Multi-glycoside of *Tripterygium wilfordii* Hook. f. reduces proteinuria through improving podocyte slit diaphragm dysfunction in anti-Thy1.1 glomerulonephritis

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Podocyte
Slit diaphragm
Nephrin
Podocin

**Abstract**

*Ethnopharmacological relevance:* Multi-glycoside of *Tripterygium wilfordii* Hook. f. (GTW) has been proved clinically effective in reducing proteinuria in chronic kidney disease in China. However, the mechanisms involved are still unclear. In this study we examined the effects of GTW at the different dosages on proteinuria and podocyte slit diaphragm (SD) dysfunction in anti-Thy1.1 glomerulonephritis (GN).

**Materials and methods:** Rats with anti-Thy1.1 GN were divided into 2 groups, a GTW group and a vehicle group, and sacrificed at 30 min, on day 7, and on day 14 in Experiments 1, 2 and 3, respectively. The administration of GTW at the moderate and high doses was started 3 days before or at the same time of antibody injection till sacrifice. Proteinuria was determined in Experiments 1, 2, and 3. After sacrifice, the staining intensity of SD-associated key functional molecules including nephrin and podocin, podocyte structure, mesangial change, macrophage infiltration, and blood biochemical parameters were examined, respectively. Protein and mRNA expressions of nephrin and podocin in glomeruli were also investigated. Besides, liver histological characteristics were analyzed.

**Results:** In Experiment 1, GTW pretreatment at the medium dose (75 mg/kg body weight) caused no influence on the induction of anti-Thy1.1 GN and the basal nephrin expression. In Experiment 2, the high dosage (100 mg/kg body weight) of GTW ameliorated proteinuria, the distribution of nephrin and podocin, mesangial proliferation, and the activated macrophage accumulation, as compared with vehicle group \((P<0.05)\). Additionally, it increased mRNA and protein expressions of nephrin and podocin in glomeruli on day 7, but had no influence on podocyte structure. In Experiment 3, the medium dosage (75 mg/kg body weight) of GTW improved proteinuria, the partial matrix expansion, and the distribution of nephrin and podocin on day 14, as compared with anti-Thy1.1 GN rats \((P<0.05)\). GTW at the high or moderate dose did not affect hepatic function on day 7 and on day 14.

**Conclusions:** Podocyte SD dysfunction, such as the disordered distribution and down-regulation of nephrin and podocin expression, is critically involved in the pathogenesis of anti-Thy1.1 GN induced by mAb 1-22-3. The restoration of the distribution and expression of nephrin and podocin by GTW could be an important mechanism by which GTW ameliorates proteinuria and podocyte SD dysfunction.

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**Abbreviations:** GTW, multi-glycoside of *Tripterygium wilfordii* Hook.f.; anti-Thy1.1 GN, anti-Thy1.1 glomerulonephritis; CKD, chronic kidney disease; SD, slit diaphragm; MsPGN, mesangial proliferative glomerulonephritis; mAb, monoclonal antibody; PAN, puromycin aminonucleoside; BW, body weight; KW, kidney weight; \(\alpha\)-SMA, \(\alpha\)-smooth muscle actin; PB, phosphate buffer; PAS, periodic acid-Schiff; EM, electron microscopy; BUN, blood urea nitrogen; Scr, serum creatinine; IF, immunofluorescence; LM, light microscopy; BSA, bovine serum albumin; FITC, fluorescein isothiocyanate; cDNA, complementary DNA; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PBS, phosphate-buffered saline; PI, protease inhibitors; EDTA, ethylenediaminetetraacetic acid; RT-PCR, reverse transcription polymerase chain reaction.

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1. Introduction

Proteinuria is the primary clinical manifestation in early chronic kidney disease (CKD), including mesangial proliferative glomerulonephritis (MsPGN), focal segmental glomerular sclerosis, minimal change disease, and membranous nephropathy (de et al., 2005; Palmer, 2007; Mathieson, 2005). Anti-Thy1.1 glomerulonephritis (GN), which is characterized by the significant proteinuria and the progressive glomerular lesion, is a widely used animal model for the clarification of pathogenic factors involved in MsPGN (Bagchus et al., 1986; Shimizu et al., 1999). It has been recognized that the dysfunction of slit diaphragm (SD), which bridges the neighboring foot processes of glomerular visceral epithelial cell (podocyte), is also a critical pathogenic factor implicated in the development of proteinuria (Kretzler et al., 1994; Sawai et al., 2003; Matsuoka et al., 2005). The alteration in two important podocyte SD-associated molecules, nephrin and podocin, has been shown to be closely associated with proteinuria states in MsPGN (Han et al., 2003; Schaefer et al., 2004; Migliori et al., 2005). They have been used as the markers of podocyte SD function in vivo.

