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CELL BIOLOGY – IMMUNOLOGY – PATHOLOGY

Effects of anti-TGF-β type II receptor antibody on experimental glomerulonephritis

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Background. Renal fibrosis, characterized by the accumulation of extracellular matrix (ECM), is a common histopathological feature of progressive renal disease of diverse etiology. Interaction between transforming growth factor-β (TGF-β) and TGF-β type II receptor (TGF-βIIR) may play an important role in the ongoing fibrotic process. TGF-βIIR and TGF-β have been reported to be up-regulated in human glomerulopathies. In order to block the TGF-β system, many studies have inhibited TGF-β itself, but not its receptors. Our study explored the effects of fully human monoclonal antibody against TGF-βIIR (hTGF-βIIRAb) on experimental proliferative glomerulonephritis.

Methods. hTGF-βIIRAb was generated from Xenomice. The expression of TGF-βIIR was studied by immunohistochemistry in normal and anti-Thy-1 nephritis rats. hTGF-βIIRAb or control Ab was injected intraperitoneally at day 0 and day 4 of anti-Thy-1 nephritis, and rats were sacrificed at day 7. Effects of hTGF-βIIRAb were assessed by histological and immunopathological measurements.

Results. The specificity of hTGF-βIIRAb was confirmed by ELISA and Western blot analysis. By immunostaining, TGF-βIIR expression was up-regulated in the proliferative lesions of anti-Thy-1 nephritis at day 7. In the hTGF-βIIRAb-treated group, the extent of mesangial expansion was less than that in the control group. By immunohistology, α-smooth muscle actin, fibronectin-EDA, and type I collagen were significantly reduced in the hTGF-βIIRAb-treated group.

Conclusions. Anti-TGF-βIIR antibody ameliorated ECM accumulation in anti-Thy-1 nephritis. Our data suggest that TGF-βIIR may be one of the therapeutic targets, and that fully human monoclonal antibody against TGF-βIIR may have a new therapeutic potential for renal fibrosis.

Key words: human monoclonal antibody, fibronectin-EDA, renal fibrosis, extracellular matrix, proliferative glomerular nephritis, scarring.

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Renal fibrosis may occur in etiologically diverse renal diseases, including immunologically mediated glomerulonephritis, hemodynamic disorders, and metabolic and hereditary diseases. Excessive deposition of extracellular matrix (ECM) is the characteristic feature of organ fibrosis. Lots of mediators are involved in the development of scarring and fibrotic conditions. Transforming growth factor-β (TGF-β) is known to play an important role in this process [1]. Generally, TGF-β induces the deposition of ECM via stimulation of ECM production [2], down-regulation of ECM-degrading proteases and up-regulation of protease inhibitors [3]. By these mechanisms, TGF-β is thought to be involved in the progression of renal fibrosis. Therefore, inhibition of TGF-β is considered to be one of the crucial therapeutic strategies to prevent renal fibrosis. Inhibition of TGF-β by neutralizing antibody [4], anti-sense oligonucleotide [5], decorin [6, 7], and TGF-β receptor–IgG Fc chimera [8] suppressed the accumulation of ECM in experimental proliferative glomerulonephritis induced by anti-Thy-1 antibody. However, all of these reports showed the effects of inhibition of TGF-β itself, but not TGF-β receptors.

Transforming growth factor-β regulates cellular processes by binding to three high-affinity cell-surface receptors: types I, II, and III [9]. In the extracellular space, TGF-β binds either to the type III receptor, which presents it to the type II receptor (TGF-βIIR), or directly to TGF-βIIR on the cell membrane. Then the TGF-β-TGF-βIIR complex binds to the type I receptor, and finally, phosphorylation of the type I receptor occurs [10]. Phosphorylation activates several signaling pathways, including the Smad pathway, and regulates multiple TGF-β functions [11]. In this respect, TGF-βIIR is important for the TGF-β signaling pathway. Therefore, inhibition or modification of TGF-βIIR may be another therapeutic target to prevent renal fibrosis.

Recently, a new strategy for producing fully human

monoclonal antibodies in mice was established with therapeutic potential [12, 13]. Using this technique, we developed a fully human monoclonal antibody against TGF-βIIR. Our study explored the effects of fully human anti-human TGF-βIIR antibody on the anti-Thy-1 nephritis. To our knowledge this is the first report of a direct inhibition of TGF-β receptor function in experimental glomerulonephritis.

METHODS

Cells

Mouse myeloma cells, P3/X63-Ag8.653 (CRL-1580; American Type Culture Collection, Manassas, VA, USA), were cultured in Dulbecco's modified Eagle's medium (DMEM; Nikken Biomedical, Kyoto, Japan) containing 10% fetal bovine serum (FBS; Moregate, Australia). Human embryonal kidney cells, HEK293 (CRL-1573, ATCC), were cultured in DMEM medium containing 10% FBS. Mouse/mouse hybridomas were cultured in ASF104 medium (Ajinomoto, Tokyo, Japan) supplemented with 10% FBS. All cells were cultured at 37°C with 5% CO_2.

Animals

Xenomice, which generate high-affinity fully human antibodies to multiple antigens, were maintained in the Pharmaceutical Frontier Research Laboratories (Central Pharmaceutical Research Institute, Japan Tobacco Inc.) [12, 13]. Seven-week-old female Wistar rats were purchased from Chubu Kagaku Shizai Co. Ltd. (Nagoya, Japan) and were allowed free access to food and water. The experiments were performed according to the Animal Experimentation Guide of Nagoya University School of Medicine (Nagoya, Japan).

Production and purification of soluble TGF-βIIR

HEK293 cells were transfected with the plasmid containing the cDNA for the extracellular domain (amino acids 1 to 159) of human TGF-βIIR. The recombinant soluble TGF-βIIR was purified by antibody affinity chromatography using anti-human TGF-βIIR polyclonal antibody (AF-241-NA; R&D Systems, Minneapolis, MN, USA), and then its purity was examined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and silver staining. The purified human TGF-βIIR was used for immunization.

