TJN-331 Improves Anti-glomerular Basement Membrane Nephritis by Inhibiting the Production of Intraglomerular Transforming Growth Factor-β1

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The study of experimental glomerulonephritis in rats was performed to examine the antinephritic effects of TJN-331, a new herbal compound. To clarify the action of TJN-331 ((E)-N-(3,4-dimethoxyphenethyl)-N-methyl-3-(3-pyridyl)-2-propenamide) on TGF-β1, glomeruli were isolated from rats with anti-glomerular basement membrane (GBM) nephritis and incubated for 48 h with test drugs in vitro. Next, we examined the effects of TJN-331 on rat anti-GBM nephritis induced by injection with anti-GBM serum. TJN-331 dose-dependently inhibited the increase in total and mature TGF-β1 production from nephritic glomeruli, although it did not inhibit TGF-β1 production from normal glomeruli. Administration of TJN-331, at a dose of 2 mg/kg/d, prevented proteinuria and increased crescent formation and adhesion of capillary walls to Bowman’s capsule. The increases in mature TGF-β1 protein production and TGF-β1 staining score in nephritic rats were reversed by TJN-331 treatment. These results suggest that TJN-331 inhibits proteinuria and histopathological changes in glomeruli via suppression of TGF-β1 production from inflamed glomeruli.

Key words TJN-331; glomerular basement membrane; proteinuria transforming growth factor-β

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in the in vivo assay and 0.7% dimethyl sulfoxide (DMSO) in the in vitro assay.

**Induction of the Nephritic Model and Test Drug Administration** Anti-GBM nephritis was induced in male Sprague-Dawley rats (Charles River Laboratories Japan, Yokohama, Japan) by injecting 6.5 mg goat γ-globulin in 0.25 ml of Freund’s complete adjuvant into their hind foot pads, following intravenously (i.v.) injection of 0.75 ml of goat anti-GBM GBM serum into the tail vein, as previously described. The rats were divided into 6 groups (n = 7 per group) such that the mean urinary protein excretion 20 d after anti-GBM serum injection was similar among the groups. From day 21 to the final day of the experiment (day 41), TJN-331 (0.1, 0.5, or 2.0 mg/kg) and dipyridamole (50 or 300 mg/kg) were orally administered, once daily, and the kidneys were harvested on the final day of the experiment. A total of eight groups, including untreated and nephritic control groups were administered the 0.5% CMC suspension instead of test drugs. All experiments were performed between 9:00 a.m. and 6:00 p.m. in compliance with the guidelines established by the Animal Ethics Committee of Tsumura & Co.

**Measurement of Urinary Protein Content and Serum Creatinine Level** Urinary protein excretion was determined at 20, 24, 30, 35, and 41 d after induction of anti-GBM nephritis. The rats were loaded with 8 ml of water (including the test drug solution) and placed in a stainless steel metabolic cage where urine samples were collected for 24 h under fasting conditions. Urinary protein excretion was measured using a Tonein TP-II (Otsuka Pharmaceuticals, Tokyo, Japan) model TBA 20FR automatic analyzer at 30 and 41 d. Urinary protein excretion was determined in a blinded manner for 50 randomly selected glomeruli that included the vascular pole, according to a previously published procedure. Immunostaining scores were determined in a blinded manner for 50 randomly selected glomeruli that included the vascular pole, according to a previously published procedure. Immunostaining scores were assigned based on the extent of glomerular PAS staining, according to the modified method reported by Kagami et al. All tissue evaluations were made by two evaluators in an independent and blinded manner.

0 points, no glomerular staining
1 point, weak glomerular staining
2 points, segmental staining
3 points, global staining (below 50%)
4 points, global staining (more than 50%)

Score = ∑(each score × number of glomeruli)/50

**Immunohistochemistry** Detection of α-smooth muscle actin and TGF-β1-positive regions. In the enzyme antibody method, tissue sections deparaffinized in xylene were incubated in a solution consisting of 3% hydrogen peroxide in methanol for 5 min and then washed in phosphate buffered saline (PBS). Normal goat serum was added to distilled water and applied to the tissue sections. Primary antibodies (anti-α-smooth muscle actin antibody, Medac GmbH, Hamburg, Germany and anti-TGF-β1 antibody, Austral Biologicales Co., CA, U.S.A.) were applied after 100-fold dilution in PBS, and then incubated overnight at 4 °C. After washing, a biotinylated anti-murine IgG antibody (Jackson Immunoresearch, West Grove, PA, U.S.A.) was applied. The specimen was stained after washing using the ABC Elite Kit (Vector Laboratories). α-Smooth muscle actin and TGF-β1-positive regions were evaluated on the same section, and each glomerulus was graded as follows: 0, diffuse, very weak, or absent glomerular staining, and no observable increase in staining; 1+, diffuse, weak glomerular staining with 1—25% focal increase of the glomerular tuft showing staining; 2+, 25—50% of glomerular tuft demonstrating focal, strong staining; 3+, 50—75% strong staining of the glomerular tuft in a focal manner; and 4+, >75% strong staining of the glomerular tuft.

