Full Paper

Contrasting Dose–Effects of Multi-glycoside of *Tripterygium wilfordii* Hook. f. on Glomerular Inflammation and Hepatic Damage in Two Types of Anti-Thy1.1 GlomerulonephritisYi-Gang Wan¹, Qing Zhao², Wei Sun^{3,*}, Hui-Lan Zhang⁴, Min Li¹, Qing-Xue Wei², Wei Wu¹, Li-Jun Yue¹, and Qing Wang²¹Department of Traditional Chinese Medicine, Nanjing Drum Tower Hospital, The Affiliated Hospital of Nanjing University Medical School, Nanjing 210008, China²Department of Graduate School, Nanjing University of Chinese Medicine, Nanjing 210046, China³Department of Nephrology, Jiangsu Provincial Hospital of Chinese Medicine, Affiliated Hospital of Nanjing University of Chinese Medicine, Nanjing 210029, China⁴Oriental Academy of Chinese Medicine, Kuala Lumpur 58200, Malaysia

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Abstract. Multi-glycoside, one of the extracted compounds from *Tripterygium wilfordii* Hook. f. (GTW), has been shown to be clinically effective in suppressing glomerular inflammation in chronic kidney disease. However, its clinical application is often limited by its cytotoxic actions on the liver. This study was performed to contrast the dose–effects of GTW on glomerular inflammation and hepatic damage in two types of anti-Thy1.1 glomerulonephritis (GN). Rats with acute or chronic anti-Thy1.1 GN were either left untreated (the Vehicle group) or treated with a high or low dose of GTW and sacrificed on day 7 or day 45. GTW was administrated 3 days before or at the same time as the antibody injection and lasted until sacrifice. GTW at high dose ameliorated glomerular macrophage accumulation, mesangial proliferation, proteinuria, and interleukin-2 expression in the acute anti-Thy1.1 GN model, but caused structural and functional lesions in the liver. In contrast, GTW at low dose improved activated macrophage and T lymphocyte infiltration, mesangial injury, proteinuria, and interleukin-2 and interferon- γ expressions without hepatic toxicity in the chronic model of GN induced by anti-Thy1.1 antibody. In conclusion, GTW at low dose not only effectively inhibits glomerular inflammation but also avoids severe injuries to the liver.

Keywords: anti-Thy1.1 glomerulonephritis, multi-glycoside of *Tripterygium wilfordii* Hook. f. (GTW), macrophage, T lymphocyte, cytokine

Introduction

Tripterygium wilfordii Hook. f. (TWHF) is a woody vine native to Eastern and Southern China, Korea, Japan, and Taiwan (1, 2). In China, this plant has a long history of use in inflammatory diseases. One of the extracted compounds from TWHF is a stable glycoside, known as multi-glycoside of TWHF (GTW) (3, 4). GTW has been approved by the China State Food and Drug Administra-

tion (Z32021007) for the treatment of rheumatoid arthritis and nephritis. The processing of the product was subjected to strict quality control, and the main bioactive components (5, 6), such as *Triptolide* (C₂₀H₂₄O₆), *Triptonide* (C₂₀H₂₂O₆), *Triptiolide* (C₂₀H₂₄O₇), and *Triptohairic acid* (C₂₁H₂₈O₃), were subjected to standardization. For the last 30 years, GTW at the standard low dose of 0.5 – 1 mg/kg body weight (BW) daily has been applied extensively in China for the treatment of human chronic kidney disease (CKD), as an anti-inflammatory agent (7 – 9). Interestingly, in a former randomized trial of 79 patients with various kinds of proliferative glomerulonephritis (GN), a large dose (2 mg/kg BW daily) regime of