Generation and characterization of anti-human TGF-βIIR monoclonal antibodies

Xenomice are characterized by gene targeted-inactivated mouse heavy and κ chain loci and transgenic for human heavy and κ chain loci. Thus, these mice are deficient in murine antibody production, but produce human antibodies [12, 13]. Xenomice were immunized once by subcutaneous injection of recombinant soluble TGF-βIIR (sTGF-βIIR) emulsified with an equal volume of Freund's complete adjuvant (FCA; ICN/Cappel, Aurora, OH, USA). These mice were repeatedly immunized every week for one month with recombinant sTGF-βIIR in Freund's incomplete adjuvant (ICN/Cappel). The final booster injection was performed two days prior to fusion. Popliteal lymph node cells were fused with mouse myeloma cell lines, P3/X63-Ag8.653, using PEG4000 (Life Technologies, Grand Island, NY, USA). Supernatants of the growing hybridomas were tested by direct enzyme-linked immunosorbent assay (ELISA) against human sTGF-βIIR.

A hybridoma, JMAb-45 (IgG4), was cloned and confirmed to recognize human sTGF-βIIR. Cross-reactivity of JMAb-45 against rat TGF-βIIR was checked by the immunohistochemical study on rat kidney cell lines, NRK (CRL-6509; ATCC) and on normal rat kidney tissues.

Control antibody, JMAb-77 (IgG4), was generated by immunization with keyhole limpet hemocyanin (KLH; Pierce, Rockford, IL, USA).

Direct ELISA procedure

Enzyme-linked immunosorbent assays were performed at room temperature. Each well of the 96-well plate (Nalge Nunc International, Rochester, NY, USA) was incubated for one hour with 0.2 μg sTGF-βIIR (241-R2/CF, R&D) in 50 μL phosphate-buffered saline (PBS). Each well was then blocked for two hours with Block Ace (Dainippon Pharmaceutical, Osaka, Japan). After washing with PBS containing 0.1% Tween 20 (Bio-Rad Laboratories, Hercules, CA, USA; PBS/T), each well was incubated for one hour with 0.0003 to 10 μg/mL JMAb-45 in 50 μL PBS/T. Wells were subsequently washed three times with PBS/T and incubated for one hour with 50 μL horseradish peroxidase labeled anti-human IgG antibody (Amersham Pharmacia Biotech, Buckinghamshire, UK) in PBS/T containing 0.5% bovine serum albumin (BSA fraction V; Nacalai Tesque, Kyoto, Japan). After washing four times with PBS/T, each well was incubated with 100 μL tetramethyl bendidine (Bio-Rad Laboratories). The reaction was terminated by 25 μL 0.5 N sulfuric acid, and the absorbance at 450 nm was read on a microplate reader, M-Tmax (Molecular Devices, Sunnyvale, CA, USA).

Western blot analysis

Soluble TGF-βIIR (R&D) protein was diluted in equal amounts of 2 × Tris-SDS seprasol buffer (Dai-ichi Chemical, Tokyo, Japan) under an unreduced condition and boiled for five minutes. The protein samples were run through a 10%/20% gradient acrylamide gel (Dai-ichi Chemical). Different concentrations of sTGF-βIIR samples and molecular-weight standards (7711BTS; New England BioLabs, Beverly, MA, USA) were subjected to electrophoresis for one hour at 40 mA on the vertical

gel apparatus (Bio-Rad Laboratories). The separated proteins on the SDS gel were transferred electrophoretically to an Immobilon-P polyvinylidine difluoride (PVDF) transfer membrane (Millipore, Tokyo, Japan).

After transfer, the membrane was washed in PBS/T and then incubated with Block Ace at room temperature for two hours on a shaker to block nonspecific antibody binding sites. The membrane was washed in Tris-buffered saline (TBS; Takara, Kyoto, Japan) containing 0.1% Tween 20 (TTBS) and incubated with JMAb-45, JMAb-77 or polyclonal goat anti-human TGF-βIIR antibody (R&D) for 1.5 hours at room temperature. After washing with TTBS, the membrane was incubated with anti-human Ig biotinylated sheep whole antibody (Amersham Pharmacia Biotech) or anti-goat Ig biotinylated donkey whole antibody (Amersham Pharmacia Biotech) diluted with TTBS for one hour at room temperature. After washing with TTBS, immobilized antibodies were detected by luminol reagents (ECL Western blotting reagents; Amersham Pharmacia Biotech); subsequently, the membrane was exposed to ECL hyper-film (Amersham Pharmacia Biotech) for 30 seconds in the dark to visualize the specific protein bands.

Urinary protein excretion

Rats were housed in metabolic cages overnight from day 0 to day 1, from day 2 to day 3, and from day 6 to day 7. Urinary protein was measured by a pyrogallol red method [14].

Serum creatinine level

Serum of each rat was collected at day 3 and at day 7. Serum creatinine was measured using the CRE-EN Kainos kit (Kainos, Tokyo, Japan).

Experimental protocol

Experimental proliferative glomerulonephritis was induced in female Wistar rats by intravenous injection of the monoclonal antibody (mAb) 1-22-3 (mouse IgG3) [15]. Thirty-nine rats were divided into three groups. Rats of group I ($N = 22$) and II ($N = 22$) were injected intravenously with 500 μg of mAb 1-22-3 in 1.0 mL saline as described previously [15]. Group I rats were intraperitoneally injected with anti-TGF-βIIR antibody (JMAb-45, 5 mg/kg) at one hour and four days after mAb 1-22-3 injection. Rats of group II were intraperitoneally injected with anti-KLH antibody (JMAb-77, 5 mg/kg) instead of anti-TGF-βIIR antibody at the same time points. An additional 15 rats were injected intravenously with 1.0 mL of saline at day 0 and studied as normal controls (group III). At day 1, six rats of group I, six group II, and five group III rats were sacrificed. At day 3, six rats of group I, six group II, and five group III rats were sacrificed. At day 7, the remaining rats were sacrificed. The experimental protocol is summarized in Table 1. Tissues for light microscopy were fixed with methacarn overnight, dehydrated through graded ethanol series and embedded in paraffin. Histologic sections of kidneys were stained with periodic acid-Schiff (PAS) and hematoxylin and eosin (HE). A part of the tissues was snap frozen in liquid nitrogen for immunostaining.

Table 1. Experimental protocol

Group	Reagent injection	Treatment	Number of rats		
			Day 1	Day 3	Day 7
I	mAb 1-22-3 IV	JMAb-45 IP	6	6	10
II	mAb 1-22-3 IV	JMAb-45 IP	6	6	10
III	Saline IV	None	5	5	5

Abbreviations are: IV, intravenous injection; IP, intraperitoneal injection.

