These proteoglycans have been implicated in many of the crucial steps connected with renal fibrosis, including the control of collagen deposition, and regulation of growth factors.

Since HGF may be an important modulator of fibrosis in the renal interstitium, we examined the effects of HGF on proteoglycan synthesis in the renal interstitial fibroblasts, and found that HGF can enhance the synthesis of both secreted and cell-associated proteoglycan synthesis. Because both ion exchange chromatography and enzyme digestion studies suggested subtype-specific regulation of proteoglycans by HGF, we further characterized the changes in proteoglycan core protein expression and found evidence for up-regulation of the core proteins biglycan and decorin. Since these two proteoglycans are both of the CSPG/DSPG class, the results of these studies at the molecular (mRNA) level were consistent with the biochemical findings. Confirmatory Western blots of proteoglycan core proteins were also performed on human cells (NHF) because the antibodies used (LF-51 and LF-136) were raised against the human proteins, and these results were also consistent with the results using NRK-49F cells.

Both biglycan and decorin are members of the small leucine-rich proteoglycan (SLRP) family, and are preferentially expressed in the renal interstitium [25, 26]. These proteoglycans are noted for their ability to interact with growth factors and cytokines. Decorin is of clinical interest, first, because it has been shown to be able to inactivate TGF-β both in vitro and in vivo, and second, because expression of decorin in the renal interstitum has been suggested to be a prognostic marker for renal disease progression [9, 10, 27]. Hildebrand et al [28] showed that both biglycan and decorin can bind to TGF-β and can inhibit binding of the growth factor to Mv 1 Lu cells. Of interest, the binding between TGF-β and biglycan or decorin was increased by removal of the proteoglycan GAG side chains, suggesting that the interaction is mediated through the core proteins [9, 28]. In this study, we determined whether biglycan can modulate the actions of TGF-β1 in NRK-49F fibroblasts and found that biglycan alone does not cause a significant change in fibroblast

proliferation, but showed the ability to attenuate the proliferative actions of TGF-β1 on fibroblasts. This finding is relevant since TGF-β1 is thought to play a central role in the progression of renal disease, and the long-term overproduction of this growth factor is thought to be a major event in the pathogenesis of interstitial fibrosis [29]. TGF-β has been shown to enhance the expression of biglycan in different cells, including fibroblasts, arterial smooth muscle cells, and mesangial cells [30–32]. These findings are compatible with the view that TGF-β–induced up-regulation of biglycan may act as a negative feedback loop to limit excessive action of this growth factor in these tissues, but further in vivo studies are required in this area.

As mentioned in the introduction, data from recent studies have suggested that HGF may have antagonistic actions to TGF-β, particularly in terms of renal fibrosis. Concerning the mechanisms of the interaction between HGF and TGF-β, it has been suggested that HGF may exert at least a part of its actions via down-regulation of TGF-β itself [5], as well as by up-regulation of extracellular matrix–degrading enzymes such as matrix metalloproteinase (MMP)-1, and MMP-9 [33]. The results of this study suggest the possibility that HGF may also interact with TGF-β in the renal interstitium by regulating TGF-β–modulatory proteoglycans.

Concerning potential mechanisms of the HGF-induced increase in gene expression of biglycan, our results suggested the involvement of transcriptional control at a region between –1212 and –686 relative to the transcription start site. Of interest, this region contains several putative regulatory elements, including interleukin-6 (IL-6) responsive elements, CCAAT/enhancer-binding protein (C/EBP), and activator protein-2 (AP-2) [21]. Potential transcription factors which have been reported to be affected by HGF include ETS1, nuclear factor-κB (NK-κB), AP-1, and activating transcription factor-2 (ATF-2) [34, 35], but the possibility that HGF could act by a different transcription factor cannot be excluded and requires further assessment.

HGF is currently anticipated to be of potential therapeutic benefit for the treatment of renal diseases, and indeed clinical trials using HGF for the treatment of vascular disease have already been started [36]. It is relevant that HGF can up-regulate decorin because decorin itself is a candidate for treatment of chronic renal failure [10, 11]. Although most in vivo studies have shown that HGF can reduce renal interstitial fibrosis [5–7], one report using transgenic mice that overexpress HGF noted detrimental effects such as increased focal segmental glomerulosclerosis, as well as renal hyperplasia and renal cysts [37]. Therefore, further studies are required to clarify the advantages and disadvantages of treatment strategies using HGF alone, compared with direct treatment with TGF-β–inhibitory proteoglycans (biglycan or decorin) for the treatment of progressive renal disease.

CONCLUSION

The results of this study suggest (*1*) that renal interstitial fibroblasts express the HGF receptor, which undergoes tyrosine phosphorylation after agonist treatment; (*2*) that HGF can activate ERK and p38 MAPK pathways in these cells; (*3*) that HGF up-regulates proteoglycans of the CSPG/DSPG class, namely biglycan and decorin; and (*4*) that the HGF-mediated effects involve both ERK and p38 MAPK-mediated pathways, as well as subtype-specific increase in gene expression through transcriptional control. Since proteoglycans have important growth-regulatory effects in the renal interstitium, these results may be important for furthering our understanding of how HGF can be of therapeutic value in inhibiting the progression of renal interstitial changes.

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Therapeutic effect of all-trans retinoic acid on rats with anti-GBM antibody glomerulonephritis

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Therapeutic effect of all-trans retinoic acid on rats with anti-GBM antibody glomerulonephritis.

Background. All-trans retinoic acid (ATRA) has antiproliferative and anti-inflammatory effects and is currently used in the treatment of leukemia and dermatologic diseases. We tested the therapeutic potential of ATRA on anti-glomerular basement membrane (GBM) glomerulonephritis rats.

Methods. Glomerulonephritis was induced in male Wistar-Kyoto rats on day 0 by an intravenous injection of antirat GBM antibody. On day 14 after the induction of anti-GBM glomerulonephritis, some rats were sacrificed ($N = 5$). Another 10 rats were divided into two groups: the vehicle group ($N = 5$) and the ATRA treated group ($N = 5$). ATRA was orally administrated from day 14 to day 27 after disease induction. Blood pressure, body weight, urinary protein excretion, and blood chemistry was determined on days 1, 14, 21, and 27. Kidney samples were obtained on day 28. The kidneys were examined with periodic acid-Schiff staining (PAS) and immunohistochemistry using antibodies against the proliferative cell nuclear antigen (PCNA), rat monocyte and macrophage (ED-1), and α-smooth muscle actin (α-SMA). Glomerular RNA was extracted from isolated glomeruli, and reverse transcription (RT) followed by polymerase chain reaction (PCR) was performed.

Results. ATRA administration produced a 55% reduction of proteinuria in glomerulonephritis rats. Light microscopic analysis revealed severe necrosis/crescent formation (>50% of the glomerulus) affecting 34% of glomeruli in vehicle rats, whereas ATRA treatment reduced the glomeruli showing severe change to 14%. ATRA also significantly reduced PCNA-positive cells, ED-1–positive cells and α-SMA–positive area in the glomeruli. RT-PCR analyses revealed that a wide variety of genes including inflammation related [tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), and CCAAT enhancer-binding protein δ (C/EBPδ)], cell proliferation–related [platelet-derived growth factor (PDGF)] and fibrosis-related [transforming growth factor-β1 (TGF-β1), type I collagen, and α-SMA) genes were suppressed in the glomeruli of ATRA-treated rats.

Conclusion. ATRA administration significantly reduced severe necrosis/crescent formation and urinary protein excretion in glomerulonephritis rats. Suppression of a wide variety of gene expression may partly explain the mechanism of ATRA's antiproliferative and anti-inflammatory effects. These data suggest a novel therapeutic application of ATRA toward glomerulonephritis.

Key words: crescentic glomerulonephritis, phenotypic change, AP-1, c-fos, c-jun, C/EBPδ.

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Crescentic glomerulonephritis is a disease that rapidly progresses to renal failure in humans. In Wistar-Kyoto rats, administration of a small dose of anti-glomerular basement membrane (anti-GBM) antibody induces severe necrotizing glomerulonephritis with crescent formation [1].

Retinoic acids, which are biologically active derivatives of vitamin A, are necessary for normal growth, maintenance of tissues, reproduction, immune response, and survival [2]. Retinoic acid receptors belong to the supergene family of ligand-inducible transcriptional regulatory factors that includes steroid hormone, thyroid hormone, and vitamin D_3 receptors as well as the peroxisome proliferator–activated receptors (PPAR) and others [3]. Retinoid receptors are nuclear receptors, which enter the cell nucleus only after a ligand has been bound. They bind to specific sequence elements on the promoters of responsive genes, which allow the retinoid receptors to directly modulate gene transcription [4].

Retinoic acids are not complete newcomers in the area of human therapy. In dermatology, retinoids have been used for the treatment of acne, psoriasis, and neoplastic processes [5].

Recently, in anti-Thy1.1 nephritis, treatment of nephritic rats with all-trans retinoic acid (ATRA) or isotretinoin (13-cis RA) effectively limited renal damage and mesangial cell proliferation [6]. The retinoic acids attenuated the increase in glomerular cell proliferation, glomerular transforming growth factor-β1 (TGF-β1) expression, and urinary albumin excretion. These results indicate that the properties of retinoic acids to down-

regulate inflammatory and proliferative responses makes them attractive potential candidates for therapeutic use in renal disease.

In the present study, we tested the therapeutic efficacy of ATRA on anti-GBM antibody–induced renal injury. We started the ATRA therapy at 14 days after disease induction, when the disease is fully developed.