Tripterygium wilfordii Hook. f. is a medicinal plant used for various immune and inflammatory diseases in China. One of the extracts from this plant is a stable glycoside, known as mita-glycoside of Tripterygium wilfordii Hook. f. (GTW) (Qian et al., 1995; Ma et al., 2007; Brinker et al., 2007). GTW has been approved by the China State Food and Drug Administration (Z320210077) for the treatment of rheumatoid arthritis and glomerulonephritis. The processing of the product was subjected to strict quality control, and the main bioactive components, specified in Table 1 were subjected to standardization. For recent 30 years, GTW has been applied extensively in China for the treatment of proliferative glomerulonephritis (Li, 1981; Li and Liu, 2003; Wan et al., 2003). In the previous studies, we have described that GTW dose-dependently ameliorated the injurious process such as proteinuria and mesangial injury in two types of anti-Thy1.1 GN model induced by monoclonal antibody (mAb) 1-22-3. In addition, we have revealed that the effect of GTW is closely related to its actions on the several inflammatory cytokines, such as transforming growth factor (TGF)-β, platelet-derived growth factor (PDGF), monocyte chemoattractant protein (MCP)-1, interleukin (IL)-2, and interferon (IFN)-γ (Wan et al., 2005, 2010). Interestingly, a recent study demonstrated that Triptolide, one of major active components of GTW, also ameliorated podocyte injury and proteinuria in puromycin aminonucleoside (PAN)-induced rat nephropathy (Zheng et al., 2008). Because there were no the obvious inflammatory responses in this model of glomerular disease, the effect of GTW on proteinuria could be mediated by its direct action on podocyte. Therefore, it would be interesting to examine whether GTW could have an effect on podocyte SD dysfunction in anti-Thy1.1 GN.

In this study, we investigated the distribution and expression of nephrin and podocin, as well as its correlation with proteinuria and mesangial lesion in an acute rat model of anti-Thy1.1 GN. Furthermore, we examined whether the beneficial effect of GTW at the different dosages on proteinuria could be related to its actions on these molecules.

2. Materials and methods

2.1. Animals, drug, and anti-Thy1.1 mAb

All experiments were performed using female Wistar rats weighing from 190 to 220 g, purchased from the Experimental Animal Center of The Affiliated Hospital of Nanjing University Medical School (Nanjing, China). The experimental protocol was approved by the Animal Ethics Committee of Nanjing University Medical School. GTW was kindly provided by Jiangsu Meitong Pharmaceutical Co., Ltd. of Jiangsu Province (Taizhou, China). It was solubilized in distilled water and administrated via gavage. The mAb 1-22-3 (IgG3) and other important antibodies were given by Drs. Fujio Shimizu and Hiroshi Kawachi (Department of Cell Biology, Institute of Nephrology, Niigata University Graduate School of Medical and Dental Sciences, Niigata, Japan).

2.2. Experimental protocol

2.2.1. Experiment 1

The objective of Experiment 1 was to analyze whether GTW pre-treatment at the medium dose interferes with the binding of mAb 1-22-3 to glomeruli, the fixation of complement, and the expression of nephrin in glomeruli. Eight rats were each given 75 mg/kg body weight (BW) of GTW or a vehicle (distilled water) from 3 days before the injection of 500 μg of mAb 1-22-3, and sacrificed 30 min after the injection. Temporary urine samples were collected from the injection of mAb 1-22-3 to sacrifice. Urine protein concentrations were determined by colorimetric dye-binding assay (Bio-Rad, Oakland, CA, USA) using bovine serum albumin (BSA) as a standard. After cardiac puncture, kidneys were removed to be stained with fluorescein isothiocyanate (FITC)-conjugated anti-mouse IgG3 (Dakopatts, Glostrup, Denmark) to detect bound mAb 1-22-3, or with FITC-conjugated anti-rat C3 (Cappel, West Chester, PA, USA), or FITC-conjugated anti-rabbit immunoglobulins (for anti-nephrin) (Dakopatts, Glostrup, Denmark).

2.2.2. Experiment 2

Fourteen rats were randomly divided into two groups (n=7 rats/group) and orally given 100 mg/kg BW of GTW (high dose) or the vehicle (distilled water, 5 ml/kg) from 3 days before the injection of 500 μg of mAb 1-22-3, respectively. MsPGN was induced by a single injection with 1 ml saline containing 500 μg of mAb 1-22-3. Twenty-four-hour urine samples were collected before and on days 1, 3, 5, and 7 after the injection of mAb 1-22-3. Afterward weighing BW of each rat, all animals were sacrificed on day 7. The blood was drawn from the heart. Serum creatinine (Scr), blood urea nitrogen (BUN), alanine aminotransferase (ALT), and aspartate aminotransferase (AST) levels were measured. Kidneys were removed, weighed, cut into portions and used for an examination by a light microscopy (LM) or immunofluorescence (IF) or electron microscopy (EM), and for the preparation of glomerular RNA. Additionally, a part of liver was extracted for the histological investigation. Semi-quantitative RT-PCR and Western blot were carried out on glomerular mRNA and protein of each rat for nephrin and podocin. Moreover, the infiltrative macrophages in glomeruli were determined by IF staining using specific mAbs against rat macrophages antigens. Five rats without injection of mAb 1-22-3 were used as the normal control.