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GTW treatment markedly promoted the remission of glomerular inflammatory changes (10). Recent in vitro and in vivo studies showed that, *Triptolide*, one of the major active components of GTW, and GTW itself dose-dependently inhibit T lymphocyte and macrophage activation and secretion of inflammatory cytokines such as interleukin (IL)-2, interferon (IFN)- γ , and monocyte chemoattractant protein (MCP)-1 (11, 12) in the cultured T lymphocyte and in an early mesangial proliferative glomerulonephritis (MsPGN) model. It seems to suggest that GTW could suppress glomerular inflammatory responses in vivo and in vitro in a dose-dependent manner. However, the beneficial effects of GTW at an oral overdose in the kidney are always accompanied by adverse events in the liver. Xu et al. (13) reported that serum transaminase was increased in various degrees in some patients with CKD after GTW treatment at 1 mg/kg BW daily dose or higher for about 2 months. Admittedly, the clinical applicable scope of GTW is undoubtedly limited because of these unfavorable outcomes. Consequently, it is very important to find the suitable dose of GTW, at which GTW improves glomerular inflammation without causing liver injury. Notably, however, there are two questions remaining to be unanswered, namely, whether GTW at low dose could ameliorate glomerular inflammation effectively, as well as GTW at high dose, and whether GTW at high dose could cause severe structural and functional lesions in the liver under renal inflammatory state.

It is known that the progressive state of CKD both in animal models and humans is characterized by the infiltration of inflammatory cells and the proliferation of mesangial cells (14, 15). The density of macrophages and T lymphocytes, especially the activated macrophages and T lymphocytes in glomeruli, predicts the disease progression in MsPGN (16–18). Therefore, these cells could be targeted for therapeutic purposes and for exploring the mechanism of anti-inflammatory agents against MsPGN. Anti-Thy1.1 GN, induced by the injection of anti-Thy1.1 monoclonal antibody (mAb) 1-22-3, is commonly used as the model of human MsPGN (19–21). It is reported that, the accumulation of macrophages and T lymphocytes into glomeruli is one of the hallmarks of anti-Thy1.1 GN and plays an important pathogenetic role (22). Indeed, the involvement of ED3⁺ macrophages and CD4⁺ T helper lymphocytes in the development of the anti-Thy1.1 GN model induced by the injection of mAb 1-22-3 has been reported. In addition, the critical roles of the activated ED3⁺ cell and CD4⁺ cell, as well as the related inflammatory mediators, such as IL-2 and IFN- γ , in the chronic anti-Thy1.1 GN model induced by two consecutive injections of mAb 1-22-3 in an interval of 2 weeks (23, 24) has also been documented. Thus we be-

lieve that comparing glomerular ED3⁺ cell and CD4⁺ cell activation between two types of anti-Thy1.1 GN model would be a successful way to identify the anti-inflammatory effectiveness and mechanism of GTW at the different doses in vivo.

In this report, using the acute and chronic types of anti-Thy1.1 GN model induced by mAb 1-22-3, we contrasted the effects of different doses of GTW on glomerular inflammation and hepatic damage in the acute and chronic models of GN induced by anti-Thy1.1 antibody. Our findings thus provide useful information about the optimum therapeutic dose of GTW treatment in vivo.

Materials and Methods

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 administered via gavage. The mAb 1-22-3 (IgG3) and other important antibodies were given to us 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).

Experimental protocol

The experimental procedure is shown in Fig. 1. In Experiment 1, 21 rats were randomly divided into 3 groups ($n = 7$ for each group), the acute anti-Thy1.1 GN treated with high-dose (100 mg/kg BW) GTW group (the HD-GTW group), the acute anti-Thy1.1 GN treated with low-dose (50 mg/kg BW) GTW group (the LD-GTW group), and the untreated acute anti-Thy1.1 GN group (the Vehicle group). Rats were given via gavage a high dose or low dose of GTW or the vehicle (distilled water, 5 ml/kg BW) daily from 3 days before the injection of 500 μ g of mAb 1-22-3. All rats were sacrificed on day 7 after the induction of the acute anti-Thy1.1 GN model. One-hour after, rats were deprived of food, and 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 and livers were removed, weighed, cut into portions, and examined by light microscopy (LM), immunofluorescence (IF), or electron microscopy (EM), and for the preparation of glomerular RNA and protein.