We found that urinary protein excretion was reduced in ATRA-treated group along with a significant attenuation of glomerular injury. A wide variety of glomerular gene expression was suppressed in ATRA-treated group, which may partly contribute to the therapeutic efficacy of ATRA on renal disease.

METHODS

Experimental protocol

Male Wistar-Kyoto rats, aged 12 weeks, were used in the present study. Glomerulonephritis was induced in 15 rats on day 0 by a single intravenous injection of 25 μL/100 g body weight of anti-rat GBM antiserum as previously reported [7]. Five rats were sacrificed on day 14. We divided the remaining 10 rats into two groups: the vehicle group, the anti-GBM glomerulonephritis without treatment ($N = 5$), and the ATRA-treated group ($N = 5$), the anti-GBM glomerulonephritis group treated by ATRA. ATRA was mixed with sesame oil (9 mg/mL) and was orally administrated at a dose of 30 mg/kg body weight using gavage tube once daily from day 14 to day 27. Blood pressure and body weight was determined on days 1, 14, 21, and 27. Blood pressure was measured by blood pressure monitor for rats and mice model MK-1100 (Muromachi Kikai Co., Ltd., Tokyo, Japan).

Proteinuria and creatinine determination

For determination of urinary protein excretion, rats were placed in metabolic cages and urine was collected for 24 hours on days 1, 14, 21, and 27. Blood samples were taken from tail vein on the same time points. Urinary protein concentration was determined by pyrogallol red-molybdate complex method using a Micro TP-test WAKO (Wako Pure Chemical Industries, Ltd., Osaka, Japan) [8]. Serum and urinary creatinine concentration was determined by Jaffe method using a Creatinine-test WAKO (Wako Pure Chemical Industries, Ltd.).

RNA isolation from glomeruli

Both vehicle-treated rats and ATRA-treated rats were sacrificed on day 28. Both kidneys were removed and partially preserved for histologic analysis. Glomeruli were isolated by differential sieving as described previously [9]. Glomerular total RNA was extracted from isolated glomeruli by the acid guanidinium thiocyanate-phenol-chloroform method [10].

Table 1. The design of primers

	Sense primer	Antisense primer	Annealing temperature	Cycle
GAPDH	5'-AATGCATCCTGCACCACCAA-3'	5'-GTAGCCATATTCATTGTCATA-3'	55	28
TNF-α	5'-TACTGAACTTCGGGGTGATTGGTCC-3'	5'-CAGCCTTGTCCCTTGAAGAGAACC-3'	64	28
IL-1β	5'-TGATGTTCCCATTAGACAGC-3'	5'-GAGGTGCTGATGTACCAGTT-3'	55	28
TGF-β1	5'-CTTCAGCTCCACAGAGAAGAACTGC-3'	5'-CACGATCATGTTGGACAACTGCTCC-3'	64	28
MCP-1	5'-ATGCAGGTCTCTGTCACG-3'	5'-CTAGTTCTCTGTCATACT-3'	55	32
Collagen I	5'-AACGGCAAGGTGTTGTGCGATG-3'	5'-AGCTGGGAGCAAAGTTTCCTC-3'	58	32
α-SMA	5'-CTCTTCCAGCCATCTTTCATT-3'	5'-GCATTTGCGGTGGACAATGGA-3'	58	28
ICAM-1	5'-CTGGAGAGCACAAACAGCAGAG-3'	5'-AAGGCCGCAGAGCAAAAGAAGC-3'	58	26
PDGF	5'-CTCCTTTGATGATCTTCAGCG-3'	5'-GGGTGTGCTTAAACTTTCGG-3'	58	25
c-fos	5'-AGCCGACTCCTTCTCCAGCAT-3'	5'-AGGTGCGTGGGCTGCCAAAAT-3'	58	28
c-jun	5'-GCCTGATCATCCAGTCCAGCA-3'	5'-GCTCCTGAGACTCCATGTCGA-3'	58	28
C/EBPδ	5'-GCAGACAGTGGTGAGCTTGG-3'	5'-AAGCATGCGCAGTCTTCC-3'	55	32

Abbreviations are: GADPH, glyceraldehyde-3-phosphate dehydrogenase; TNF-α, tumor necrosis factor-α; IL-1β, interleukin-1β; TGF-β1, transforming growth factor-1β; MCP-1, monocyte chemoattractant protein-1; α-SMA, α-smooth muscle actin; ICAM-1, intercellular adhesion molecule-1; PDGF, platelet-derived growth factor; C-EBSδ, CCAAT enhancer-binding protein δ.

Semiquantitative reverse transcription-polymerase chain reaction

Reverse transcription (RT) was performed as follows: 4 μL first-strand RT buffer was added to 0.4 μg of total RNA from isolated glomeruli [final concentration of 50 mmol/L Tris (hydroxymethyl) aminomethane hydrochloride, pH 8.3, 75 mmol/L KCl, and 3 mmol/L $MgCl_2$], 2.5 μL H_2O, 0.5 μL RNase inhibitor (55 U), 1 μL of 10 mmol/L deoxynucleotide mixture, 1 μL random primer [0.02 A260 absorbance units of hexadeoxyribonucleotide mixture (p(dN)6) per reaction], 2 μL of 0.1 mol/L dithiothreitol, and 1 μL Moloney murine leukemia virus reverse transcriptase (MMLV transcriptase) (Gibco BRL, Gaithersburg, MD, USA). Reaction tubes were incubated at 30°C for 10 minutes and 42°C for 40 minutes. At the end of the incubation, the reaction was stopped by heating at 95°C for 5 minutes to inactivate MMLV.

Polymerase chain reaction (PCR) was performed as follows: 0.4 μL of 10 μmol/L forward and reverse primer was added to 1 μL of the RT reaction mixture, 2 μL of 10 × buffer (final concentration of 10 mmol/L Tris HCl, pH 8.3, 50 mmol/L KCl, 1.5 mmol/L $MgCl_2$, and 0.001% gelatin), 14.5 μL H_2O, 1.6 μL of 2.5 mmol/L deoxynucleoside triphosphate (dNTP) mix, and 0.1 μL of Taq polymerase.

The primers that we used are listed in Table 1. The primers for glyceraldehyde-3-phosphate dehydrogenase (GAPDH), interleukin-1β (IL-1β), tumor necrosis factor-α (TNF-α), TGF-β1, and monocyte chemoattractant protein-1 (MCP-1) were used as described previously [11]. The primers for type I collagen (collagen I) were used as previously reported [12]. The primers for intercellular adhesion molecule-1 (ICAM-1) were used as previously reported [13]. The primers for rat c-fos, c-jun and α-smooth muscle actin (α-SMA) were constructed based on rat c-fos, c-jun and α-SMA nucleotide sequences. The primers for platelet-derived growth factor (PDGF) and CCAAT enhancer-binding protein δ (C/EBPδ) were designed based on mouse PDGF and C/EBPδ nucleotide sequences.

All PCR was performed using a thermal cycler, PCR System 9700 (Perkin Elmer, Wellesley, MA, USA), using the following parameters. After initial denaturation for 5 minutes at 95°C, 25 to 32 cycles of sequential steps denaturation was performed at 95°C for 1 minute, annealing at 55 to 64°C for 1 minute, extension at 72°C for 2 minutes, followed by a final incubation at 72°C for 7 minutes. The primers and PCR conditions for each primer set are summarized in Table 1.