**TGF-β1 Protein Determination** Next, we attempted to determine the effects of TJN-331 on production of TGF-β1 protein in anti-GBM nephritis in vivo. Anti-GBM nephritis was induced, and TJN-331 (0.5, 2.0, or 5.0 mg/kg) or dipyridamole (50 or 300 mg/kg) was subsequently administered to the anti-GBM nephritic rats from Day 1 to Day 41 after induction of nephritis. Kidneys were removed from the rats on the last day of the experiment (1 h after the last administration on Day 41) after ventrotomy, under pentobarbital anesthesia to isolate the glomeruli. Glomeruli from individual rats were isolated using a graded sieving technique, as detailed below. Glomeruli were washed twice in fresh RPMI-1640 serum-free media and then cultured at a density of 2000 glomeruli/ml/rat for 48 h. The amount of total or mature TGF-β1 protein in the dissociated glomeruli was then measured according to the following method.

**In Vitro Studies** To investigate the direct effects of TJN-331 on the production of TGF-β1 protein and TGF-β1 signal transduction-associated gene expression (TGF-β1, and Smad 2, 3), we performed an experiment in which TJN-331 was added to the culture medium of isolated glomeruli obtained from rat anti-GBM nephritic rats. Because both total and mature TGF-β1 contents are detectable in glomeruli, we used rat glomeruli to measure the in vitro action of TJN-331 on the production of TGF-β1 protein. Kidneys were harvested 1 d after induction of anti-GBM nephritis. Abdomens of the rats were opened under pentobarbital anesthesia and both kidneys were removed, stored in 50-ml centrifuge tubes with serum-free RPMI-1640 medium at 4 °C, minced using surgical scissors, passed sequentially through meshes of 212, 150, and 75 μm, and glomeruli that remained on the 75-μm mesh were collected using a pipette. Isolated glomeruli were washed twice with serum-free RPMI-1640 medium after confirming the isolation of individual cells by microscopic observation. Fresh serum-free RPMI-1640 medium was added to adjust the concentration to 2000 glomeruli/ml. The suspension was dispersed in 350-μl aliquots in 48-well plates so as to obtain the same number of glomeruli in each well. TJN-331 (final concentrations: 1, 5, 10, 50 μmol/l) prepared in RPMI-1640 (0.7% DMSO) was added in 50-μl aliquots (n = 5). Medium containing 0.7% DMSO was added to the controls. The glomeruli were cultured in a CO2 incubator for 48 h. Based on the results of preliminary experiments, we determined the amounts of TGF-β1 at both 24 and 48 h after isolation of glomeruli; we were able to clearly confirm a measurable increase 48 h after isolation. Sedimented
glomeruli were then used for the quantitation of activated TGF-β1. Glomeruli were frozen at −80°C for 1 h and then thawed at the time of quantitation to destroy glomerular proteins. Fresh serum-free RPMI-1640 media (300 µl) was added to the destroyed glomeruli. The quantitation of total and mature TGF-β1 was conducted using the TGF-β1 enzyme-linked immunosorbent assay (ELISA) kit (Amersham Biosciences KK, Tokyo, Japan). Measurement of total TGF-β1 was performed following the addition of HCl to each sample.

Isolated glomeruli were homogenized and total RNA extracted using the RNeasy Universal Tissue kit (Qiagen, Valencia, CA, U.S.A.), according to the manufacturer’s protocol. Optical density of total RNA was determined using ND-1000 (NanoDrop Ltd., Wilmington, DE, U.S.A.). Each sample was then diluted to 100 ng/µl. Diluted total RNA was incubated at 70°C for 5 min and then cooled on ice. The total RNA (1000 ng) was reverse transcribed using TaqMan Reverse Transcription reagents (Applied Biosystems, Foster City, CA, U.S.A.), according to the manufacturer’s protocol. Quantitative polymerase chain reaction (PCR) assays were performed using TaqMan Universal PCR Master Mix (Applied Biosystems) and a Prism 7900HT Sequence Detection System (Applied Biosystems). mRNA expression was normalized using ribosomal protein S29 (Rps29) as an endogenous control to correct differences in the amount of total RNA added in each reaction. These differences were expressed by the Ct value, where \[\text{Ct} = 2^{(\text{A} - \text{B})/\text{B}}, \] A is the number of cycles needed to reach threshold for the housekeeping gene (Ct threshold cycle) and B is the number of cycles needed for the target gene. All oligonucleotide primers and fluorogenic probe sets for TaqMan real-time PCR were obtained from Applied Biosystems (glyceraldehyde-3-phosphate dehydrogenase: Rn99999916_s1, TGF-β1: Rn00572010_m1, Smad2: Rn00569900_m1, Smad3: Rn00565331, Plasminogen: Rn00585167_m.