2.2.3. Experiment 3

Most procedures were carried out as Experiment 2, but 14 rats were orally given 75 mg/kg BW of GTW (medium dose) or vehicle after the injection of 500 μg of mAb 1-22-3, respectively. Twenty-four-hour urine samples were collected before and on days 1, 3, 5, 7, 10, and 14 after the injection of mAb 1-22-3. All rats were sacrificed on day 14 after the induction of GN. The blood was drawn from

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**Table 1** The main bioactive ingredients of GTW.

<table>
<thead>
<tr>
<th>Chemical name</th>
<th>Formula</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Triptolide</td>
<td>C20H24O6</td>
<td>Kupchan et al. (1972)</td>
</tr>
<tr>
<td>Triptonide</td>
<td>C20H22O6</td>
<td>Kupchan et al. (1972)</td>
</tr>
<tr>
<td>Triptolide</td>
<td>C20H24O6</td>
<td>Kupchan et al. (1972)</td>
</tr>
<tr>
<td>Triptohaeric acid</td>
<td>C21H28O3</td>
<td>Nakano et al. (1998)</td>
</tr>
</tbody>
</table>
the heart. Scr, BUN, ALT, and AST levels were measured as well. Kidneys were removed, weighed, cut into portions and used for an assessment by LM or IF, and then, for examining the distribution of nephrin and podocin in glomeruli.

2.3. Light microscopy examination

Renal and liver tissue samples for light microscopic assessment were fixed with 10% neutral buffered formalin, embedded in paraffin, cut into 3 μm sections and stained with a periodic acid-Schiff (PAS) or a hematoxylin and eosin (HE) reagent. Semiquantitative morphological studies of glomerular lesion were carried out by randomly selecting 30 full-sized glomeruli (80–100 μm) from each specimen. The sections were analyzed in a double-blinded manner and the degree of glomerular mesangial matrix expansion was scored from 0 to 4+ according to the percentage of glomerular involvement, as described by Raji et al. (1984). The total number of cells in glomeruli was also counted in a blinded protocol and computed for 30 glomeruli for each kidney.

2.4. Electron microscopy investigation

Renal tissue samples for electron microscopic assessment were fixed in 2.5% glutaraldehyde in 0.1 mol/L phosphate buffer (PB) for several days at 4°C. After washing in PB and post-fixing in 1% OsO4 for 2 h, the fixed material was dehydrated through an ethanol-propylene oxide series and embedded in Araldite M. The ultrathin sections were prepared and stained with uranyl acetate and lead citrate, and then, investigated and photographed under a JEOL JEM-1011 transmission electron microscope (Tokyo, Japan).

2.5. Immunofluorescence assay

Renal tissue samples for IF studies were snap-frozen in pre-cooled n-hexane and stored at −70°C. Frozen sections were cut into 3 μm thick with a cryostat and stained with anti-collagen type I (Chemicon, Temecula, CA, USA) or anti-α-smooth muscle actin (anti-α-SMA; IgG2a; Sigma, St. Louis, MO, USA) antibodies. FITC-conjugated anti-rabbit IgG and FITC-conjugated anti-mouse IgG2a (Southern Biotechnology Associates, Birmingham, AL, USA) were used as secondary antibodies (Dako A/S). The degree of collagen type I and α-SMA staining was scored from 0 to 4+ in 30 randomly selected glomeruli according to the method described by Floege et al. (1998).

In order to evaluate the degree of podocyte SD dysfunction, the cryostat sections were incubated with rabbit anti-α-nephrin and rabbit anti-rat podocin (Southern Biotechnology Associates, Inc., Birmingham, AL, USA), and then, stained with FITC-conjugated anti-rabbit immunoglobulins. After that, we scored the staining patterns of nephrin and podocin in accordance with the method described by Macconi et al. (2000). A score was assigned to each individual glomerulus in the tissue section. The score 0, 0.5, and 1.0 were used, respectively, for the continuous distribution along glomerular capillary wall, the heterogeneous distribution along glomerular membrane, and the markedly discontinuous distribution. The final score per section was then calculated as weighted mean: score = Σ(N1 × 0 + N2 × 0.5 + N3 × 1)/(N1 + N2 + N3), where N1, N2, and N3 (i = 1–3) is the number of glomeruli in each category. The score were assigned blindly. On average, more than 100 glomeruli per section were evaluated.