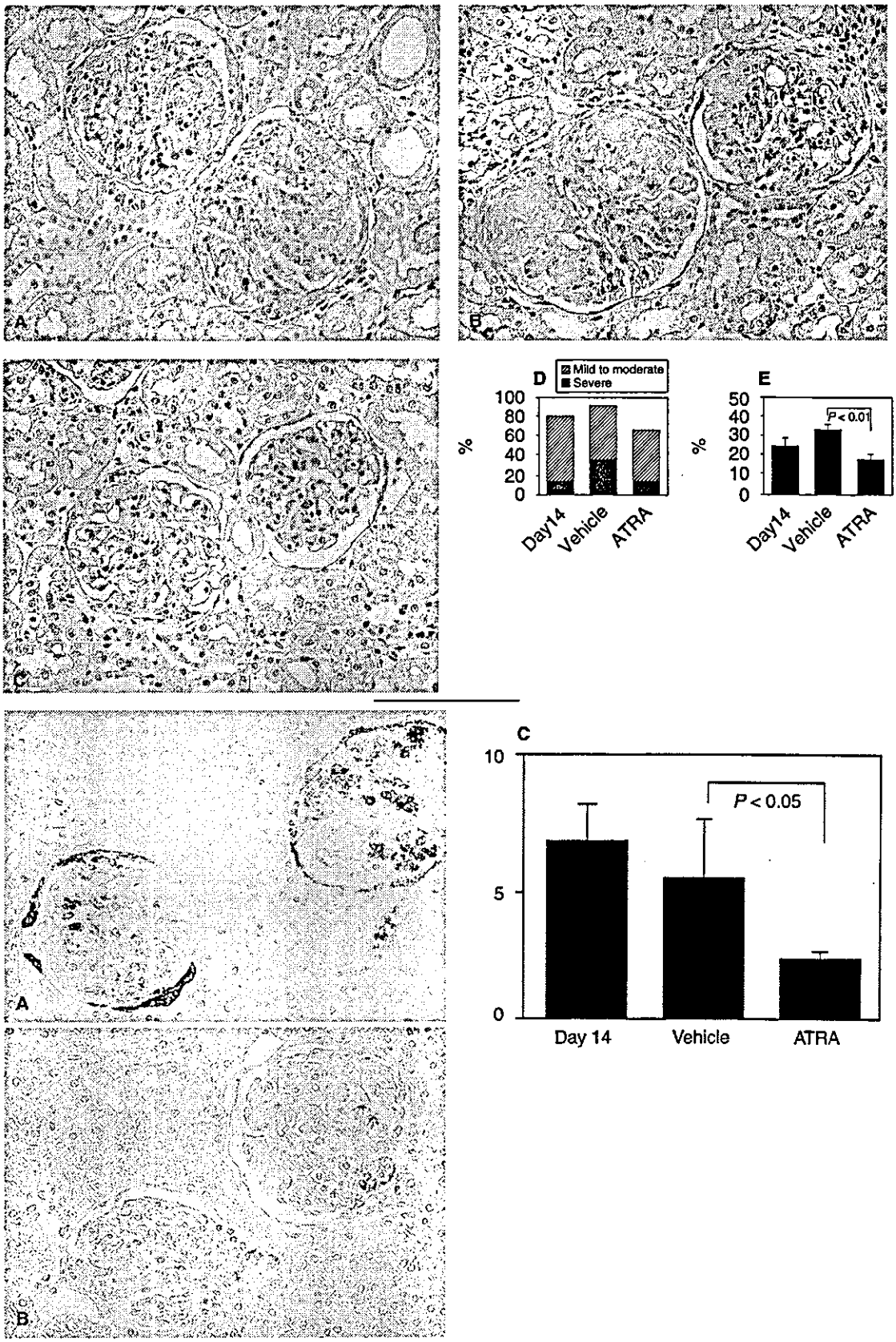
The PCR products were separated by electrophoresis on 2.0% agarose gels and visualized by ethidium bromide staining. Each experiment included the amplification of GAPDH, and the intensities of cDNA bands were quantified with the computing densitometry Image Quant (Molecular Dynamics, Sunnyvale, CA, USA), and were normalized to those of the GAPDH band as reported previously [12].

Histologic examination

The kidneys were perfused with cold autoclaved phosphate-buffered saline (PBS) and were removed. Tissues for microscopic examination were fixed with 4% paraformaldehyde overnight and then dehydrated by graded ethanol, then paraffin embedded. Thin section was examined with periodic acid-Schiff (PAS) staining as described previously [14]. Fifty glomeruli per section were randomly selected and were assessed. Glomerular appearance was graded as normal, mild to moderate, or severe injury (>50% of glomerulus affected by necrosis/crescent formation), and results were expressed as percentage of glomeruli examined. At the same time, glomerular area and necrosis/crescent formation area were quantitatively measured under high-power magnification (×400) by using computer-aided manipulator program (Macscope; Mitani Corporation, Fukui, Japan), and the percentage of the glomerular necrosis/crescent formation area was calculated.

Immunohistochemistry

PBS-perfused slices (4 μm) of renal tissue obtained from comparable renal areas in all rats were fixed in methacarn solution (methanol 60%, chloroform 30%, and acetic acid 10%) and processed using the direct or indirect immunoperoxidase technique. The endogenous peroxidase activity in tissue sections was blocked by incubating in PBS with 3% hydrogen peroxide for 30 minutes. Tissue sections were preincubated with goat or horse serums diluted 1:20 with PBS for 30 minutes to block the nonspecific staining and were then incubated with the primary antibodies for 60 minutes at room temperature. Glomerular cell proliferation was assessed by staining with 19A2 (Coulter Corp., Hialeah, FL, USA), a mouse monoclonal immunoglobulin M (IgM) antibody to the proliferative cell nuclear antigen (PCNA). To assess the invasion of macrophages, mouse IgG antirat monocyte and macrophage (ED-1) antibody was used (Serotec, Inc., Raleigh, NC, USA). To stain α-SMA, mouse IgG anti-SMA monoclonal antibodies were used (Immunotech S.A., Cedex, Marseilles, France). To stain C/EBPδ, rabbit IgG anti-C/EBPδ monoclonal antibodies (M-17) was used (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). Sections were then processed using an avidin-biotinylated peroxidase complex method (Vectastain ABC kit; Vector Laboratories, Inc., Burlingame, CA, USA) with diaminobenzidine as the chromogen. The sections were counterstained with methyl green. Sections labeled with PCNA and ED-1 monoclonal antibodies were scored by the number of PCNA-positive cells and ED-1–positive cells within the glomerulus. Count-


A
B
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Mild to moderate
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Day14
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Day 14
Vehicle
ATRA

Fig. 1. Effect of all-trans retinoic acid (ATRA) administration on anti-glomerular basement membrane (anti-GBM) glomerulonephritis. Periodic acid-Schiff (PAS) staining of kidney on day 14 in anti-GBM glomerulonephritis rats (*A*), on day 28 in vehicle-treated rats (*B*), and ATRA-treated rats (*C*). The severity of glomerular injury was graded as normal, mild to moderate, or severe (>50% of glomerulus affected by necrosis/crescent formation) (*D*). Vehicle-treated rats showed 89% injured glomeruli, with severe segmental necrosis/crescent formation affecting 34% of glomeruli. ATRA-treated rats showed 67% injured glomeruli, with severe change in 14%. Glomerular necrosis/crescent formation area was quantitatively analyzed by computer-aided manipulator program (*E*). The area was 24.9 ± 3.9% on day 14. The area on day 28 in vehicle rats was 33.1 ± 5.7% and 17.9 ± 4.4% in ATRA-treated rats.

ing of the marker positive cells was performed under high-power (× 400) microscopy. The number of PCNA-positive cells and ED-1–positive cells per glomerulus was determined by the observation of randomly selected 50 glomeruli for each animal. All scoring was performed on blinded slides by one of the authors. Glomerular α-SMA–positive and C/EBPδ area was quantitatively analyzed by using computer-aided manipulator program, Macscope (Mitani Corporation), as described previously [15, 16].

Statistical analysis

The results were given as means ± standard deviation. The differences between vehicle-treated and ATRA-treated groups were tested using the Student *t* test. Statistically significant differences between groups were defined as P values less than 0.05.

RESULTS

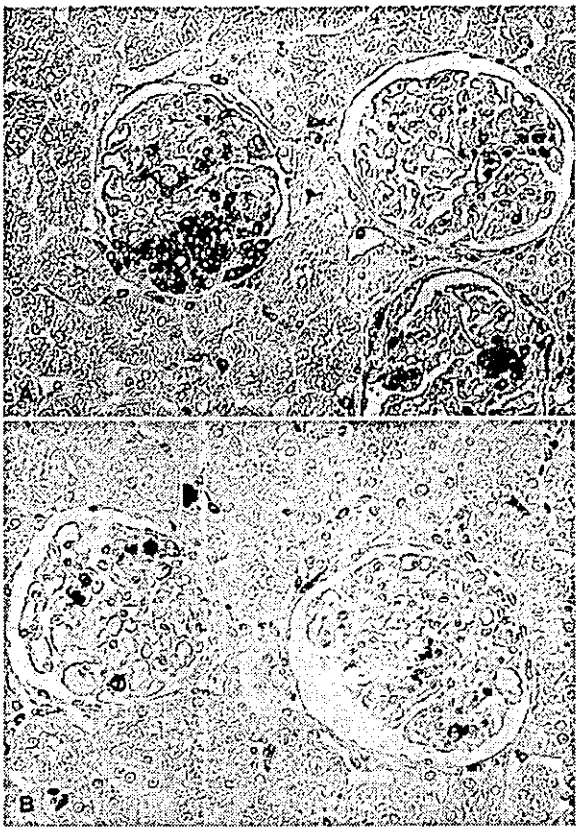
ATRA reduced renal injury

We examined whether ATRA administration could ameliorate the renal injury of anti-GBM glomerulonephritis rats. On day 14, light microscopy of kidney tissue of anti-GBM glomerulonephritis rats showed diffuse necrotizing glomerulonephritis affecting 81% of glomeruli, with severe segmental necrosis/crescent formation (>50% of the glomerulus) affecting 14% of glomeruli. On day 28, vehicle-treated rats showed diffuse necrotizing glomerulonephritis affecting 89% of glomeruli, with severe necrosis/crescent formation affecting 34% of glomeruli. ATRA-treated rats showed diffuse necrotizing glomerulonephritis affecting 67% of glomeruli, with severe change in 14% (Fig. 1D). Computer-aided quantitative analysis revealed that glomerular necrosis/crescent formation area was also reduced from 33.1 ± 5.7% to 17.9 ± 4.4% ($P < 0.01$) (Fig. 1E). We examined whether ATRA could decrease the glomerular cell proliferation assessed by the PCNA expression. The number of PCNA-positive cells per glomerulus in ATRA-treated rats was significantly reduced (2.2 ± 0.43) compared to that in vehicle-treated glomerulonephritis rats (5.2 ± 2.2) (Fig. 2). We examined macrophage infiltration by the number of ED-1–positive cells per glomerulus. The number of ED-1–positive cells was significantly reduced in ATRA-treated rats compared with vehicle-treated rats (5.2 ± 0.96 vs. 9.3 ± 2.4, respectively) (Fig. 3). To examine the degree of phenotypic change of glomerular cells to myofibroblasts, we measured the α-SMA–positive area of glomeruli. The α-SMA–positive area was significantly reduced from 8.3 ± 1.3% to 3.7 ± 0.3% by ATRA administration (Fig. 4). To examine protein levels of C/EBPδ in glomerulus, we performed the immunostaining of C/EBPδ and measured the C/EBPδ-positive area of glomeruli. The C/EBPδ-positive area was significantly reduced from 21.9 ± 4.2% to 14.2 ± 3.5% by ATRA administration (Fig. 5) on day 28. Of note, there was no immunostaining of C/EBPδ in normal rat glomeruli, suggesting its involvement in the pathologic process in glomeruli (Fig. 5).