Immunostaining

Two-micrometer-thick frozen sections were cut by a cryostat, air dried, and fixed in acetone at room temperature for 10 minutes. For analysis of glomerular macrophage infiltration, sections were incubated with mAb ED1 (anti-rat-macrophage/monocyte, mouse IgG1; BMA), followed by FITC-labeled goat anti-mouse IgG antibody (Cappel Laboratory, West Chester, PA, USA) as the second reagent.

For immunohistological analysis, endogenous peroxidase activity was inhibited with 0.1% NaN_3 and 0.3% hydrogen peroxide in PBS and nonspecific protein-binding sites were blocked with normal goat serum. The sections were incubated with mAb 1A4 [anti-α-smooth muscle actin (α-SMA) antibody, mouse IgG2a; Dako, Glostrup, Denmark], IST-9 (anti-cellular fibronectin EDA domain, mouse IgG1; Harlan Sera-lab, Sussex, UK), or rabbit polyclonal anti-collagen I (Chemicon International, Temecula, CA, USA), followed by a conjugate of polyclonal goat anti-mouse IgG antibodies or goat anti-rabbit IgG antibodies, horseradish peroxidase and dextran backbones (EnVision™ System; Dako) as the second reagent. To detect the localization of TGF-βIIR in rat and mouse kidney, endogenous biotin present in the renal tissues was blocked with avidin/biotin blocking kit in two successive steps (Vector, Burlingame, CA, USA). Then tissues were incubated with JMAb-45. Immobilized human antibodies were detected by biotin-avidin-immunoperoxidase technique using Vectastain ABC kit human IgG (Vector). Finally, the enzyme activity of horseradish peroxidase was detected using 3-amino-9-ethyl-carbazole (Dako) or 3,3′-diaminobenzidine tetrahydrochloride liquid system (Dako). Negative controls were performed by replacement of first step antibodies by isotype-matched mAb or species-matched antibody.

Morphometric analysis of histology and immunohistology

For each kidney and for each type of staining, cross sections of 20 different glomeruli and twenty different

tubulointerstitial areas of 0.1 mm^2 were examined as described previously [16]. The total number of nuclei in each glomerulus was counted using tissues stained by PAS. The average number of cells/glomerulus was calculated and used as a representative number. The number of macrophages was counted, and the average numbers per glomerulus were calculated. In each glomerulus, ratio of area positively stained for α-SMA, fibronectin-EDA, or type I collagen to total glomerular area was measured by computer-aided planimetry using Mac Scope (Mitani, Fukui, Japan) and expressed as a percentage. Similarly, the ratio of positively stained area in 0.1 mm^2 of tubulointerstitial area was measured [16].

Statistical analysis

All values are provided as mean ± SD. Statistical analysis was performed by one-way analysis of variance (ANOVA). When significant difference was present, statistical analysis was further performed using the Scheffe *F* test between two groups. Significant difference was set when the *P* value was less than 0.05 (5%).

RESULTS

Characterization of fully human anti-TGF-βIIR monoclonal antibody

ELISA. The direct ELISA for sTGF-βIIR showed that anti-TGF-βIIR antibody, JMAb-45, detected sTGF-βIIR in a dose-dependent manner from 0.01 to 10 μg/mL of JMAb-45 concentration (Fig. 1A). Control antibody (JMAb-77) did not react with sTGF-βIIR (Fig. 1A)

Western blot analysis. Western blotting showed that JMAb-45 specifically detected proteins of 20 to 30 kD. The molecular size of the band reacted with JMAb-45 was similar to that identified by other polyclonal anti-TGF-βIIR antibody (R&D; Fig. 1B).

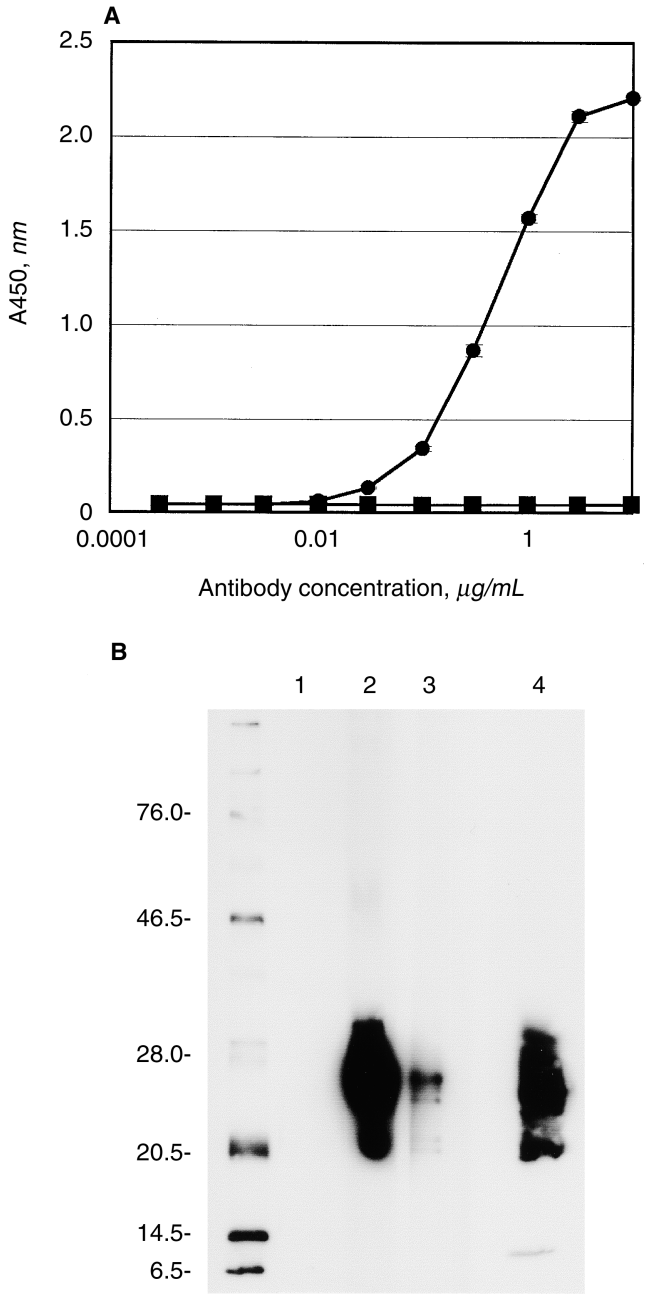


Fig. 1. Direct enzyme-linked immunosorbent assay (ELISA) and Western blot analysis. (*A*) Direct ELISA shows specific detection of sTGF-βIIR by JMAb-45 (●) in a dose-dependent manner, but not by JMAb-77 (■). (*B*) Western blot analysis shows 20-30 kD sTGF-βIIR broad band detected by JMAb-45 and anti-TGF-βIIR (R&D), but not by JMAb-77. Lane 1, sTGF-βIIR 0.05 μg/lane, JMAb-77 2 μg/mL; lane 2, sTGF-βIIR 0.5 μg/lane, JMAb-45 2 μg/mL; lane 3, sTGF-βIIR 0.05 μg/lane, JMAb-45 2 μg/lane; lane 4, sTGF-βIIR 0.05 μg/lane, anti-TGF-βIIR polyclonal antibody (R&D) 0.05 μg/mL, as a positive control.