Fig. 1. Experimental procedure.

RT-PCR and semiquantitative western blotting were carried out on the glomerular mRNA and protein of each rat for IL-2 and INF- γ . Moreover, the infiltrated macrophages or T lymphocytes in glomeruli was determined by IF staining using specific mAbs against rat antigens. Five rats without injection of mAb 1-22-3 were used as the normal control.

In Experiment 2, most procedures were carried out as in Experiment 1, but 10 rats were randomly divided into 2 groups ($n = 5$ for each group): the chronic anti-Thy1.1 GN treated with low-dose (50 mg/kg BW) GTW group (the LD-GTW group) and the untreated chronic anti-Thy1.1 GN group (the Vehicle group). Rats were injected intravenously with 500 μ g of mAb 1-22-3 in 1.0 ml saline through the tail. After 2 weeks, both groups of rats were injected with the same dose of mAb 1-22-3. Rats were given 50 mg/kg BW daily of GTW (low dose) or the vehicle (distilled water, 5 ml/kg BW daily) via gavage after the second injection. All rats were sacrificed on day 45 after the first injection. The blood was also drawn from the heart. Scr, BUN, ALT, and AST were measured as well. Kidneys and livers were removed, cut into portions and used for preparation of glomerular RNA and protein, as well as LM, IF, and EM studies.

Immunofluorescence

Renal tissue samples for IF studies were snap-frozen in precooled *n*-hexane and stored at -70°C . Frozen sections were cut into 3- μ m-thick slices with a cryostat and stained with anti-collagen type I (Chemicon International, Inc., 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 (DACO 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. (25).

The presence of macrophages in glomeruli was determined by IF staining using the specific mAbs against rat macrophage antigens. ED1⁺ (IgG1, reactive with the pan macrophages) and ED3⁺ (IgG2a, reactive with macrophage sialoadhesin) were purchased from Chemicon International, Inc. and Serotec (Oxford, UK), respectively. Mouse anti-rat mAb OX-19 (IgG1, anti-CD5⁺) was used as a pan T lymphocyte marker and OX-38 (IgG2a, anti-CD4⁺), as a helper T lymphocyte marker. Both OX-19 and OX-38 were precipitated from ascites using the corresponding hybridoma (European Collection of Animal Cells, Porton Down, Salisbury, UK). FITC-conjugated goat anti-mouse IgG1 (Southern Biotechnology Associates) and FITC-conjugated goat anti-mouse IgG2a were used as the 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.

Reverse transcription polymerase chain reaction (RT-PCR) studies

Glomeruli were isolated by graded sieving of more than 95% purity using the technique described by Krakower and Greenspon (26). 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, GCG CAC CCA CTT CAA GCC CT, and 3' primer, CCA CCA CAG TTG CTG GCT CA for IL-2 (27); 2) 5' primer, CCC TCT CTG GCT GTT ACT GC, and 3' primer, CTC CTT TTC CGC TTC CTT AG for IFN- γ (27). Amplification was carried out using the PC-800 programmable temperature control system (Astec, Fukuoka) through 20 to 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. The number of amplification cycles was 36 for IL-2 and 35 for IFN- γ , and 26 for the housekeeping gene glyceraldehydes-3-phosphate dehydrogenase (GAPDH) (27). 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). Results were expressed as ratios relative to GAPDH expression.