The presence of macrophage in glomeruli was determined by the specific mAbs against rat macrophage antigens. ED1 (IgG1, reactive with pan monocytes/macrophages) and ED3 (IgG2a, reactive with macrophage sialoadhesin) were purchased from Chemicon International Inc. and Serotec, respectively. FITC-conjugated goat anti-mouse IgG1 (Southern Biotechnology Associates) and FITC-conjugated goat anti-mouse IgG2a were used as secondary antibodies. The number of mononuclear cells per glomerular cross section (c/gcs) was counted in 30 randomly selected full-sized glomeruli by an observer who was unaware of the experimental protocol.

2.6. Reverse transcription polymerase chain reaction (RT-PCR) study

Glomeruli were isolated by graded sieving of more than 95% purity using the technique described by Greenspon and Krakower (1950). Isolated glomeruli were immediately disassociated by guanidinium and phenol extraction (TRIZOL; Gibco BRL, Gaithersburg, MD, USA). Complementary DNA (cDNA) was synthesized using a commercial kit (Superscript Preamplification System; Gibco BRL) following the standard protocol. PCR was performed with the following primers: (1) 5′ primer, CTG AGG CTG AAG CTT CTC, and 3′ primer, AAG AGC ACA GCC AGG GC for nephrin (Kawachi et al., 2000); (2) 5′ primer, CCT GTG AGT GGC TTC TTG TTC TC, and 3′ primer, GGA GAC GCC TCA TAG TGG TTT GCA for podocin (Kawachi et al., 2003). Amplification was carried out using the PC-800 programmable temperature control system (Astec, Fukuoka, Japan) through 30–40 cycles of denaturation at 95°C for 30 s, annealing at individual temperatures for 30 s, and extension at 72°C for 1 min. The optimum number of amplification cycles used for the semiquantitative PCR was chosen based on the preliminary trial in the linear phase of amplification. Amplification of the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a positive control for intact RNA and for measuring the efficiency of RT. The PCR products were electrophoresed on 1.2% agarose gel containing 0.0001% ethidium bromide in TAE buffer, and band intensities were determined by image analysis using a Macintosh computer and the Densitometry program (Densitograph, ATTO, Tokyo, Japan). All results were corrected for the amount of mRNA of GAPDH as an internal standard. Data are shown as ratios relative to the normal rat expression, and are expressed as mean ± S.E. of these independent experiments.

2.7. Semiquantitative Western blot analysis

Western blot analysis with sequentially solubilized glomerular lysates was performed basically according to the method previously described (Kawachi et al., 2000, 2003). Glomeruli from the rats of anti-Thy-1.1 GN (day 7) were isolated with phosphate–buffered saline (PBS) including protease inhibitors (PI) and sequentially solubilized with 1% Triton X–100, RIPPA buffer (0.1% SDS, 1% sodium deoxycholate, 1% Triton X–100, 0.15 mol/L NaCl, and 0.01 mol/L ethylenediaminetetraacetic acid (EDTA) in 0.025 mol/L Tris–HCl, pH7.2) with PI, and separated into Triton X–100-soluble (T), RIPPA-soluble (R), and RIPPA-insoluble (S) fractions. Equal amounts of these sequentially solubilized fractions were subjected to SDS-PAGE with 7.5% or 10% acrylamide gel, according to the method of Laemmli (1970) and transferred to a PVDF membrane (Bio-Rad) by electrophoretic transblotting for 30 min using Trans-Blot SD (Bio-Rad). After blocking with BSA, the strips of membrane were exposed to rabbit anti-α-nephrin, anti-α-rat podocin, or anti-α β-actin antibody purchased from Sigma–Aldrich (St. Louis, MO, USA). They were washed and incubated with alkaline phosphatase-conjugated anti-rabbit immunoglobulins (Bio Source International, Tago Immunologicals, Camarillo, CA, USA). The reaction was developed with an alkaline phosphatase chromogen kit (5-bromo-4-chloro-3-indolyl phosphate p-toluidine salt/ nitro blue tetrazolium; Biomedica, Foster City, CA, USA). The density of the positive bands was quantitated by Densitograph (ATTO, Tokyo, Japan). This procedure was carried out using the technique described by Greenspon and Krakower (1950). Isolated glomeruli were immediately disassociated by guanidinium and phenol extraction (TRIZOL; Gibco BRL, Gaithersburg, MD, USA). Complementary DNA (cDNA) was synthesized using a commercial kit (Superscript Preamplification System; Gibco BRL) following the standard protocol. PCR was performed with the following primers: (1) 5′ primer, CTG AGG CTG AAG CTT CTC, and 3′ primer, AAG AGC ACA GCC AGG GC for nephrin (Kawachi et al., 2000); (2) 5′ primer, CCT GTG AGT GGC TTC TTG TTC TC, and 3′ primer, GGA GAC GCC TCA TAG TGG TTT GCA for podocin (Kawachi et al., 2003). Amplification was carried out using the PC-800 programmable temperature control system (Astec, Fukuoka, Japan) through 30–40 cycles of denaturation at 95°C for 30 s, annealing at individual temperatures for 30 s, and extension at 72°C for 1 min. The optimum number of amplification cycles used for the semiquantitative PCR was chosen based on the preliminary trial in the linear phase of amplification. Amplification of the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a positive control for intact RNA and for measuring the efficiency of RT. The PCR products were electrophoresed on 1.2% agarose gel containing 0.0001% ethidium bromide in TAE buffer, and band intensities were determined by image analysis using a Macintosh computer and the Densitometry program (Densitograph, ATTO, Tokyo, Japan). All results were corrected for the amount of mRNA of GAPDH as an internal standard. Data are shown as ratios relative to the normal rat expression, and are expressed as mean ± S.E. of these independent experiments.
Fig. 1. Effects of GTW pretreatment at the medium dose (75 mg/kg BW) on the induction of anti-Thy1.1 glomerulonephritis (GN) and the basal expression of nephrin. Representative immunofluorescence findings of monoclonal antibody (mAb) 1-22-3 (A and B), C3 (C and D) and nephrin (E and F) in glomeruli (original magnification, 200×).