Urinary protein excretion

At day 1 and day 3, the urinary protein of groups I and II was increased when compared with the normal group; however, those were not significantly different between groups I and II (day 1, group I, 11.7 ± 4.6 mg/day; group II, 11.3 ± 6.3 mg/day; group III, 2.9 ± 0.9 mg/day; day 3, group I, 38.3 ± 22.7 mg/day; group II, 27.6 ± 19.9 mg/day; group III, 3.0±0.9 mg/day; Fig. 2A). At day 7, the urinary protein of group I was significantly less than that of group II (53.2 ± 22.9 mg/day vs. 90.7 ± 25.8 mg/day, $P < 0.005$). The urinary protein excretion of group III was significantly less than that of groups I and II (3.0 ± 1.0 mg/day, $P < 0.005$ vs. group I, $P < 0.0001$ vs. group II; Fig. 2A).

Serum creatinine level

At day 3, the serum creatinine level was not different among the three groups (group I, 0.44 ± 0.02 mg/dL; group II, 0.45 ± 0.03 mg/dL; group III, 0.45 ± 0.02 mg/dL). At day 7, the serum creatinine level of group II was significantly elevated when compared with that of groups I or III (group I, 0.65 ± 0.13 mg/dL; group II, 0.78 ± 0.09 mg/dL; group III, 0.60 ± 0.02 mg/dL, $P < 0.005$

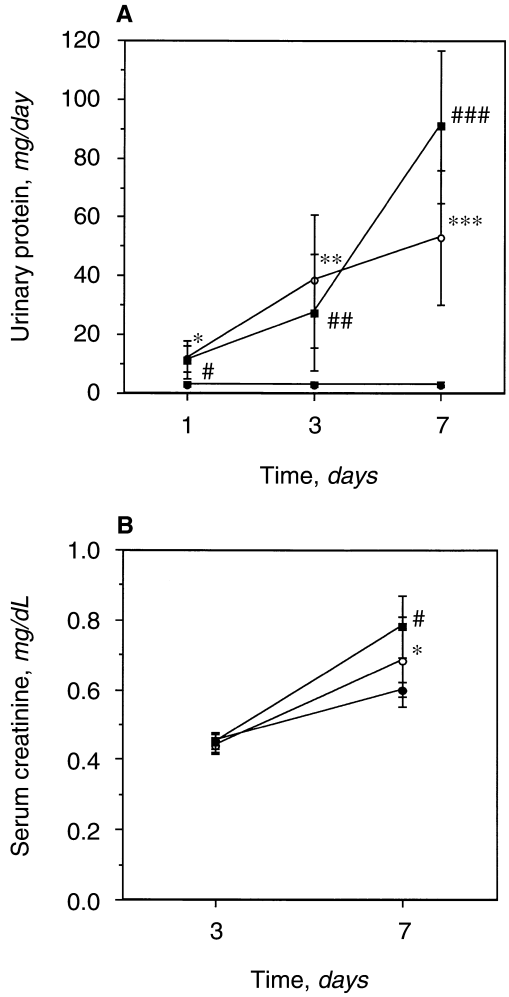


Fig. 2. Urinary protein excretion and serum creatinine levels in (○) group I, (■) group II, and (●) group III. (*A*) At days 1 and 3 the urinary protein excretion was not different between groups I and II. At day 7, urinary protein excretion was significantly increased in group II compared to group III. Urinary protein excretion was significantly reduced by injection of JMAb-45 (group I) compared with group II, but was still larger than that of group III (*$P < 0.05$ vs. group III; #$P < 0.05$ vs. group III; **$P < 0.05$ vs. group III; ##$P < 0.05$ vs. group III; ***$P < 0.005$ vs. group II; $P < 0.005$ vs. group III; ###$P < 0.0001$ vs. group III, respectively). (*B*) At day 3, serum creatinine level among three groups was not significantly different. At day 7, serum creatinine level of group II was significantly increased compared with group III (#$P < 0.05$ vs. group III). Serum creatinine level was significantly suppressed by injection of JMAb-45 (group I, *$P < 0.05$ vs. group II). The difference between group I and group III was statistically not significant.

group II vs. groups I or III), while those were not significantly different between groups I and III (Fig. 2B).

TGF-βIIR expression

In normal rat kidney, TGF-βIIR was expressed mainly by glomerular epithelial cells and was faintly detected in the apical sites of tubular epithelial cells (Fig. 3A). In anti-Thy-1 nephritis, the expression of TGF-βIIR was up-regulated in glomerulus at day 7, especially by proliferated mesangial cells (Fig. 3B). We also examined the expression of TGF-βIIR in normal murine kidney. The expression pattern was similar to that of normal rat kidney (data not shown).

Light microscopic findings

At day 1 and day 3, almost all glomeruli in group I and group II showed mesangiolysis (Fig. 4). These findings were not different between group I and group II. At day 7, mesangial area of group II was expanded in almost all glomeruli (Fig. 3D). In several glomeruli, microaneurysm formation, the advanced form of mesangiolysis, was still observed. These pathological findings were comparable to the findings reported previously in anti-Thy-1 nephritis at day 7 [15]. On the other hand, in group I the extent of mesangial expansion was less than that of group II (Fig. 3C).