Effects of ATRA on glomerular gene expression

Histologic examination demonstrated that ATRA administration ameliorated glomerular injury in anti-GBM glomerulonephritis. To explore the underlying mechanisms of this therapeutic efficacy, we examined the effects of ATRA administration on glomerular gene expression of wide variety of genes, including inflammation-related, cell proliferation–related, and fibrosis-related genes by semiquantitative PCR. TNF-α and IL-1β mRNAs were significantly reduced in ATRA-treated rats (Fig. 6). TGF-β1, type I collagen, and α-SMA mRNA was reduced in ATRA-treated rats compared with vehicle-treated rats (Fig. 7). PDGF, MCP-1, and ICAM-1 mRNA was also significantly suppressed in ATRA-treated rats (Fig. 8). Additionally, c-fos and c-jun mRNAs, which are components of activated protein 1 (AP-1), were significantly reduced in ATRA-treated rats (Fig. 9). C/EBPδ mRNA was significantly reduced in ATRA-treated rats (Fig. 10),

Fig. 2. Effect of all-trans retinoic acid (ATRA) administration on glomerular cell proliferation. Representative immunohistochemical photomicrographs of glomeruli stained for proliferating cell nuclear antigen (PCNA) on day 28 in vehicle-treated rats (*A*), and ATRA-treated rats (*B*). Cell proliferation was quantified with the number of PCNA-positive cells per glomerulus (*C*). The number was 6.5 ± 1.3 in anti glomerular basement membrane (anti-GBM) glomerulonephritis on day 14. The number in vehicle-treated rats was 5.2 ± 2.2 and 2.2 ± 0.43 in ATRA-treated rats ($P < 0.05$).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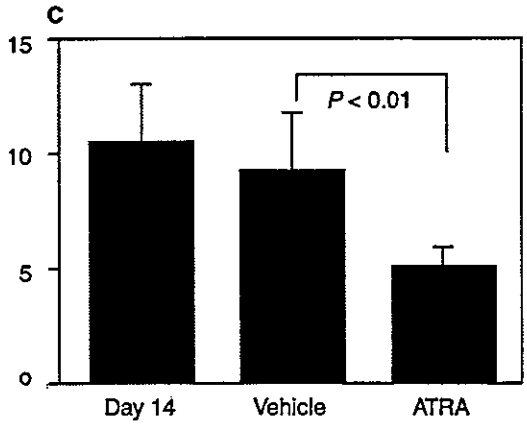
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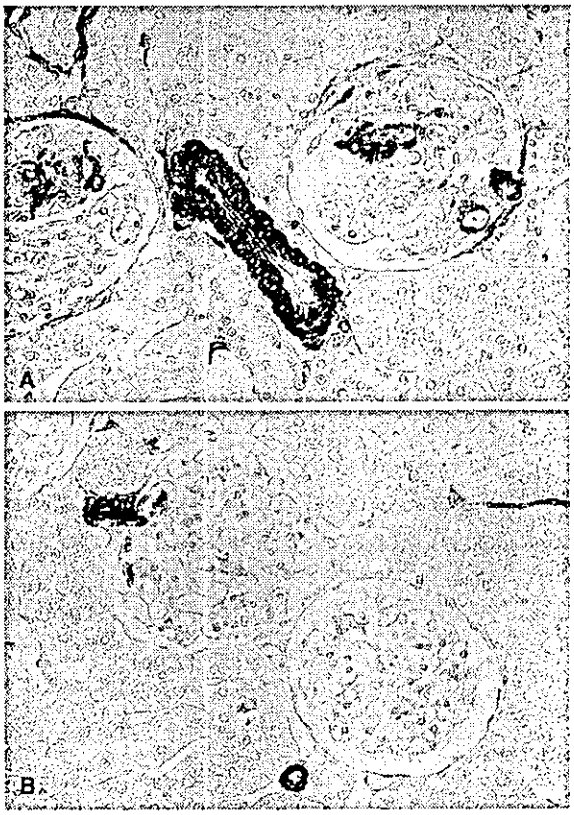
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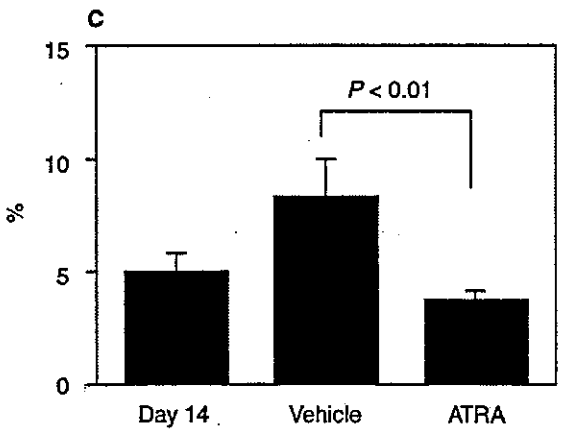
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Fig. 3. Effect of all-trans retinoic acid (ATRA) administration on macrophage infiltration. Representative immunohistochemical photomicrographs of glomeruli stained for ED-1 on day 28 in vehicle-treated rats (*A*), and ATRA-treated rats (*B*). Macrophage infiltration was quantified with the number of ED-1–positive cells per glomerulus (*C*). The number was 10.4 ± 2.0 in anti-glomerular basement membrane (anti-GBM) glomerulonephritis on day 14. The number in vehicle-treated rats was 9.3 ± 2.4 and 5.2 ± 0.96 in ATRA-treated rats ($P < 0.01$).

which coincides with the changes in glomerular immunostaining of C/EBPδ as shown in Figure 5.

Body weight

There was no significant difference in body weight between the vehicle-treated and ATRA-treated rats (day 1, 300 ± 9.6 g vs. 301 ± 9.0 g; day 14, 339 ± 11.8 g vs. 334 ± 7.4 g; day 27, 350 ± 14.6 g vs. 342 ± 11.0 g).

Urinary protein excretion

The rate of urinary protein excretion was markedly increased after injection of anti-rat GBM antibody both in vehicle- and ATRA treated glomerulonephritis rats before the commencement of ATRA treatment. In ATRA-treated group, the rate of urinary protein excretion was significantly reduced. On day 21, 7 days after the commencement of ATRA therapy, the rate of proteinuria was reduced to 46% of that of vehicle-treated rats in ATRA-treated rats and the suppression was continued until day 27 (Fig. 11). Creatinine clearance or serum creatinine levels were not different between vehicle- and ATRA-treated groups (data not shown).

Blood pressure

There was no significant difference in blood pressure between the vehicle-treated and ATRA-treated groups just before the commencement of ATRA administration. On day 21, 7 days after ATRA treatment was started, blood pressure was not different, but on day 27, 14 days after the beginning of ATRA administration, blood pressure was significantly lower in ATRA-treated rats compared to vehicle-treated rats (Fig. 12).

DISCUSSION

In the present study, intravenous injection of antirat GBM antibody induced necrotizing glomerulonephritis affecting 81% of glomeruli and severe segmental necrosis/crescent formation affecting 14% of glomeruli on day 14, and severe segmental necrosis/crescent formation increased to 34% of glomeruli on day 28 in vehicle-treated rats. In this anti-GBM glomerulonephritis rat model, acute glomerular injuries show a peak around day 15 and then sclerotic/fibrotic glomerular lesions develop thereafter [17]. The percentages of affected glomeruli in the present study are similar to those in the previous report [18]. Anti-GBM glomerulonephritis rats showed about 100 mg/day of proteinuria on day 14, which is comparable to the level of proteinuria induced by the same anti-GBM antibody [19]. In our experiment, ATRA administration significantly reduced renal injury and urinary protein excretion. In immunohistochemical study, ATRA reduced the number of PCNA or ED-1–positive cells in glomeruli. We also found that ATRA suppressed mRNA expression of proliferation and inflammatory-related genes, which may partly contribute to ATRA's antiproliferative and anti-inflammatory effects on glomerular injury.

The substantial local macrophage proliferation within Bowman's space in crescentic lesion has already been documented in anti-GBM glomerulonephritis rats by double staining of ED-1 and PCNA [20]. We stained PCNA and ED-1 on consecutive sections and found considerable overlapping of ED-1–positive cells in PCNA-positive cells, although we did not perform double staining. We speculate that some population of PCNA-positive cells in glomeruli was ED-1–positive macrophages.