Semiquantitative western blot analysis

Glomeruli from the rats of the acute (day 7) or chronic anti-Thy1.1 GN (day 45) were isolated with phosphate-buffered saline (PBS) including protease inhibitors (PI); sequentially solubilized with 1% Triton X-100, RIPA buffer (0.1% SDS, 1% sodium deoxycholate, 1% Triton X-100, 0.15 M NaCl, and 0.01 M ethylenediaminetetraacetic acid (EDTA) in 0.025 M Tris-HCl, pH 7.2) with PI; and separated into Triton X-100-soluble (T), RIPA-soluble (R), and RIPA-insoluble (S) fractions. The RIPA-insoluble fraction was solubilized with SDS-PAGE sample buffer (2% SDS, 10% glycerol, and 5% 2-mercaptoethanol in 0.0625 M Tris-HCl, pH 6.8) (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 et al. (28) and transferred to a PVDF membrane (Bio-Rad, Oakland, CA, USA) 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-rat IL-2, anti-rat IFN- γ , or anti-rat 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, Inc., 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). This procedure was carried out three times. The ratio of the 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 the mean $\pm$ S.E.M. of these independent experiments.

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- μ m sections, and stained with a periodic acid-Schiff (PAS) or a hematoxylin and eosin reagent. Semiquantitative morphological studies of glomerular lesions were carried out by randomly selecting 30 full-sized glomeruli (80–100 μ m) 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 Raij et al. (29). The total number of cells in the glomeruli was also counted in a blinded protocol and computed for 30 glomeruli for each kidney.

Electron microscopy investigation

Renal and liver tissue samples for electron microscopic assessment were fixed in 2.5% glutaraldehyde in 0.1 M 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 ethanolpropylene oxide series and embedded in Araldite M. The ultra-thin sections were prepared and stained with uranyl acetate and lead citrate and then, investigated and photographed under a JEM-1011 transmission electron microscope (JEOL, Tokyo).

Kinetics of urinary protein excretion

On days 1, 3, 5, and 7 in Experiment 1 or on days 1, 3, 14, 25, 35, and 45 in Experiment 2, 24-h urine samples were collected after the induction of the acute and chronic Thy1.1 GN models. In both experiments, urine samples were also collected before the injection for determining the amount on day 0. Urine protein concentrations were determined by colorimetric assay (Bio-Rad) using bovine serum albumin (BSA) as the standard.

Statistical analyses

Statistical significance was evaluated using the Mann-Whitney U test and one-way analysis of variance (ANOVA). Values were expressed as the mean $\pm$ S.E.M.

Differences at $P < 0.05$ were considered significant. Data were analyzed using Stat View for Macintosh (Abacus Concepts, Berkeley, CA, USA).

Results

Dose-effects of GTW on infiltrated glomerular inflammatory cell

At the beginning of Experiment 1, we compared the effects of GTW at high dose or low dose on the infiltration of ED1⁺ macrophage in the same acute anti-Thy1.1 GN model. Significant increase in the number of ED1⁺ cell (4.47 ± 0.25 cells/cross section) was observed in glomeruli on day 7 after the acute anti-Thy1.1 GN induction in the Vehicle group. GTW at 100 mg/kg BW daily dose significantly reduced the number of ED1⁺ cells (2.33 ± 0.23 cells/cross section) in glomeruli of the HD-GTW group, whereas GTW at 50 mg/kg BW daily dose did not affect that (4.18 ± 0.92 cells/cross section) in the LD-GTW group during the course of the same acute anti-Thy1.1 GN.