α-SMA expression and ECM deposition in glomerulus

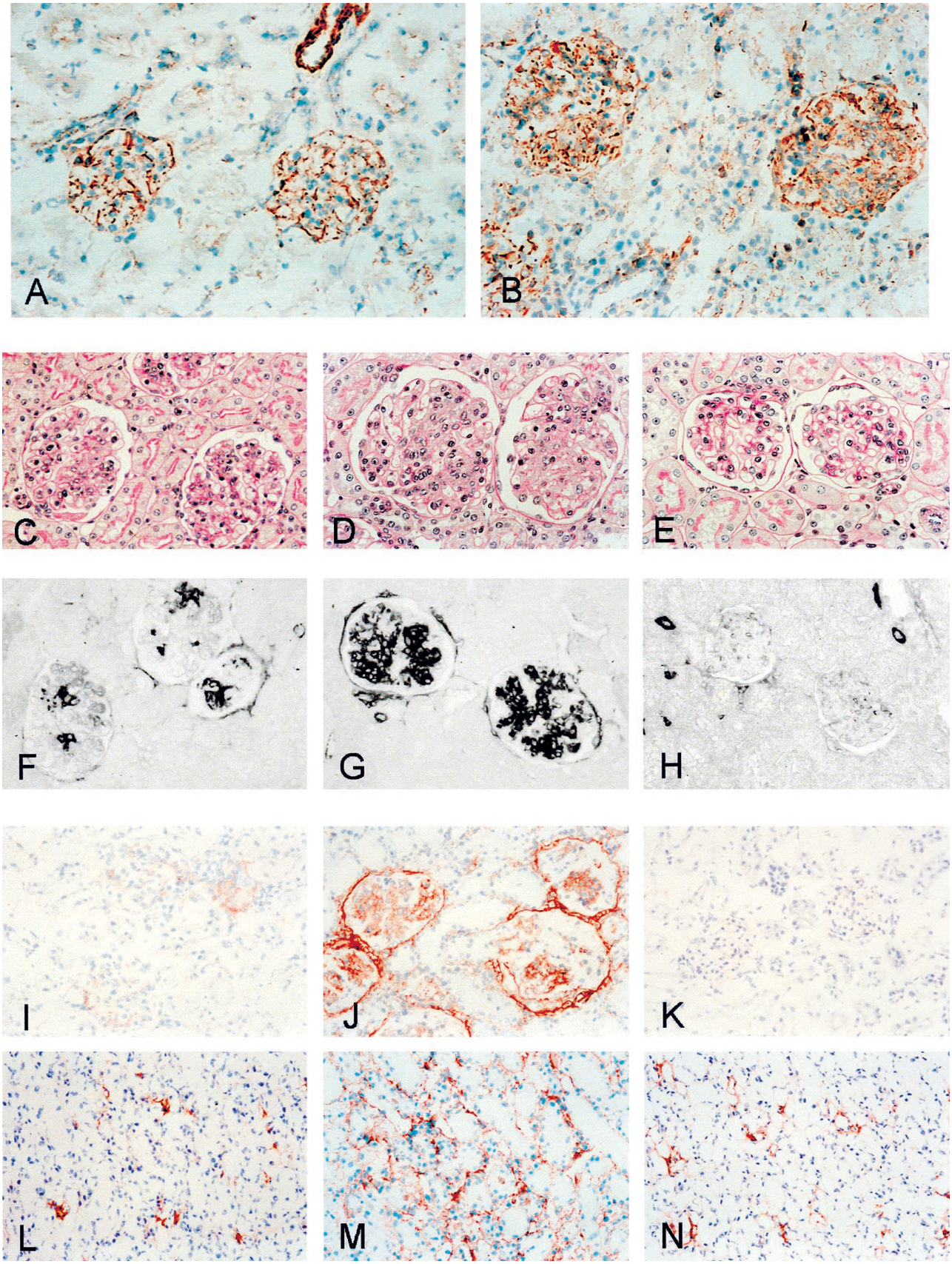
By immunohistochemistry, α-SMA expression was confined to blood vessels, but was not detected in the glomeruli of group III rat (Fig. 3H). In group II rats, the α-SMA-positive area was significantly increased (Fig. 3G). Expression of α-SMA in group I (Fig. 3F) was significantly decreased when compared with group II (Fig. 3G). Using a computer-aided analyzer, α-SMA expression was quantitatively measured. α-SMA expression in group I was significantly suppressed when compared to that in group II. Rats of group III did not express α-SMA in glomeruli (Fig. 5A).

Fibronectin-EDA was not detected or weakly positive in the glomerulus from group III rats (Fig. 3K). In group II, fibronectin-EDA was markedly increased in mesangial area and in periglomerular area (Fig. 3J). In contrast, the glomerular staining of fibronectin-EDA was dramatically decreased in rats of group I (Fig. 3I). The results obtained from type I collagen staining showed similar results. Quantitative data from the computer-aided analyzer are shown in Figure 5A.

α-SMA expression and ECM deposition in interstitium

In group III rats, the α-SMA expression was confined to blood vessel walls. In groups I and II, α-SMA was faintly but significantly stained in a part of tubular epithelial cells.