It is now well established that glomerular macrophage infiltration is closely related to the progression of renal injury [21]. We found that ATRA administration significantly suppressed the glomerular macrophage infiltration. Glomerular expression of ICAM-1 and MCP-1 was also significantly suppressed in ATRA-treated rats. Up-regulation of glomerular ICAM-1 and MCP-1 expression was previously demonstrated in anti-GBM glomerulonephritis rats [7, 22]. Administration of anti-ICAM-1 antibody [22] or anti-MCP-1 antibody [7] significantly suppressed macrophage infiltration and urinary protein excretion in this model. These results suggest that enhanced expression of ICAM-1 and MCP-1 play a crucial role in renal injury

Fig. 4. Effect of all-trans retinoic acid (ATRA) administration on glomerular α-smooth muscle action (α-SMA) expression. Representative immunohistochemical photomicrographs of glomeruli stained for α-SMA on day 28 in vehicle-treated rats (*A*), and ATRA-treated rats (*B*). Five animals were analyzed for each group and 50 randomly selected high-power fields were quantitated with a computer-aided image manipulator and averaged to obtain the value for each animal(*C*). α-SMA–positive area in anti-GBM glomerulonephritis on day 14 was 4.7 ± 1.1%. The area in vehicle-treated rats was 8.3 ± 1.3% and 3.7 ± 0.3% in ATRA-treated rats ($P < 0.01$).

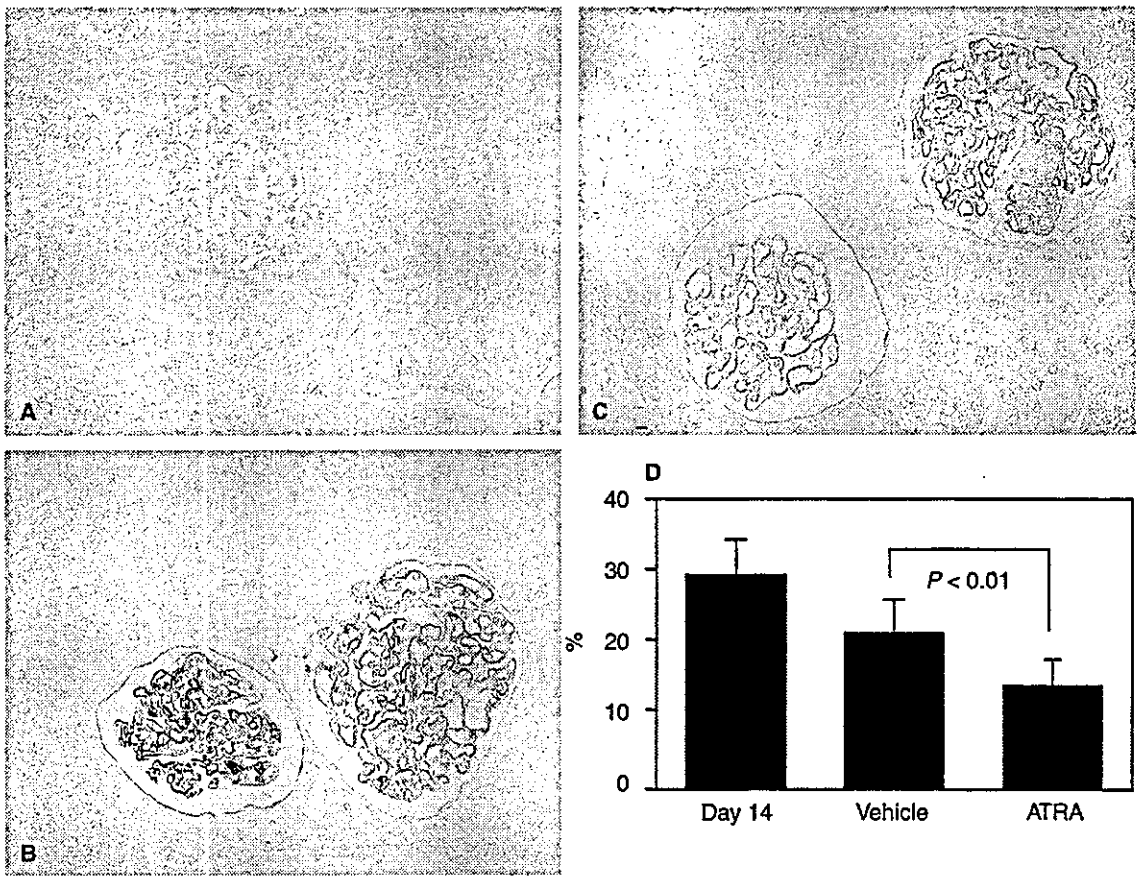

Fig. 5. Effect of all-trans retinoic acid (ATRA) administration on glomerular CCAAT enhancer-binding protein δ (C/EBPδ) expression. Representative immunohistochemical photomicrographs of glomeruli stained for C/EBPδ on day 28 in normal rats (*A*), vehicle-treated rats (*B*), and ATRA-treated rats (*C*). Five animals were analyzed for each group and 50 randomly selected high-power fields were quantitated with a computer-aided image manipulator and averaged to obtain the value for each animal(*D*). C/EBPδ-positive area in anti-glomerular basement membrane (anti-GBM) glomerulonephritis on day 14 was 30.4 ± 3.9%. The area in vehicle-treated rats was 21.9 ± 4.2% and 14.2 ± 3.5% in ATRA-treated rats ($P < 0.01$).

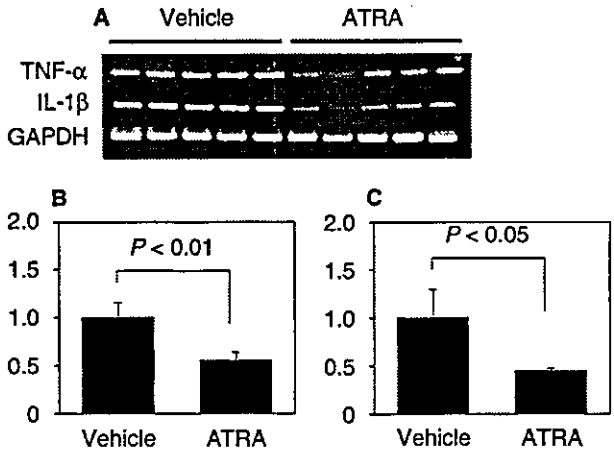

Fig. 6. Effects of all-trans retinoic acid (ATRA) administration on proinflammatory glomerular gene expression in anti-glomerular basement membrane (anti-GBM) glomerulonephritis rats. (*A*) Ethidium bromide–stained gels. Vehicle-treated rats and ATRA-treated rats. The level of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was not significantly different. Tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β) mRNA expression was significantly reduced by ATRA administration. The intensities of cDNA bands were quantified with the computing densitometry and normalized by GAPDH. (*B*) TNF-α mRNA was decreased to 55% and (*C*) IL-1β mRNA was decreased to 44% in ATRA-treated rats compared with vehicle-treated rats.

of anti-GBM glomerulonephritis. The reduction of renal macrophage infiltration and proteinuria in our experiment may partly be due to the attenuation of ICAM-1 or MCP-1 mRNA expression. Proinflammatory cytokines such as TNF-α, IL-1β, and nitric oxide are known to activate macrophages. ATRA decreased the level of nitric oxide and TNF-α in macrophage cell line [23]. In our study, glomerular TNF-α and IL-1β mRNA expression was reduced in ATRA-treated rats. This result suggests that ATRA reduces such proinflammatory cytokines and attenuates macrophage activity. The attenuation of macrophage infiltration and activation may be one of the un-

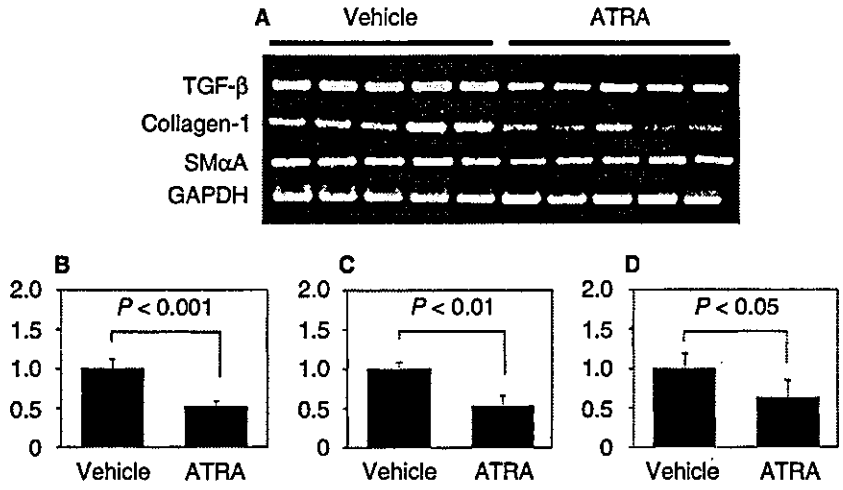

Fig. 7. Effects of all-trans retinoic acid (ATRA) administration on fibrosis related glomerular gene expression in anti-glomerular basement membrane (anti-GBM) glomerulonephritis rats. (*A*) Ethidium bromide–stained gels. ATRA administration significantly reduced transforming growth factor-β1 (TGF-β1) (−49%) (*B*), type I collagen (−47%) (*C*), and α-smooth muscle actin (α-SMA) (−38%) (*D*) mRNA expression. GAPDH is glyceraldehyde-3-phosphate dehydrogenase.