Subsequently, we assessed the effects of GTW at different doses on macrophages and T lymphocytes in glomeruli between the acute and chronic anti-Thy1.1 GN models. The number of macrophages indicated by the presence of ED3⁺ cells as well as ED1⁺ cells in glomeruli increased and peaked on day 7 after a single injection of mAb 1-22-3 in the acute anti-Thy1.1 GN model. Contrary to these results, in Experiment 2, the number of CD5⁺ and CD4⁺ T lymphocytes on day 45 in the chronic anti-Thy1.1 GN model induced by two injections of mAb 1-22-3 was obviously higher than those in the acute anti-Thy1.1 GN model. Comparing dose-effects of GTW on the active inflammatory cells between two anti-Thy1.1 GN models at sacrifice time, GTW at the high dose of 100 mg/kg BW daily reduced glomerular accumulation of ED1⁺ macrophages (pan macrophages: the HD-GTW group, 2.33 ± 0.23 vs. the Vehicle group, 4.47 ± 0.25 cells/cross section, $P < 0.05$) and ED3⁺ macrophage (activated macrophages: the HD-GTW group, 2.23 ± 0.22 vs. the Vehicle group, 4.45 ± 0.87 cells/cross section, $P < 0.05$) (Fig. 2), but did not affect glomerular infiltration of CD5⁺ and CD4⁺ T lymphocytes (data not shown). On the other hand, GTW at the low dose of 50 mg/kg daily also greatly reduced glomerular accumulation of ED3⁺ macrophages (activated macrophages: the LD-GTW group, 2.34 ± 0.35 vs. the Vehicle group, 4.31 ± 0.52 cells/cross section, $P < 0.01$) and CD4⁺ T lymphocytes (helper T lymphocytes: the LD-GTW group, 1.85 ± 0.17 vs. the Vehicle group, 2.63 ± 0.14 cells/cross section, $P < 0.01$) (Fig. 3), and had no significant influence on the number of ED1⁺ macrophages and CD5⁺ T lymphocytes (data not shown).

In brief, GTW at high dose could reduce infiltrated glomerular macrophages in the acute anti-Thy1.1 GN model. By comparison, GTW at low dose could effectively improve the infiltration of the activated macrophages and T lymphocytes in glomeruli in the chronic anti-Thy1.1 GN model as well.

Dose-effects of GTW on mRNA and protein expressions of glomerular inflammatory cytokines

RT-PCR showed that, the expressions of IL-2 and IFN- γ at mRNA level in the acute anti-Thy1.1 GN model were significantly higher than those in the chronic anti-Thy1.1 GN model. In Experiment 1, GTW at the high dose of 100 mg/kg BW daily reduced glomerular expression of mRNA for IL-2 (45.32%, compared to the Vehicle group, $P < 0.05$), but not that of IFN- γ (Fig. 4). In Experiment 2, GTW at the low dose of 50 mg/kg BW daily significantly suppressed the mRNA expression of IL-2 (67.57%, compared to the Vehicle group, $P < 0.01$). In addition, it tended to inhibit the mRNA expression of IFN- γ (24.21%, compared to the Vehicle group, $P < 0.05$) on day 45 (Fig. 5). Consistently, semiquantitative western blot analysis of extracts from isolated glomeruli also revealed that protein expressions of IL-2 (44.83%, compared to the Vehicle group, $P < 0.01$) and IFN- γ (20%, compared to the Vehicle group, $P < 0.05$) were markedly down-regulated after GTW treatment with the low dose of 50 mg/kg BW daily in the chronic anti-Thy1.1 GN model on day 45 (Fig. 6).

In short, GTW at low dose could effectively down-regulate the mRNA and protein expressions of IL-2 and IFN- γ in the chronic anti-Thy1.1 GN model, as well as GTW at high dose in the acute anti-Thy1.1 GN model.

Dose-effects of GTW on glomerular injury and proteinuria

In Experiment 1, we also contrasted the effects of GTW at high dose or low dose on proteinuria and renal function in the same acute anti-Thy1.1 GN model. In the Vehicle group, proteinuria quickly developed after mAb 1-22-3 injection from day 1 to day 7. Compared with the Vehicle group, proteinuria was significantly diminished in both the HD-GTW and LD-GTW groups after the acute anti-Thy1.1 GN induction. Moreover, the level of proteinuria in the HD-GTW group was significantly lower than that in the LD-GTW group on day 7 (proteinuria: the HD-GTW group, 22.15 ± 17.39 mg vs. the LD-GTW group, 77.97 ± 31.50 mg). On the other hand, the level of Scr in both GTW treated groups did not change (Scr: the HD-GTW group, 0.30 ± 0.04 mg/dl vs. the LD-GTW group, 0.32 ± 0.05 mg/dl) except the reduction of BUN in the HD-GTW group (BUN: the HD-GTW group, 21.84 ± 3.83 mg/dl vs. the LD-GTW group,