In group III, fibronectin-EDA was weakly positive in the tubulointerstitial area (Fig. 3N). In group II, expression of fibronectin-EDA was increased especially in outer medulla (Fig. 3M). The expression in group I (Fig. 3L) was significantly less than that of group II. The expression of type I collagen was similar with that of fibronectin-EDA. Quantitative analysis showed that the differences in the fibronectin-EDA and type I collagen-positive area were statistically significant among the three groups (Fig. 5B).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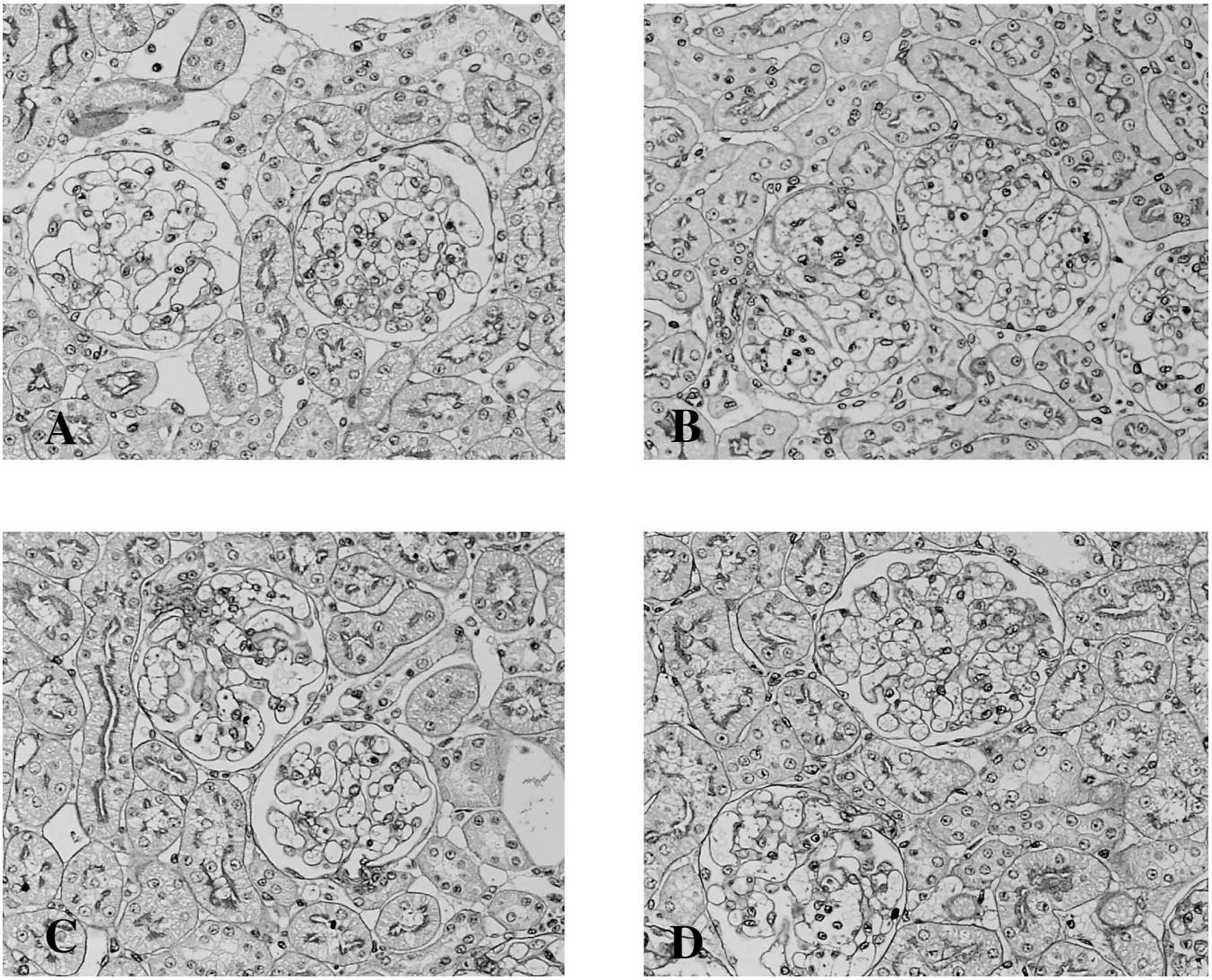

Fig. 4. Light microscopic findings at day 1 and day 3. Light microscopic findings at day 1 and day 3 are shown by PAS stain (×400). Panel *A* shows group I at day 1; *B*, group II at day 1; *C*, group I at day 3; *D*, group II at day 3. At day 1 and day 3, almost all glomeruli showed mesangiolysis and cellular infiltration in both groups. These findings were not different between two groups.

Number of total glomerular cells and glomerular macrophages

At day 1 and day 3, the number of total glomerular cells was significantly decreased in groups I and II compared with group III, but there was no difference between group I and group II. At day 7, the number of total glomerular cells was significantly increased in groups I and II compared with group III. The number of total glomerular cells in group I was slightly decreased when compared with group II ($P < 0.01$; Fig. 6A).

Glomerular macrophages were rarely detected in group III. Through all periods of this experiment, the

Fig. 3. Light microscopic findings and expression of TGF-βIIR, α-SMA, and fibronectin-EDA at day 7. Immunostaining micrograph shows TGF-βIIR expression in normal rat kidney (*A* ×400) and anti-Thy-1 nephritis rat kidney at day 7 (*B* ×400). TGF-βIIR was expressed in glomerulus, especially in glomerular epithelial cells, and faintly in tubular epithelial cells in normal rat kidney (A). TGF-βIIR expression was increased in both glomerulus and tubules in Thy-1 nephritis at day 7 (B). Light microscopic appearance at day 7 (PAS stain ×400 *C*, *D*, *E*: C, group I; D, group II; E, group III). The mesangial area was expanded in group II (D) compared with group III (E). Injection of anti-TGF-βIIR antibody (group I) suppressed mesangial expansion (C) compared to group II (D). Immunostaining micrograph shows α-SMA expression in glomerulus (*F*, *G*, *H*, ×200), fibronectin-EDA expression in glomerulus (*I*, *J*, *K*, ×200) and in interstitium (*L*, *M*, *N*, ×200) (group I is in panels F, I, L; group II, panels G, J, M; group III, panels H, K, N). The expression of α-SMA and fibronectin-EDA was not detected or weakly positive in group III (H, K, N) and increased in group II (G, J, M). The expression of both proteins in group I (F, I, L) was significantly reduced when compared to group II.