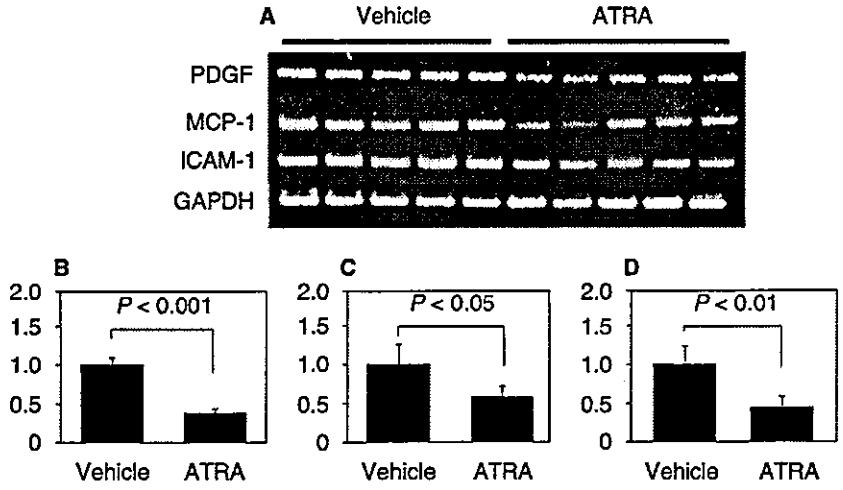

Fig. 8. Effect of all-trans retinoic acid (ATRA) administration on proliferation-related and macrophage infiltration–related glomerular gene expression in anti-glomerular basement membrane (anti-GBM) glomerulonephritis rats. (*A*) Ethidium bromide–stained gels. ATRA administration significantly reduced platelet-derived growth factor (PDGF) (−63%) (*B*), monocyte chemoattractant protein-1 (MCP-1) (−42%) (*C*), and intercellular adhesion molecule-1 (ICAM-1) (−56%) (*D*) mRNA expression. GADPH is glyceraldehyde-3-phosphate dehydrogenase.

derlying mechanisms of the therapeutic effect of ATRA on anti-GBM antibody glomerulonephritis.

ATRA was shown to down-regulate H_2O_2-induced [24] and fetal bovine serum (FBS)-stimulated [25] expression of c-fos and c-jun in cultured mesangial cells. In our study, glomerular c-fos and c-jun mRNAs were reduced in ATRA-treated rats in vivo. Previous study demonstrated that down-regulation of c-fos and c-jun mRNA levels suggests a mechanism for anti-AP-1 activity by ATRA [25]. Thus, the blunted gene expression of c-fos and c-jun mRNA in ATRA-treated rats is supposed to result in the down-regulation of AP-1 activity in the glomeruli. MCP-1 expression is reported to be partly regulated by AP-1 activation [26, 27]. MCP-1 production stimulated by IL-1β was suppressed via inhibition of nuclear factor-kappa B (NF-κB) and AP-1 activation [28]. On the other hand, overexpression of AP-1 protein induced ICAM-1 gene expression [29]. Taken together, down-regulation of ICAM-1 and MCP-1 in ATRA-treated rats may at least partly be explained by the suppressive effects of ATRA on c-fos and c-jun mRNAs. There have been some reports demonstrating that AP-1 regulates TGF-β gene expression in variety of cells, including mesangial cells and tubular cells [30–33]. TGF-β1 mRNA expression was decreased in ATRA-treated rats in our study, which might be related to the suppression of AP-1 component genes. Morath et al [34] also suggested that the beneficial effects of ATRA on anti-Thy1.1 nephritis may be due to a suppression of renal TGF-β1. TGF-β is supposed to play a central role in the progression of tissue fibrosis via stimulation of extracellular matrix components (ECM) synthesis and cellular phenotypic change to myofibroblasts. α-SMA is one of the typical molecular markers of myofibroblasts and is known to be induced by TGF-β [35]. The reduction of TGF-β1 mRNA expression may result in the reduction of glomerular

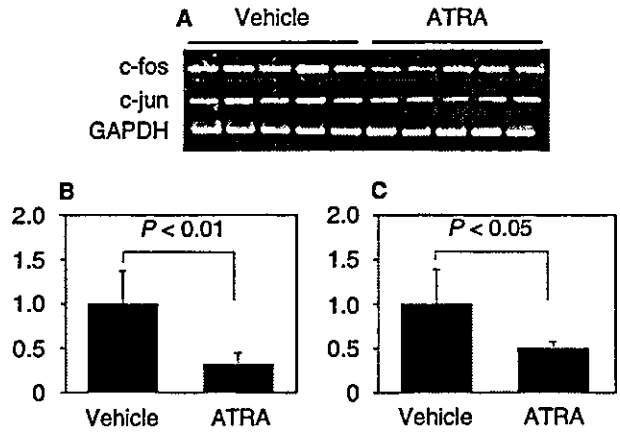

Fig. 9. Effect of all-trans retinoic acid (ATRA) administration on c-fos and c-jun mRNA in anti-glomerular basement membrane (anti-GBM) glomerulonephritis rats. (*A*) Ethidium bromide-stained gels. The level of c-fos (*B*) and c-jun (*C*) mRNA expression was significantly reduced by ATRA administration (−69% and −49%, respectively). GAPDH is glyceraldehyde-3-phosphate dehydrogenase.

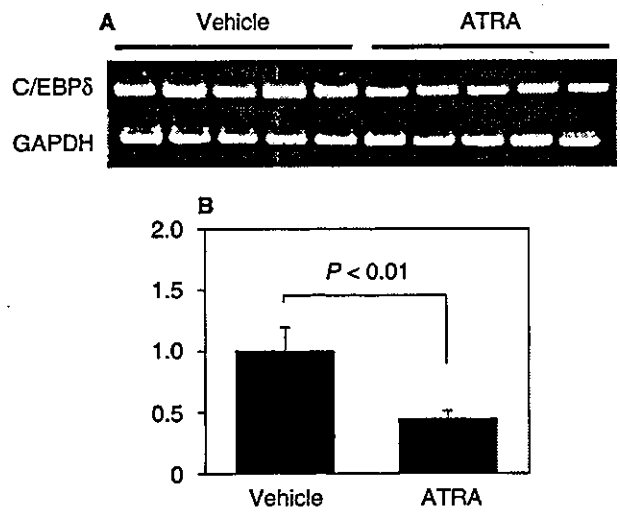

Fig. 10. Effect of all-trans retinoic acid (ATRA) administration on CCAAT enhancer-binding protein δ (C/EBPδ) mRNA in anti-glomerular basement membrane (anti-GBM) glomerulonephritis rats. (*A*) Ethidium bromide-stained gels. The level of C/EBPδ mRNA (*B*) expression was significantly reduced by ATRA administration (−56%). GAPDH is glyceraldehyde-3-phosphate dehydrogenase.

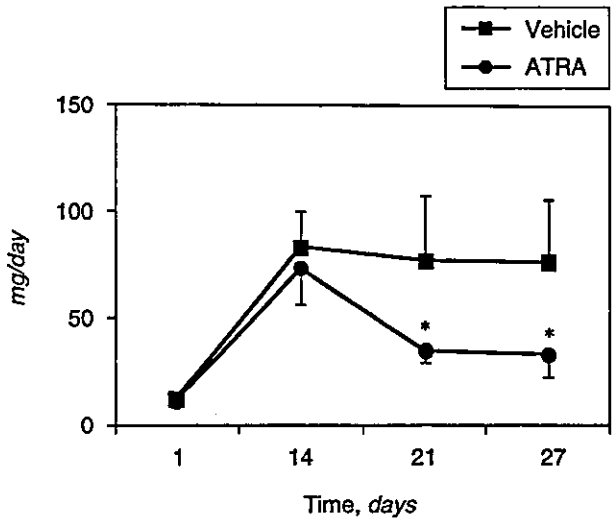

Fig. 11. Effect of all-trans retinoic acid (ATRA) administration on urinary protein excretion in anti-glomerular basement membrane (anti-GBM) glomerulonephritis rats. Urinary protein excretion was decreased to 46% of untreated levels 7 days after ATRA administration. $*P < 0.05$ vehicle vs. ATRA treated rats.

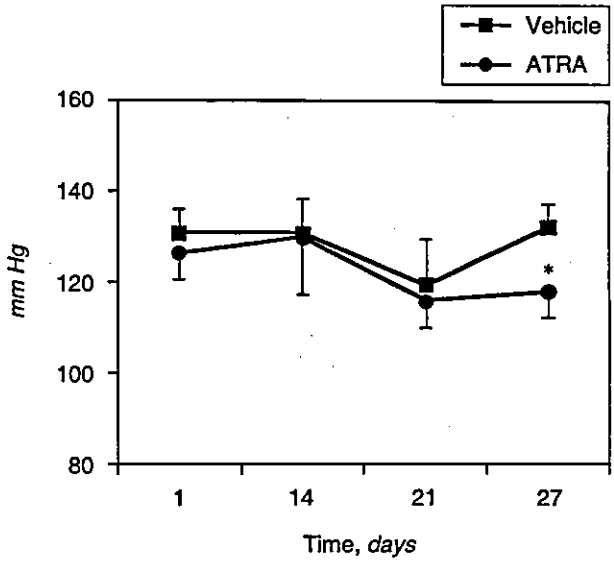

Fig. 12. Effect of all-trans retinoic acid (ATRA) administration on blood pressure in anti-glomerular basement membrane (anti-GBM) glomerulonephritis rats. There was no difference between two groups 7 days after ATRA administration, but blood pressure was significantly lower in ATRA-treated group 14 days after the beginning of ATRA administration. $*P < 0.05$ vehicle vs. ATRA-treated rats.

myofibroblast expansion revealed by α-SMA expression and reduction of type I collagen mRNA expression.