out three times. The ratio of densitometric signal of the molecules examined to that of β-actin was determined. Data are shown as ratios relative to control findings and expressed as mean ± S.E. of these independent experiments.

2.8. Statistical analysis

Values were expressed as mean ± S.E. Statistical significance was evaluated using the Mann–Whitney U test. A result was considered significant if Pvalue was <0.05 using Stat View for Windows (Abacus Concepts, Berkeley, CA, USA).

3. Results

3.1. Effects of GTW pretreatment on the induction of anti-Thy1.1 GN and the basal expression of nephrin

In Experiment 1, no difference in the staining intensity of bound mAb 1-22-3, rat C3, or nephrin in glomeruli was observed between the rats pretreated with GTW at the medium dose (75 mg/kg BW) (Fig. 1B, D, and F) and with vehicle (Fig. 1A, C, and E). Hence, there was no the negative influence of GTW pretreatment on the induction of Thy1.1 GN and the basal expression of nephrin.

3.2. Effects of the high dose of GTW on proteinuria, the distribution of nephrin and podocin, mesangial proliferation, podocyte structure, and the expression of nephrin and podocin in anti-Thy1.1 GN on day 7

In Experiment 2, the significant reduction of proteinuria on days 1, 5 and 7 was observed in the group treated by GTW at the high dosage (100 mg/kg BW) during 7 days (Table 2). Scr, BUN, BW and kidney weight (KW) of the rats were summarized in Table 3. GTW treatment slightly reduced serum levels of BUN on day 7. The BW and KW of the rats at the time of sacrifice (day 7) were not greatly altered by GTW. The representative findings of glomeruli
in the rats treated with or without GTW were shown (total cell number/30 glomerular cross sections; GTW group, 70.12 ± 2.31 vs. vehicle group, 87.55 ± 2.31, \( P < 0.05 \). matrix expansion; GTW group, 1.83 ± 0.21 vs. vehicle group, 2.22 ± 0.29, \( P < 0.05 \). α-SMA expression; GTW group, 1.93 ± 0.28 vs. vehicle group 2.30 ± 0.33, \( P < 0.05 \)).

Mesangial cell proliferation and matrix expansion were detected in vehicle group (Fig. 2B), whereas light micrograph findings of GTW group appeared to be ameliorated (Fig. 2C). The intense staining of α-SMA was detected in mesangial area in vehicle group (Fig. 2E), whereas it was decreased in GTW group (Fig. 2F). Fig. 2A and D

Table 2

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Pre</th>
<th>Day 1</th>
<th>Day 3</th>
<th>Day 5</th>
<th>Day 7</th>
</tr>
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<tbody>
<tr>
<td>Normal</td>
<td>5</td>
<td>3.91 ± 1.40</td>
<td>3.40 ± 1.67</td>
<td>4.21 ± 1.74</td>
<td>3.90 ± 0.93</td>
<td>3.38 ± 0.93</td>
</tr>
<tr>
<td>Vehicle</td>
<td>7</td>
<td>4.01 ± 1.45</td>
<td>57.01 ± 15.44</td>
<td>191.92 ± 52.86</td>
<td>156.58 ± 60.39</td>
<td>97.21 ± 16.43</td>
</tr>
<tr>
<td>GTW</td>
<td>7</td>
<td>3.67 ± 1.26</td>
<td>46.51 ± 31.61*</td>
<td>122.14 ± 43.11</td>
<td>96.27 ± 57.23*</td>
<td>58.83 ± 19.58*</td>
</tr>
</tbody>
</table>

\* \( P < 0.05 \) vs. vehicle group. The data are expressed as mean ± S.E.
Table 3
Effects of GTW at the high dose (100 mg/kg body weight) on serum creatinine, serum blood urea nitrogen, body weight, and kidney weight in anti-Thy1.1 glomerulonephritis on day 7.