Fig. 2. High-dose effects of GTW on glomerular macrophage accumulation in the acute anti-Thy1.1 GN model. Immunofluorescence findings of staining for ED1⁺ (A – C) and ED3⁺ macrophages (D – F) in glomeruli of rats treated with vehicle (B, E) or GTW (C, F) on day 7 after mAb 1-22-3 injection are shown. The findings in panels A and D are those in normal rats. The increased infiltration of ED1⁺ (B) or ED3⁺ (E) macrophages observed in the Vehicle group was suppressed in the HD-GTW group (C, F) (original magnification 400 ×). G and H) Data are expressed as the mean ± S.E.M. (n = 7 or 5). ***P* < 0.01 vs. normal group and #*P* < 0.05 vs. the Vehicle group.

29.79 ± 4.99 mg/dl) during the course of the acute anti-Thy1.1 GN.

Then, we checked the effects of GTW at the different doses on glomerular injury and proteinuria between the acute and chronic anti-Thy1.1 GN models. A comparison of glomerular injury evaluated by mesangial morphological change, α -SMA and collagen type I staining score, serum level of creatinine and BUN, and proteinuria of rats on day 7 and on day 45 was summarized, respectively. In Experiment 1, GTW at the high dose of 100 mg/kg BW slightly improved mesangial proliferation, decreased the intense staining of α -SMA (Table 1), and reduced proteinuria during 7 days (Fig. 7). In Experiment 2, GTW at the low dose of 50 mg/kg BW obviously ameliorated extracellular matrix (ECM) expansion, lessened the intense staining of collagen type I (Table 1), reduced Scr, BUN (Table 2), and lowered proteinuria

during 45 days (Fig. 8). Besides, the kidney weight (KW) and BW of rats at the time of sacrifice were not greatly altered by GTW treatment with the different doses in either the acute anti-Thy1.1 GN model or in the chronic anti-Thy1.1 GN model (Table 2).

In sum, GTW could dose-dependently ameliorate glomerular injury and proteinuria in the same acute anti-Thy1.1 GN model. GTW at low dose could effectively promote the recovery of glomerular lesion and reduced proteinuria in the chronic anti-Thy1.1 GN model as well.

Dose-effects of GTW on hepatic damage

In Experiment 1 and 2, the data of hepatic function in clinical practice, including serum ALT and AST, in two types of anti-Thy1.1 GN model were examined. Additionally, we investigated, by LM and EM, the histological

Fig. 3. Low-dose effects of GTW on glomerular macrophage and T lymphocyte infiltration in the chronic anti-Thy1.1 GN model. Findings of immunofluorescent staining for ED3⁺ (A – C) and CD4⁺ cells (D – F) in glomeruli of rats treated with vehicle (B, E) or GTW (C, F) on day 45 after mAb 1-22-3 two-injection and normal rats (A, D) are shown. The increased accumulation of ED3⁺ (B) or CD4⁺ (E) cells as observed in the Vehicle group was decreased in the LD-GTW group (C, F) (original magnification 400 ×). G and H) Data are expressed as the mean ± S.E.M. (n = 5). ***P* < 0.01 vs. normal group and ^{##}*P* < 0.01 vs. the Vehicle group.

and ultramicrostructural characteristics of liver tissue in the rats treated with GTW at the different doses. Our results showed that, after GTW treatment with the high dose of 100 mg/kg BW daily for 7 days, ALT in the HD-GTW group and the Vehicle group was 52.61 ± 6.48 and 49.26 ± 7.62 U/l, respectively, and AST in the HD-GTW group and the Vehicle group was 59.59 ± 9.89 and 63.84 ± 10.59 U/l, respectively. Although the level of serum ALT was a little increased in the HD-GTW group compared to the acute anti-Thy1.1 GN model rats without GTW treatment, the difference between two groups was not significant. By contrast, after GTW treatment with the low dose of 50 mg/kg BW daily during 45 days, neither ALT (the LD-GTW group, 38.74 ± 12.02 U/l vs. the Vehicle group, 39.22 ± 7.39 U/l) nor AST (the LD-GTW group, 44.70 ± 8.62 U/l vs. the Vehicle group, 43.94 ± 4.52 U/l) in blood was obviously increased in

both groups.