Myofibroblasts are recognized as the key to understand the reconstruction and excessive matrix formation in injured tissue. We have been investigating the phenotypic change from renal cells to myofibroblasts in the process of progressive renal diseases in animal models and human glomerulonephritis [15, 35, 36]. α-SMA is a typical molecular marker of myofibroblasts, and we hypothesized that the molecular mechanisms underlying the induction of α-SMA in myofibroblasts are closely related to the molecular pathophysiology of progressive renal disease leading to renal fibrosis. We speculated any factor that promotes α-SMA expression might play an important role for transdifferentiation to myofibroblast and progression of tissue injury. Then, we identified C/EBPδ as a major transcription factor that induces phenotypic change in renal disease. We also found that C/EBPδ-deficient mice show a significant reduction in

α-SMA expression along with substantial amelioration in renal damage in Habu venom glomerulonephritis or unilateral ureteral obstruction. In the present study, we demonstrated a significant reduction of both C/EBPδ and α-SMA mRNA or protein expression in glomeruli from ATRA-treated anti-GBM glomerulonephritis rats. This observation and our results of other study using C/EBPδ-deficient mice further support the role of C/EBPδ in myofibroblast formation [(manuscript in preparation) abstract; Takeji M, et al, *J Am Soc Nephrol* 13:295A, 2002]. We speculate that the suppression of C/EBPδ mRNA is at least part of the molecular mechanism of therapeutic effect of ATRA in anti-GBM glomerulonephritis.

It was already demonstrated that ATRA lowered the high blood pressure in anti-Thy1.1 glomerulonephritis rats [6]. In our experiment, blood pressure of ATRA-treated rats was significantly lower compared with vehicle-treated rats 14 days after ATRA administration, although no significant difference was observed after 7 days of treatment. Interestingly, it was recently shown that ATRA might have an inhibitory effect on renin-angiotensin system (RAS) in the kidney [37]. Thus, the lower blood pressure at 14 days after ATRA administration may be related to the suppression of RAS component genes, and alternatively, be secondary to the amelioration of renal injury by ATRA treatment.

CONCLUSION

We have demonstrated therapeutic effects of ATRA on anti-GBM antibody glomerulonephritis rats revealed by histologic changes, urinary protein excretion, and blood pressure. A wide variety of disease-related gene expression in glomeruli was blunted in ATRA-treated rats, which may explain the molecular mechanisms of therapeutic effects of ATRA in glomerulonephritis rats. Clinical feasibility of ATRA treatment in progressive renal diseases is expected to be established with further investigation.

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Short Report

Peroxisome proliferator-activated receptor γ C161T polymorphisms and survival of Japanese patients with immunoglobulin A nephropathy

Song J, Sakatsume M, Narita I, Goto S, Omori K, Takada T, Saito N, Ueno M, Gejyo F. Peroxisome proliferator-activated receptor γ C161T polymorphisms and survival of Japanese patients with immunoglobulin A nephropathy.
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Peroxisome proliferator-activated receptor γ (PPARγ) plays an important role in lipid metabolism, insulin sensitivity, atherogenesis, and immune regulation. A genetic polymorphism (C161T) at exon 6 of PPARγ gene (*PPARG*) was reported to be associated with the onset of coronary artery disease. However, there has been no report of an association with renal disease. Genomic DNAs were isolated from 225 Japanese patients with histologically confirmed immunoglobulin A nephropathy (IgAN). The *PPARG* C161T genotype was determined by polymerase chain reaction-restriction fragment length polymorphism. The association of the polymorphism with renal prognosis in IgAN patients was analyzed using the Kaplan–Meier method and Cox proportional hazard regression model. The *PPARG* polymorphism was not associated with the renal survival rate. However, when patients were stratified into those either with or without hypertension at the time of diagnosis, the renal survival of the CT/TT genotypes was significantly better in those without hypertension than those with the CC genotype. We report that the *PPARG* C161T polymorphism is associated with the survival of IgAN patients without hypertension. The T allele of the polymorphism might have a protective effect on the progression of IgAN.

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Immunoglobulin A nephropathy (IgAN) is a worldwide common renal disease, where one-third of the patients progress to end-stage renal disease (ESRD) (1–3). The pathogenesis of IgAN and the mechanism of individual differences of renal survival rates are still unclear (4). An accumulating body of evidence suggests that genetic factors may determine the susceptibility to the development and the progression of this disease (5–7). To date, high blood pressure, severe proteinuria, and severe histological appearance of renal biopsy have been identified as poor prognostic factors (1, 8).

The peroxisome proliferator-activated receptors (PPARs) are ligand-activated transcription factors and members of the nuclear receptor superfamily (9). There are three isoforms of PPAR namely α, β, and γ, which are differentially expressed in different types of cells (10). PPARs are involved in various metabolic processes. Among these isoforms, PPARγ is involved in the control of inflammation and atherosclerosis as well as lipid and glucose metabolism (10–12). PPARγ is predominantly expressed in adipose tissues, intestine, renal epithelium, and the immune system (10, 11, 13). Some ligands, such as pioglitazone, rosiglitazone, and troglitazone, are well known as a remedy against insulin resistance. In kidney diseases, the activation of PPARγ by these ligands suppresses the proliferation of mesangial cells and the activity of

plasminogen activator inhibitor-1 and transforming growth factor-β, resulting in amelioration of the progression of glomerulosclerosis in a non-diabetic model (14). This also inhibits the progressive accumulation of mesangial matrices in Zucker fatty rats, which exhibit insulin resistance, glucose intolerance, and hyperinsulinemia (15). Moreover, it is possible that PPARγ affects the immunological machinery involved in renal injury in IgAN as PPARγ controls the function of immune cells such as T cells, B cells, and macrophages (10, 12, 16–18). PPARγ activators have been shown to exert an anti-inflammatory activity by inhibiting the expression of proinflammatory genes such as cytokines and chemokines (19, 20).

Thus, the polymorphisms in the PPARγ gene (*PPARG*) may be good candidates for contributing to the genetic basis of the susceptibility to the progression of IgAN. This study investigated the role of *PPARG* C161T polymorphism in the survival of Japanese patients with IgAN. The polymorphism is located at exon 6, which is commonly shared by all the three isotypes of PPARγ, γ1, γ2, and γ3 (10, 21, 22). As it is not clear how each isoform is related to the pathogenesis of renal diseases, the DNA variants with the common exons of *PPARG* could be more informative than isoform-specific mutations. Therefore, we explored the *PPARG* C161T polymorphism in the present study. This polymorphism has been studied in European patients with coronary artery disease (CAD), and the T allele showed a beneficial effect on the onset of CAD (23). In this paper, we report that the T allele of the C161T polymorphism is a predictor of better survival in Japanese patients with IgAN who did not have hypertension at the time of diagnosis.

Materials and methods

Patients

The ethics committee of our institution approved the protocol for the study, and informed written consent for the genetic studies was obtained from all participants. Genomic DNA of peripheral blood cells was isolated using an automatic DNA isolation system (NA-1000, Kurabo, Osaka, Japan) from 225 Japanese patients with IgAN. Schonlein–Henoch purpura and secondary IgAN such as hepatic glomerulosclerosis were excluded from the analysis. Diagnosis of IgAN was based on the finding of renal biopsy that revealed the presence of dominant or co-dominant glomerular mesangial deposition of IgA as being assessed by an immunofluorescence examination. The mean age of the patients was 36.9 years, and all patients had been observed for more than 2 years. Clinical characteristics of the patients with IgAN including age, gender, duration of observation (in months), level of urinary protein excretion (g/day), serum creatinine (sCr, mg/dl), and 24-h creatinine clearance (ml/min) were investigated. Hypertension was defined by the use of one or more antihypertensive medications and/or the office blood pressure of ≥140 mmHg systolic or 90 mmHg diastolic. The primary endpoint or ESRD was defined as when patients underwent first hemodialysis. The administration of glucocorticoids, antihypertensive agents, and angiotensin-converting enzyme inhibitors was also recorded for each patient.

Histological scores

Histopathological findings were classified according to the classification described previously (24). A single pathologist evaluated all specimens by light microscopy in a blinded manner. Glomerular changes were scored for each glomerulus, and the average score of each patient was calculated. The scores of cell proliferation and matrix increase in the mesangium were graded into five from zero (minimal change) to four (diffuse, global, and marked). Other glomerular changes, including global sclerosis, segmental sclerosis, crescent formation and endocapillary proliferation, as well as tubulointerstitial lesions and sclerotic changes of small arteries, were graded from zero to four according to their incidence. Grades zero to four represent an incidence of lesions, 0–4%, 5–24%, 25–49%, 50–74%, and 75–100%, respectively.

Determination of the genotypes

The polymerase chain reaction (PCR) of the genomic DNA was performed to amplify a 200-bp fragment in exon 6 of the *PPARG* using the primer pair 5′-CAAGACAACCTGCTAGC-3′ and 5′-TCCTTGTAGATCTCCTGCAG-3′. The reaction mixture contained ×1 PCR buffer, 1.5 mmol/l of $MgCl_2$, 200 mmol/l of deoxynucleotide triphosphates, 1 U of Taq polymerase (Takara, Kyoto, Japan), 10 pmol of each primer, and 50–100 ng of genomic DNA. The PCR amplification reaction consisted of a cycle at 94 °C for 1 min, followed by 34 cycles of denaturation at 94 °C for 30 s, annealing at 56 °C for 30 s, and extension at 72 °C for 1 min. A final extension was performed at 72 °C for 5 min. The PCR products were digested with restriction endonuclease *Bbr*PI (Toyobo, Tokyo, Japan) at 37 °C overnight and run on 12% polyacrylamide gel followed by ethidium bromide staining. This

resulted in two fragments (120 and 80 bp) for 161C alleles and one fragment (200 bp) for 161T alleles that lacked the restriction site (Fig. 1).