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Scr (mg/dl) ± S.E.</th>
<th>BUN (mg/dl) ± S.E.</th>
<th>BW (g) ± S.E.</th>
<th>KW (g) ± S.E.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>5</td>
<td>0.31 ± 0.05</td>
<td>25.22 ± 1.50</td>
<td>210.20 ± 6.42</td>
<td>0.87 ± 0.04</td>
</tr>
<tr>
<td>Vehicle</td>
<td>7</td>
<td>0.30 ± 0.04</td>
<td>31.69 ± 3.84a</td>
<td>215.00 ± 7.07</td>
<td>0.91 ± 0.05</td>
</tr>
<tr>
<td>GTW</td>
<td>7</td>
<td>0.30 ± 0.04</td>
<td>21.84 ± 3.83b</td>
<td>212.86 ± 6.99</td>
<td>0.90 ± 0.07</td>
</tr>
</tbody>
</table>

Abbreviations: Scr, serum creatinine; BUN, serum blood urea nitrogen; BW, body weight; KW, kidney weight. The data are expressed as mean ± S.E.

a $P < 0.05$ vs. normal group.
b $P < 0.01$ vs. vehicle group.

Fig. 4. Effects of GTW at the high dose (100 mg/kg body weight) on the distribution of nephrin and podocin in anti-Thy1.1 glomerulonephritis (GN) on day 7. The panel showed the staining scores for nephrin and podocin. The data are expressed as mean ± S.E., and $** P < 0.01$ vs. vehicle group.

shows the findings of the normal rats without the injection of mAb 1-22-3.

The staining of nephrin (Fig. 3A) and podocin (Fig. 3D) in normal glomeruli was observed clearly and continuously like a linear pattern on podocyte. However, in vehicle group (Fig. 3B and E), the staining of both molecules was weak and discontinuous in almost all glomeruli on day 7. In contrast, GTW treatment kept nephrin and podocin staining at near normal levels (Fig. 3C and F). The scores for nephrin (GTW group, 0.40 ± 0.05 vs. vehicle group, 0.66 ± 0.09, $P < 0.01$) and podocin (GTW group, 0.37 ± 0.10 vs. vehicle group, 0.60 ± 0.07, $P < 0.01$) staining were summarized in Fig. 4. The staining patterns were scored in accordance with the methods described by Macconi et al. (2000). In addition, GTW treatment also ameliorated glomerular accumulation of the activated ED3+ macrophages (GTW group, 2.98 ± 0.40 vs. vehicle group, 5.13 ± 0.63 cells/cross section, $P < 0.05$).

Electron microscopy investigation showed no podocyte shape changes such as edema cytoplasm and foot processes effacement in vehicle group (Fig. 5A) and GTW group (Fig. 5B) on day 7. Semiquantitative RT-PCR showed that the treatment of GTW up-regulated the expression of mRNA for podocin (16% to vehicle group, $P < 0.05$) together with the tendency to increase the expression of nephrin (22% to vehicle group) in glomeruli (Fig. 6). Consistently, Western blot analysis of extracts from isolated glomeruli also revealed that protein expression of nephrin...
and podocin was markedly up-regulated in GTW group on day 7 (Fig. 7).

Taken together, the high dosage of GTW ameliorated proteinuria, the reduction in the distribution and expression of nephrin and podocin at both mRNA and protein levels, and mesangial proliferation in anti-Thy 1.1 GN on day 7.

3.3. Effects of the medium dose of GTW on proteinuria, matrix expansion, and the distribution of nephrin and podocin in anti-Thy1.1 GN on day 14

In Experiment 3, the data of proteinuria during the course of 14 days were depicted in Table 4. Proteinuria emerged on day 1 after mAb 1-22-3 injection, reached a peak on day 3, was then persistent from day 5 to day 7, decreased on day 10, and was normalized on day 14. The significant reduction of proteinuria was observed in the group treated by the medium dosage (75 mg/kg BW) of GTW, compared with anti-Thy1.1 GN rats, during the whole course of the observation (14 days). The decreasing effects of GTW on proteinuria were persistent both on day 7 and on day 10 in Experiment 3.