Statistical Analyses Statistical significance was analyzed by a one-way analysis of variance, followed by the Dunnett test or Steel test. Data are presented as means±standard error for each group, and significance levels of 5% and 1% are indicated in tables.

RESULTS

Effects on General Condition and Proteinuria

Body weight and urine volume in anti-GBM nephritis during the experimental periods were significantly decreased compared with saline-treated rats (data not shown). In contrast, there was no significant difference between the nephritic controls and test drugs-treated groups. Twenty days after induction of nephritis, proteinuria in nephritic rats was approximately 450 mg/dl. Rats were divided into six groups, and the mean and standard error of urinary protein excretion were similar in each group. During the experimental period, proteinuria in nephritic controls stayed within the range of 300—350 mg/dl. Administration of TJN-331 at a dose of 2.0 mg/kg/d, per os (p.o.), prevented urinary protein excretion from 30 to 41 d, and a dose of 0.5 mg/kg inhibited it at 35 d. Dipyridamole failed to inhibit proteinuria during the experimental periods (Fig. 2).

Effects on Serum Creatinine Level

At 30 and 41 d after induction of nephritis, the serum creatinine level in nephritic controls was significantly increased compared with the saline-treated group. Administration of TJN-331 and dipyridamole (50 mg/kg/d) showed a tendency to inhibit the increase in serum creatinine levels (Anti-GBM nephritic rats; 0.45±0.9 mg/dl, TJN-331 at a dosage of 2.0 mg/kg; 0.33±0.9 mg/dl, dipyridamole at a dosage of 50 mg/kg; 0.34±0.2 mg/dl at 41 d).

Effects on Crescent Formation and Adhesion in Glomeruli

Animals with anti-GBM nephritis displayed moderate crescent formation and adhesion of capillary walls to Bowman’s capsule (Fig 3A) when compared with saline treated animals, as assessed by observation of PAS-stained sections. In comparison with the saline-treated group, the crescent formation and adhesion in the anti-GBM nephritic glomeruli increased at 41 d (Fig. 4). In contrast, as seen in Fig. 3B (Photo) and Fig. 4, TJN-331 treatment at 2.0 mg/kg/d significantly inhibited the increase in the index. TJN-331 at a dose of 0.5 mg/kg/d and dipyridamole produced no obvious effects (Fig. 4).

Effects on α-Smooth Muscle Actin (SMA) and TGF-β1-Positive Areas

Only trace expression of SMA was visualized by staining in most of the glomeruli of the saline-treated group. In anti-GBM nephritis, the immunostained area increased markedly. TJN-331 treatment at a dose of 2.0 mg/kg/d significantly inhibited the increase in the SMA-positive area. Dipyridamole treatment at a dose of 300 mg/kg/d also inhibited the increase in SMA-positive area (Fig. 5A).

The expression of immunostainable TGF-β1 was observed in untreated rats with anti-GBM nephritis and was significantly reduced by the administration of TJN-331 at doses of 0.5 and 2.0 mg/kg/d (Fig. 5B). Dipyridamole failed to inhibit
the increase in the TGF-β1-positive area.

**Effects on TGF-β1 Protein Production** Total glomerular TGF-β1 production in the saline-treated group was approximately 1200 pg/ml, while total glomerular TGF-β1 production with anti-GBM nephritis was significantly higher (approx. 3000 pg/ml). TJN-331 administration showed a tendency to reduce this (Fig. 6). On the other hand, the glomerular production of mature TGF-β1 in the saline-treated group was approximately 500 pg/ml. Glomerular TGF-β1 production in the anti-GBM nephritic group was significantly increased, to 1000 pg/ml. TJN-331 at a dose of 2.0 mg/kg inhibited the increase in glomerular production of mature TGF-β1. Dipyridamole did not prevent mature TGF-β1 production (Fig. 6).

**In Vitro Effects on TGF-β1 Production** In the anti-GBM nephritic glomeruli, the addition of TJN-331 to the culture medium dose-dependently inhibited total and mature TGF-β1 production (Fig. 7A).