Statistical analysis

STATVIEW 5.0 statistical software (Abacus Concepts, Inc., Berkeley, CA) was used for statistical analyses. The χ^2 test was used when comparing allele frequencies. Hardy–Weinberg equilibrium was also tested by the χ^2 test with 1 d.f. The Kaplan–Meier method and the Cox proportional hazard regression model analyzed the time course from renal biopsy to the end point. Covariates were selected by a stepwise backward method, and the effects of these covariates were expressed by a hazard ratio (HR). A value of p less than 0.05 was considered statistically significant.

Results

The 225 patients (age, 36.3 ± 12.8 years for males and 37.4 ± 13.3 years for females) with histologically confirmed IgAN were genotyped for the *PPARG* C161T. The frequencies of CC, CT, and TT were 68.0% (n = 153), 29.3% (n = 66), and 2.7% (n = 6), respectively, and T allele frequency was 0.173. The genotype distribution was in Hardy–Weinberg equilibrium ($\chi^2 = 6.000$, $p = 0.1991$) and was not significantly different between males and females ($\chi^2 = 1.219$, $p = 0.5435$).

The clinical characteristics of the patients with IgAN are listed in Table 1. The comparison between patients with the T allele (CT/TT genotypes) and those with the CC genotype showed no significant differences in age, urinary protein, sCr, creatinine clearance, or blood pressure at the time of renal biopsy. Among the patients with IgAN, 67 (29.9%) patients progressed to ESRD during the mean follow-up duration of 104.8 ± 66.8 months. The histological changes on renal biopsy specimen were not significantly different from each other (Table 2). The difference of lipid profiles in serum was not detected, although data from all patients were not available [total cholesterol, total 206 ± 54 mg/dl (n = 193), CC *vs* CT/TT 204 ± 51 mg/dl (n = 132) *vs* 209 ± 59 mg/dl (n = 61), p = 0.630; triglyceride, total 133 ± 116 mg/dl (n = 144); CC *vs* CT/TT 140 ± 131 mg/dl (n = 104) *vs* 112 ± 64 mg/dl (n = 40), p = 0222].

The Cox proportional hazard regression model indicated that heavy proteinuria, >1 g/day and hypertension at the time of renal biopsy were independent risk factors for ESRD (Table 3). These covariates were selected by stepwise backward analysis. None of therapeutic agents such as glucocorticoids, antihypertensives, or angiotensin-converting enzyme inhibitors were selected as a significant prognostic factor by the analysis.

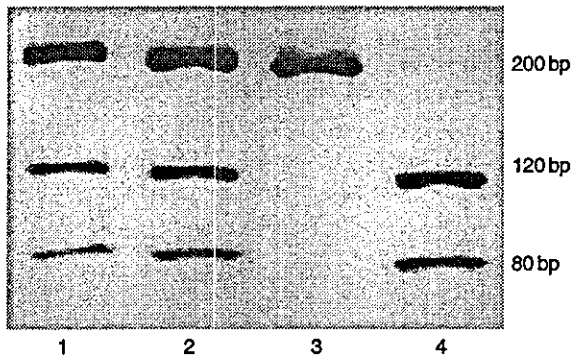

Fig. 1. The polymerase chain reaction (PCR) products of PPARγ gene (*PPARG*) digested by a *Bbr*PI-restriction enzyme. Lanes 1 and 2 are CT heterozygotes, lane 3 is a rare TT homozygote, and lane 4 shows a common CC homozygote.

The genotypes of *PPARG* C161T polymorphism did not significantly affect the renal survival (CC *vs* CT/TT, HR = 1.334, 95% confidence interval 0.768–2.316, p = 0.3059 by univariate analysis). In addition, the renal survival rate analyzed by the Kaplan–Meier method was not significantly different between the two groups (n = 225, $\chi^2 = 1.060$, p = 0.3033; Fig. 2). Next, the association of the polymorphism with the renal survival rate was examined in the stratified patients, either with or without hypertension at the time of renal biopsy (Fig. 3). In patients without hypertension (n = 143), the renal survival of patients with the CT/TT genotypes was significantly better than those with the CC genotype [Kaplan–Meier, $\chi^2 = 5.092$, p = 0.0240; Fig. 3(b)], whereas no such difference was observed in patients with hypertension [Kaplan–Meier, $\chi^2 = 0.735$, p = 0.3912; Fig. 3(a)]. We also examined the effect of the C161T polymorphism on the other stratified patients, with or without heavy proteinuria (>1 g/day) at the time of renal biopsy. The difference of the renal survival rates between the two groups was not significantly detected in either subgroups (Kaplan–Meier, $\chi^2 = 0.767$, p = 0.3812 in patients with >1 g/day of proteinuria and $\chi^2 = 0.013$, p = 0.9076 in those with less proteinuria).

Discussion

This study showed that, in the group of patients without high blood pressure at the time of diagnosis, the long-term prognosis of renal function of IgAN patients with the CT/TT genotypes of *PPARG* C161T was better than that of those with the CC genotype. The mechanism for this finding

Table 1. Clinical characteristics of patients with IgA nephropathy

	Total (n = 225)	Genotype of PPARG		p value
		CC (n = 153)	CT/TT (n = 72)	
At the time of renal biopsy				
BMI	22.6±2.8	22.6±3.0	22.4±2.6	NS
Age (years)	36.9±13.1	36.6±13.2	37.6±13.0	NS
U-protein (g/day)	1.3±1.3	1.3±1.3	1.4±1.3	NS
sCr (mg/dl)	0.94±0.36	0.91±0.32	0.97±0.40	NS
Ccr (ml/min)	90±31	91±31	88±32	NS
Blood pressure (mmHg)				
Systolic	128±18	130±19	128±17	NS
Diastolic	78±13	77±14	79±11	NS
Hypertension (%)	36.4	34.0	41.7	NS
During the observation				
Observed periods	104.8±66.8	101.1±67.3	116.3±68.8	NS
ESRD (%)	29.9	29.6	30.6	NS
Treatment				
Glucocorticoid (%)	25.7	24.6	27.9	NS
Anti-HT drugs (%)	46.2	42.5	54.2	NS
ACEI (%)	42.1	42.7	40.8	NS

ACEI, angiotensin I-converting enzyme inhibitor; anti-HT, anti-hypertensive; Ccr, creatinine clearance; ESRD, end-stage renal disease; TC, total cholesterol; TG, triglyceride; NS, not statistically significant; U-protein, urinary protein.
Hypertension: systolic >140 mmHg or diastolic >90 mmHg.
Values are mean ± SD.

Table 2. The renal histological changes and *PPARG* C161T

	Total (n=225)	Genotype of PPARG		p value
		CC (n=153)	CT/TT (n=72)	
C	1.43±0.87	1.43±0.94	1.42±0.81	NS
M	2.49±0.85	2.47±0.90	2.52±0.77	NS
GS	0.92±0.91	0.91±0.88	0.88±0.93	NS
SS	0.67±0.83	0.71±0.83	0.58±0.75	NS
Cr	0.76±0.88	0.79±0.90	0.69±0.89	NS
End	0.25±0.54	0.19±0.53	0.36±0.57	NS
I	0.90±0.81	0.88±0.79	0.90±0.76	NS
SmA	0.51±0.60	0.49±0.59	0.54±0.56	NS

C, mesangial cell proliferation; Cr, crescent; End, endocapillary proliferation; GS, global sclerosis; I, interstitium; M, mesangial matrix proliferation; NS, not statistically significant; SmA, small artery; SS, segmental sclerosis.

might be similar to that for the good prognosis of CAD in patients with the CT/TT genotypes (23), because CAD was suggested to share some common factors with IgAN for progression, such as insulin resistance, atherosclerosis, and aberrant macrophage function (12, 25). The parameters of lipid metabolism, such as total cholesterol and triglyceride, a parameter of obesity, BMI, and history of hypertension, were not influenced by the *PPARG* C161T polymorphism

Table 3. Cox proportional hazards regression model

Variable	p value	HR	95% CI
U-protein >1.0/day	0.0003	3.004	1.655–5.454
HT at the time of renal biopsy	0.0098	1.897	1.167–3.084

HR, hazard ratio; U-protein, urinary protein; HT, hypertension. Covariates were selected by stepwise backward analysis.

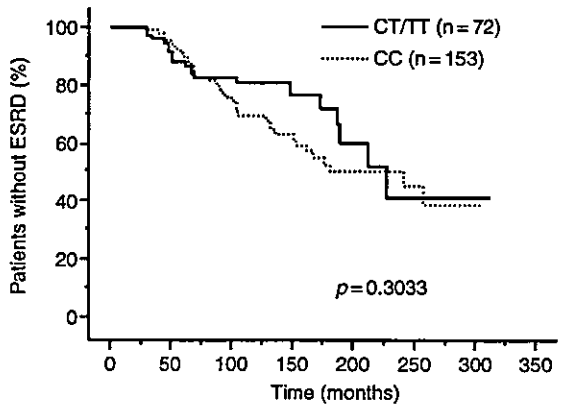

Fig. 2. Renal survival of all immunoglobulin A nephropathy (IgAN) patients. The renal survival rates of patients with CC or CT/TT genotypes were not significantly different (Kaplan–Meier, Log rank test, p = 0.3033). ESRD, end-stage renal disease.