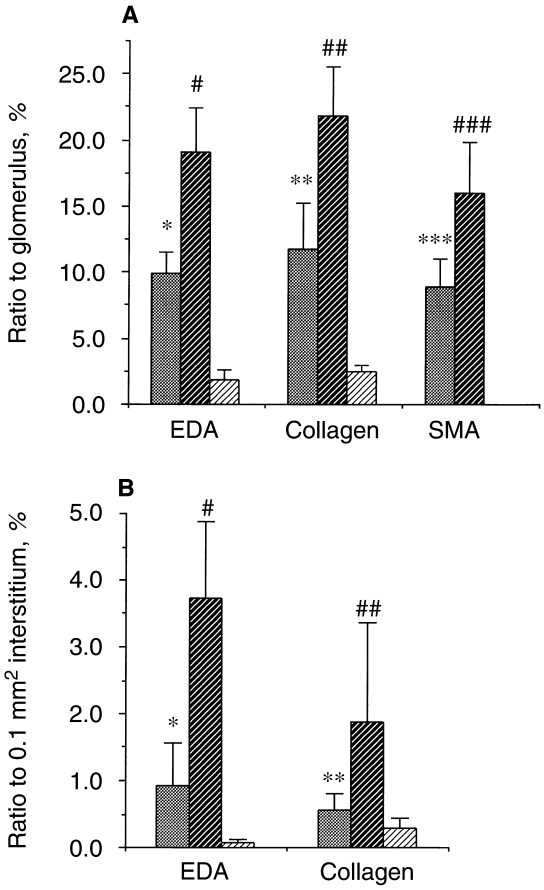

Fig. 5. Quantitative measurement of α-smooth muscle actin (α-SMA), fibronectin-EDA and type I collagen in glomeruli and in interstitium. Symbols are: (■) group 1; (▨) group II; (▨) group III. (*A*) In glomeruli, the positively stained areas for α-SMA, fibronectin-EDA and type I collagen were significantly increased in group II compared with group III (fibronectin-EDA, #$P < 0.0001$; type I collagen, ##$P < 0.0001$; α-SMA, ###$P < 0.0001$). The positively stained area for these proteins was significantly reduced in group I when compared to group II (fibronectin-EDA, *$P < 0.0001$; type I collagen, **$P < 0.0001$; α-SMA, ***$P < 0.005$, respectively). (*B*) The ratio of positively stained area for fibronectin-EDA or type I collagen in a randomly selected tubulointerstitial compartment (0.1 mm^2) was significantly increased in group II compared with group III (fibronectin-EDA, #$P < 0.0001$; type I collagen, ##$P < 0.05$). The positively stained area for both proteins was significantly reduced in group I when compared with group II (fibronectin-EDA, *$P < 0.0001$; type I collagen, **$P < 0.05$, respectively).

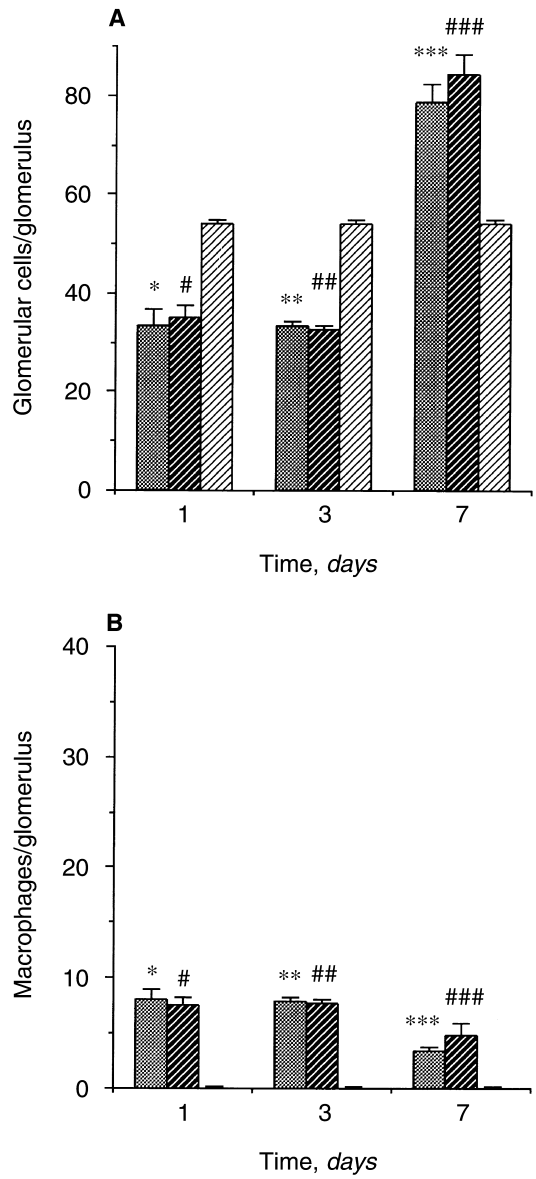

Fig. 6. Total glomerular cells and macrophages. Symbols are: (■) group 1; (▨) group II; (▨) group III. (*A*) At day 1 and day 3, the number of total glomerular cells in group I and II was significantly decreased compared with group III, but there was no difference between group I and group II. At day 7, the number of total glomerular cells in groups I and II was significantly increased compared with group III, and that of group I was reduced compared with group II. The difference between each of the two groups was statistically significant (*$P < 0.0001$ vs. group III; #$P < 0.0001$ vs. group III; **$P < 0.0001$ vs. group III; ##$P < 0.0001$ vs. group III; ***$P < 0.01$ vs. group II, $P < 0.0001$ vs. group III; ###$P < 0.0001$ vs. group III, respectively). (*B*) The number of glomerular macrophages in groups I and II was significantly increased compared to group III through all periods. There was no difference between group I and group II at day 1 and day 3, but that of group I was reduced compared to group II at day 7 (*$P < 0.0001$ vs. group III; #$P < 0.0001$ vs. group III; **$P < 0.0001$ vs. group III; ##$P < 0.0001$ vs. group III; ***$P < 0.005$ vs. group II, $P < 0.0001$ group III; ###$P < 0.0001$ vs. group III).

number of glomerular macrophages in groups I and II was significantly increased compared with group III. Infiltration of glomerular macrophages in group I was not different at day 1 and day 3, but significantly inhibited at day 7 when compared with group II (Fig. 6B).

DISCUSSION

Several targeting points and methods have been reported to prevent renal fibrosis through inhibition of TGF-β signaling pathway: (*1*) direct inhibition of TGF-β synthesis by application of antisense oligonucleotides [5];

(*2*) TGF-β inhibition by neutralizing antibody [4] or by natural inhibitor of TGF-β, decorin [6, 7]; (*3*) soluble chimeric TGF-β receptor [8], which competitively block the binding of TGF-β to its receptors; and (*4*) TGF-β intracellular signaling pathway inhibition by specific inhibitor for Rho-associated coiled-coil forming protein kinase (ROCK) (abstract; Nagatoya et al, *J Am Soc Nephrol* 11:534A, 2000), which is the downstream of Rho, an another TGF-β signaling pathway [17, 18]. Also, angiotensin-converting enzyme (ACE) inhibitors and angiotensin II type I receptor (AT1) antagonists, which have already been clinically used, are known to be renoprotective agents by reducing TGF-β expression as well as decrease of intraglomerular capillary pressure [19–21]. Dietary protein restriction reduces TGF-β expression as well [22]. However, there have been no reports demonstrating that blocking of TGF-β receptor function prevents renal fibrosis. Our studies showed for the first time that direct inhibition of TGF-βIIR is effective to prevent ECM deposition using rat anti-Thy-1 nephritis.