The representative findings of glomeruli by LM and IF in the rats treated with or without GTW were shown in Fig. 8. The severe matrix expansion (Fig. 8A) and the intense staining of collagen type I (Fig. 8C) were detected in vehicle group, but attenuated in GTW group (Fig. 8B and D) (matrix expansion; GTW group, 1.78 ± 0.28 vs. vehicle group, 2.17 ± 0.32, P < 0.05. collagen type I expression; GTW group, 1.68 ± 0.28 vs. vehicle group, 2.12 ± 0.16, P < 0.05). Consequently, proteinuria was also ameliorated in GTW group except for the partial mesangial matrix expansion. No difference in the staining intensity of nephrin (Fig. 8E and F) and podocin (Fig. 8G and H) in glomeruli was observed between the rats treated with GTW (Fig. 8F and H) and with vehicle (Fig. 8E and G) on day 14.

In short, the medium dosage of GTW improved proteinuria and the partial matrix expansion in glomeruli, and that it promoted the recovery of the distribution of nephrin and podocin in anti-Thy 1.1 GN on day 14.

3.4. Side effects of the high or medium dose of GTW on liver tissue

In Experiments 2 and 3, the data of liver cell lesions in clinical practice, including serum ALT and AST on day 7 and on day 14 were examined, respectively. Additionally, we investigated, by LM, the histological characteristics of liver tissue in the rats treated with GTW (Fig. 9B) at the high dosage (100 mg/kg BW) and without GTW (Fig. 9A). Our results showed that, after GTW treatment for 7 days, ALT in GTW group and vehicle group was 52.61 ± 6.48 U/L and 49.26 ± 7.62 U/L, AST in GTW group and vehicle group was 59.59 ± 10.59 U/L and 45.69 ± 7.68 U/L, respectively. After 14 days, ALT in GTW group and vehicle group was 49.06 ± 17.58 U/L and 39.51 ± 8.59 U/L, AST in GTW group and vehicle group was 56.27 ± 13.63 U/L and 45.98 ± 7.68 U/L, respectively.

The histological findings of liver tissue in the rats treated with GTW at the high dosage in Experiment 2 showed there was no marked pathological change except for the local inflammatory cell accumulation around hepatic lobules and bile ducts (Fig. 9B). So, GTW at the concentrations used for this investigation did not affect hepatic function in anti-Thy 1.1 GN on day 7 and on day 14.

3.5. Kinetic characteristics of mesangial alteration, the distribution of nephrin, and urinary protein at 30 min, on day 7, and on day 14 after the induction of anti-Thy 1.1 GN

The kinetic characteristics of mesangial alteration, the distribution of nephrin, and urinary protein at different time points after the induction of anti-Thy1.1 GN is shown in Fig. 10. At 30 min following the injection of mAb 1-22-3, only slight urinary protein could be detected (Fig. 10G). No changes in mesangium (Fig. 10A) and distribution of nephrin (Fig. 10D) were observed. On day 7, urinary protein reached peak (Fig. 10C), which was associated with the marked mesangial proliferation (Fig. 10B) and the obvious discontinuous distribution of nephrin (Fig. 10E). On day 14, urinary protein returned to normal level (Fig. 10G) through GTW treatment. Concomitantly, nephrin distribution also returned too near basal level (Fig. 10F). Interestingly, the mesangial lesion (Fig. 10C) was still marked at this time, in spite of some amelioration in GTW group. These results suggested that there is no direct causal relationship between SD dysfunction and mesangial lesion in this model.
Fig. 8. Effects of GTW at the medium dose (75 mg/kg body weight) on matrix expansion and the distribution of nephrin and podocin in anti-Thy1.1 glomerulonephritis (GN) on day 14. Photomicrographs of PAS staining (A and B), immunofluorescence micrographs of collagen type I (C and D), nephrin (E and F), and podocin (G and H) (original magnification, 200×).
as an interaction with nephrin (Tryggvason et al., 2006). As two key functional molecules in podocyte SD, nephrin is located at the outer leaflet of plasma membranes of podocyte SD, while podocin is involved in the development of proteinuria in several models of MsPGN. In an anti-Thy1.1 GN induced by clone OX-7, the abnormal expression of nephrin and podocin was reported, which has been considered to be closely associated with the preservation of nephrin expression and distribution of nephrin and podocin in glomeruli. In other words, the anti-proteinuric effect of GTW was strongly related with the amelioration of SD dysfunction in anti-Thy1.1 GN induced by mAb 1-22-3.

In some kinds of proteinuric glomerular disease models, such as experimental MsPGN induced by mAb OX-7 (Mii et al., 2009), PAN-induced nephropathy (Zheng et al., 2008), and passive Heymann nephritis (Chen et al., 2010), there was the obvious foot process effacement with the decreased nephrin and podocin expression. However, we did not find any podocyte structural changes in this model of anti-Thy1.1 GN on day 7 in Experiment 2. So, we further asked how GTW could reduce proteinuria without affecting podocyte shape? This observation might demonstrate that the change in the abundance of nephrin and podocin in podocyte SD, but not that of podocyte structure, was responsible for the heavy proteinuria in this early glomerular disease induced by mAb 1-22-3. GTW might reduce proteinuria through elevating nephrin and podocin expression. Furthermore, in Experiment 1, we found that GTW pretreatment at the medium dose did not influence the basal level of nephrin expression at normal rats. It appears that it is less likely that GTW had a direct effect on nephrin expression.