**In Vitro Effects on TGF-β1 and TGF-β1 Signal Transduction-Associated Gene Expression** The mRNA expression of TGF-β1 in anti-GBM nephritic glomeruli was significantly increased, compared with saline-treated rats. However, administration of TJN-331 failed to inhibit the increase in TGF-β1 mRNA expression (Fig. 7B). In addition, as seen in Fig. 8, decreased expression of Smad 2 or Smad 3 mRNA in nephritic glomeruli was observed, compared with saline-treated rats. Addition of TJN-331 to the culture medium significantly reversed it. Plasminogen mRNA expression in glomeruli was not altered by anti-GBM serum injection, but TJN-331 significantly inhibited the expression of plasminogen mRNA.
DISCUSSION

The present study demonstrates that administration of TJN-331 improves the proteinuria and histopathological changes in glomeruli of anti-GBM nephritic rats and the mechanism of action of TJN-331 may involve inhibition of TGF-β1 production or activation. These results indicate that TJN-331 may be effective for glomerulonephritis.

In this study, we showed that the addition of TJN-331 to cultured glomeruli derived from nephritic animals inhibited the increase in TGF-β1 protein production, but not mRNA expression. Poncelet et al.\(^\text{(12)}\) demonstrated that expression of Smad 2 and 3 mRNA in cultured human mesangial cells was decreased until 24—48 h after treatment with TGF-β1. In our study, Smad 2 and 3 mRNA expression was decreased in nephritic glomeruli compared with the saline-treated group. Poncelet et al.\(^\text{(12)}\) and our findings indicated that decrease in Smad 2 or 3 mRNA gene expression in nephritic glomeruli may be a negative feedback loop via activation of TGF-β1 signaling in nephritic glomeruli. Because TJN-331 also inhibited the decrease in expression of Smads mRNA, TJN-331 may block the increase in TGF-β1 protein content or maturation of TGF-β1 in the cultured glomeruli rather than an inhibition of signal transduction directly.

The detailed mechanism of TJN-331 inhibition of TGF-β1 production remains unclear. It is well known that plasmin\(^\text{(13,14)}\) has been identified as activating TGF-β1 under in vitro conditions. Latent TGF-β1 is stabilized by dimerization...
of the amino-terminal glycopeptides and further disruption of the amino-terminal glycopeptides releases mature, biologically activated TGF-β1. Plasmin has been shown to activate latent TGF-β1 from several sources. TJN-331 inhibited the expression of plasminogen mRNA in nephritic glomeruli. A possible mechanism of TJN-331 on TGF-β1 activation may involve intraglomerular plasmin synthesis. Additionally, we determined the gene expression of thrombospondin-1, a representative TGF-β1 activator,15) in nephritic glomeruli. TJN-331 failed to inhibit thrombospondin-1 mRNA (data not shown). Further investigation to clarify the action mechanisms of TJN-331 is necessary.

Our results demonstrated that TJN-331 prevented proteinuria and histopathological changes, such as crescent formation and adhesion. Additionally, administration of TJN-331 inhibited the increase in glomerular TGF-β1-positive area and mature TGF-β1 protein production from anti-GBM nephritic glomeruli. Together with the results of the in vitro assay, these results indicate that TJN-331 ameliorated anti-GBM nephritis, via inhibition of TGF-β1 production, leading to reduced extra capillary proliferation (e.g., crescent formation and adhesion) in glomeruli.

The reason for increase in urinary protein excretion may be mediated by an increase in TGF-β1 in nephritic rats remains poorly understood. There are no reports clarifying the effects of TGF-β1 on proteinuria in a nephritic condition. In the present study, we found that TJN-331 already inhibited proteinuria 5 d after initiation of administration. Increase in proteinuria is one of the most important factors influencing the expression of the TGF-β1 gene in tubular epithelial cells.16) These observations suggest that the effects of TJN-331 on proteinuria may be mediated by an improvement in the function of tubular cells, due to reduced histopathological damage, as a result of inhibiting TGF-β1 production.

In Japan, dipyridamole is widely used as a therapeutic drug for glomerulonephritis. It is believed that treatment with dipyridamole may have antiplatelet action. However, treatment with dipyridamole was not effective in this protocol, although we have previously reported the efficacy of dipyridamole in this model.9) In this experiment, we administered test drugs from day 21 to day 41 after induction of anti-GBM nephritis, and proliferative changes in glomeruli had already been induced, but intraglomerular acute inflammation may have gradually decreased. Thus, we suggest that if dipyridamole had been administered since initiation of nephritis, dipyridamole would have been more effective in this model. There is no prior evidence that dipyridamole acts as a TGF-β1 synthesis inhibitor. Previously established development of renal injury may have been primarily mediated not by platelet activation but by TGF-β1, leading to the accumulation of extracellular matrix.

In summary, administration of TJN-331 was more effective against anti-GBM nephritis, than dipyridamole. A possible mechanism of action of TJN-331 may involve inhibition of plasminogen gene expression, followed by inhibiting plasmin production.

REFERENCES