The present work tested the effects of a fully human monoclonal antibody against TGF-βIIR in a rat anti-Thy-1 nephritis. First, we studied expression of TGF-βIIR in the kidneys from normal rats and anti-Thy-1 nephritis rats at day 7. In the normal condition, TGF-βIIR is strongly expressed in glomerular visceral epithelial cells and weakly in the apical site of tubular epithelial cells. Recently, TGF-β 2 and 3 have been shown to be expressed in glomerular visceral epithelial cells as well as TGF-βIIR [16, 23]. Interaction of TGF-β and TGF-βIIR may contribute to maintain the structure and function of glomerular capillary wall in normal rat glomerulus. Administration of 1-22-3 monoclonal antibody to rats leads to proliferative glomerulonephritis [15], which is characterized by complement-dependent injury of mesangial cells, followed by mesangial proliferation [15, 24, 25]. Then transient ECM deposition is observed. Previous studies demonstrated that TGF-β was a crucial factor for accumulation of ECM in this model at day 7 [4–8, 16]. TGF-β was increased in the mesangial proliferative lesions and peaked at day 7 [16], whereas expression of TGF-βIIR has not been studied yet. As expected, TGF-β IIR was dramatically up-regulated in the mesangial proliferative lesions, tubular epithelial cells, and interstitial cells of the kidneys from anti-Thy-1 nephritis obtained at day 7. In this experiment, rats were divided into three groups to explore the effects of the fully human anti-TGF-βIIR monoclonal antibody (JMAb-45). In group I, TGF-βIIR antibody apparently reduced the deposition of fibronectin-EDA and type I collagen. Physiological and histological findings at day 1 and day 3 were not different between group I and group II. Therefore, we thought that the injection of JMAb-45 did not influence on the early phase of this model, and believed that the difference in ECM deposition at day 7 was dependent on TGF-βIIR blockade. In addition, the number of total glomerular cells was slightly but significantly decreased when compared to group II. In general, several investigators reported that, in vitro, TGF-β was an inhibitor of mitogenesis in glomerular cells [26, 27]. In an in vivo situation, however, the effects of TGF-β on glomerular cell proliferation are still controversial. Introduction of TGF-β gene into the rat glomerular mesangium was reported to induce not only ECM deposition, but also modest mitogenesis of resident cells [28]. These findings might explain that blocking for TGF-βIIR reduced the total number of glomerular cells at day 7. Further studies are needed to determine the in vivo effects of TGF-β on the glomerular cell proliferation.

Transforming growth factor-β was reported to have an anti-inflammatory function [9, 29]. In our study, the number of macrophages in early phase was not different between group I and group II. Therefore, we thought that JMAb-45 did not influence on macrophage infiltration at early time point of this experiment. However, macrophage infiltration was significantly inhibited at day 7. While the difference was small (group I, 3.4 ± 0.3 macrophages/glomerulus; group II, 4.8 ± 1.2 macrophages/glomerulus), we cannot ignore the possibility that JMAb-45 might influence macrophage infiltration. More investigations are required to define this question.

Urinary protein excretion was significantly decreased in group I compared with group II. In other studies the decrease of urinary protein excretion was indicated in anti-Thy-1 nephritis animals treated with decorin administration or with decorin gene therapy [6, 7], which attenuated TGF-β. Heparan sulfate proteoglycans (HSPG) are components of glomerular basement membrane (GBM), and HSPG alterations may contribute to the GBM's permeability. One of the mechanisms leading to a reduced HSPG function is depolymerization by oxygen or nitrogen radicals [30]. TGF-β was reported to increase GBM permeability via reactive oxygen species (ROS) production of glomerular resident cells [31]. It is possible that ROS production might be reduced by anti-TGF-βIIR antibody treatment. Serum creatinine also was decreased in group I rats. Improvement of glomerular morphological and functional changes by JMAb-45 might contribute to the improved creatinine level. In addition, tubulointerstitial damage ameliorated by anti-TGF-βIIR antibody injection might have influenced on serum creatinine level [32].

Transforming growth factor-β is well known as a major promoter of myofibroblast differentiation. In mesangial cells, TGF-β induces α-SMA expression (abstract; Imai et al, *J Am Soc Nephrol* 5:782, 1994) [33] and enhances synthesis of ECM including fibronectin-EDA [34]. Anti-EDA monoclonal antibody specifically blocks TGF-β–induced expression of α-SMA but not that of plasminogen activator inhibitor-1 [35]. In anti-Thy-1 nephritis at day 7, JMAb-45 reduced α-SMA expression as well as

fibronectin-EDA. Taken together, suppression of fibronectin-EDA deposition by TGF-βIIR antibody via inhibition of TGF-β signaling pathway might lead to the decrease in myofibroblast differentiation of mesangial cells.

The Xenomice used in this experiment are murine immunoglobulin (Ig) knockout and human Ig transgenic mice [12, 13] capable of producing human antibodies against human antigens. The usefulness of mouse monoclonal antibodies [36] or mouse/human chimera antibodies [37] could be restricted in the clinical setting because of the immune response against mouse antibodies [38] or by the difficulty in obtaining high affinity chimera antibody. Antibodies from Xenomice are able to overcome these problems. JMAb-45 is a fully human monoclonal antibody and is expected for the therapy of human diseases in the future, though more studies are required before clinical use.

In summary, our data showed that blocking for TGF-βIIR is effective in reducing the deposition of ECM in rat anti-Thy-1 nephritis. Direct inhibition of TGF-βIIR may be an additional therapeutic strategy to block TGF-β signaling pathway.

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APPENDIX

Abbreviations used in this article are: ACE, angiotensin-converting enzyme; α-SMA, α-smooth muscle actin; AT1, angiotensin II type 1 (receptor); ECL, enhanced chemiluminescence; ECM, extracellular matrix; ELISA, enzyme-linked immunosorbent assay; FBS, fetal bovine serum; FCA, Freund's complete adjuvant; GBM, glomerular basement membrane; HE, hematoxylin and eosin; hTGF-βIIR, human monoclonal antibody against transforming growth factor-β type II receptor; HSPG, heparan sulfate proteoglycan; JMAb-45, hybridoma for IgG4; KLH, keyhole limpet hemocyanin; PAS, periodic acid Schiff; PBS, phosphate-buffered saline; PBS/T, phosphate-buffered saline plus Tween 20; ROCK, Rho-associated coiled-coil forming protein kinase; ROS, reactive oxygen species; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; sTGF-βIIR, soluble transforming growth factor-β type II receptor; TBS, Tris-buffered saline; TGF-β, transforming growth factor-β; TGF-βIIR, transforming growth factor-β type II receptor; TTBS, Tris-buffered saline plus Tween 20.

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