In Experiments 2 and 3, our results showed that the treatment of GTW at the high and medium dosages promoted the recovery of the distribution and expression of nephrin and podocin. Moreover, the high dosage (100 mg/kg BW) of GTW decreased the intense staining of α-SMA and mesangial cell proliferation in glomeruli on day 7. The medium dosage (75 mg/kg BW) of GTW was also able to partially ameliorate theglomerular matrix expansion and the staining intensity of collagen type I on day 14. At present, it is unclear how the improvement in mesangial lesion by GTW could affect the restoration of the distribution and expression of nephrin and podocin.
Some recent studies demonstrated that podocyte injury caused or exacerbated mesangial proliferation in anti-Thy1.1 GN model (Sawai et al., 2003) and in glomerular sclerosis model (Matsusaka et al., 2005). Mii et al. (2009) also reported that, in early anti-Thy1.1 GN induced by mAb OX-7, angiotensin II receptor blockade (ARB) inhibited the acute proliferative glomerular lesions through the prevention of podocyte injuries such as the disordered distribution of nephrin and the effacement of foot process. Contrary to these reports, in this study, our results clearly showed that, there is the non-direct relationship between slit diaphragm dysfunction and mesangial lesion in anti-Thy1.1 GN model.

Macrophage plays an important pathogenic role in GN. The infiltration of macrophage in glomeruli is one of the hallmarks of various GN (Ikezumi et al., 2003; Minto et al., 2003). In which, the active ED3+ macrophages infiltrated in glomeruli were reported to be strongly correlated with mesangial damage (Ikezumi et al., 2003). Our results in this study showed that GTW treatment at the high dose (100 mg/kg BW) reduced the infiltration of the active ED3+ macrophage in glomeruli together with the amelioration of mesangial lesion in early anti-Thy1.1 GN induced by mAb 1-22-3. Hence, as to this experimental MsPGN model, the beneficial effect of GTW on mesangial lesion could be a result of the improved glomerular inflammation. Nevertheless, whether this effect of GTW on anti-inflammation is relevant to the attenuated podocyte injury remains unresolved. The connection between inflammatory cell infiltrated in glomeruli and podocyte injury has not been established yet. We supposed that GTW might have a direct regulative action on SD dysfunction in addition to anti-inflammation. A progressive glomerulonephritis model induced by the consecutive injections of PAN together with mAb 1-22-3 (Morioka et al., 2001) may be employed to clarify this point in future studies.

Previous studies have shown that the anti-proteinuria effects of GTW were dose-dependent in both human (Liu et al., 2009) and experimental glomerular nephritis (Wan et al., 2005). In this study, GTW at the dosages of 75 and 100 mg/kg BW similarly reduced proteinuria. This may be because that we have chosen a relatively high and effective concentration of GTW for this investigation. Based on our own experience, GTW, when used at the concentration more...
than 100 mg/kg BW in rats, induced adverse actions such as liver toxicity (Xu et al., 2009). Because of this reason, we have designated the therapeutic dosage at 100 mg/kg BW as high concentration, while the commonly used therapeutic concentration (75 mg/kg BW) as medium concentration in this study.

Notwithstanding the efficacy of GTW is confirmed in human clinical trials in China and abroad, unfortunately, its beneficial effects are always accompanied by the adverse events. It is reported that, serum ALT and AST was increased in various degree in some patients with CKD after GTW treatment for about 2 months (Xu et al., 2009). Although serum ALT and AST could return to normal after GTW treatment stop, the clinical applicable scope of GTW is undoubtedly limited because of this side effect. Consequently, as to GTW treatment in clinical practice, it is very important to mitigate the adverse effects on liver tissue.

Finally, to exclude the side effects of GTW on liver tissue at the dosages used in this investigation, we examined serum ALT, AST, and the histological characteristics of liver tissue. Our data showed that, GTW treatment at both high (100 mg/kg BW) and medium (75 mg/kg BW) dosages had no the obvious side actions on liver tissue within the period of this investigation. However, we believed that it seems to be a hasty conclusion if the side effect was denied because of this result in an acute rat model of anti-Thy-1.1 GN. The precise action of GTW on liver tissue requires a lot of studies in the further.

In conclusion, podocyte SD dysfunction, such as the disordered distribution and down-regulation of nephrin and podocin expression, is critically involved in the pathogenesis of anti-Thy-1.1 GN induced by mAb 1-22-3. The restoration of the distribution and expression of nephrin and podocin by GTW could be an important mechanism by which GTW ameliorates proteinuria and podocyte SD dysfunction.

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