The histological findings of liver tissue in the rats treated with GTW at high dose in Experiment 1 showed that hepatic cells were swollen, fused and dissolved, cytoplasm were loosened, hepatic plates were fractured, hepatic cords were disordered, sinusoids were dilated and discontinuous, and that a few inflammatory cells around hepatic lobules and bile ducts were infiltrated (Fig. 9: C – E). Ultramicrostructural examinations by EM revealed numerous lipid droplets in hepatocytes, slight mitochondria dilatation, and local glycogen particle deposition (Fig. 10C). By comparison, there was no marked structural change in the liver, except for a few lipid droplets in hepatocytes and inflammatory cell accumulation, in either the acute anti-Thy1.1 GN rats without GTW treatment (Fig. 9B, 10B) or in almost all the chronic anti-Thy1.1 GN rats with GTW treatment at

Fig. 4. High-dose effects of GTW on mRNA expressions of IL-2 and IFN- γ in the acute anti-Thy1.1 GN model. Semiquantitative RT-PCR amplification of each transcript was performed using the total RNA extracted from the isolated glomeruli of pooled kidneys of rats from each group on day 7. Ratios of the densitometric signals of IL-2 or IFN- γ and the internal control (GAPDH) were analyzed. Representative agarose gel electrophoretic patterns from one of three experiments are shown in panel A. The data in panel B are shown as ratios relative to GAPDH. Data are expressed as the mean $\pm$ S.E.M., [#] $P < 0.05$ vs. the Vehicle group.

low dose (Fig. 9F, 10D).

In any case, unlike GTW treatment with high dose in the acute anti-Thy 1.1 GN model, GTW at low dose did not affect hepatic function and structure in the chronic anti-Thy 1.1 GN model.

Discussion

Kawachi's group reported two types of rat models with mAb 1-22-3 showing different patterns of inflammatory alterations in glomeruli. One model shows an acute inflammatory response that is induced by a single intravenous injection of mAb 1-22-3 (19). The other model shows a chronic inflammatory lesion induced by two consecutive injections of mAb 1-22-3, given 2 weeks apart (20). Thereupon, the comparison of various inflammatory pathological characteristics between these two models could provide clues for elucidating the mechanism and therapeutic target of an anti-inflammatory agent in vivo. In the present study, we firstly established that GTW dose-dependently improved the infiltration of

Fig. 5. Low-dose effects of GTW on mRNA expression of IL-2 and IFN- γ in the chronic anti-Thy1.1 GN model. The expressions of IL-2 and IFN- γ at the mRNA level on day 45 were semi-quantified by RT-PCR. Representative agarose gel electrophoretic patterns are shown in panel A. The data in panel B are shown as ratios relative to GAPDH. Data are expressed as the mean $\pm$ S.E.M., [#] $P < 0.05$ and ^{##} $P < 0.01$ vs. the Vehicle group.

ED1⁺ cells, as the pan macrophage marker, in glomeruli and glomerular injury in the same acute anti-Thy1.1 GN, and the effects of GTW at high dose of 100 mg/kg BW daily are better. Then, we examined glomerular inflammatory cell activation and their secreted inflammatory mediator expression after GTW treatment with the different doses between the acute and chronic anti-Thy1.1 GN models.

It has been reported that ED3⁺ macrophages play a necessary role to promote the induction of inflammation through their ability to phagocytose mesangial cell debris and discharge IL-2 in the acute stage of anti-Thy1.1 GN induced by a single injection of mAb 1-22-3 (23, 30). In Experiment 1, to investigate whether the anti-inflammatory effect of GTW at high dose could be related to the improvement of the active inflammatory cell infiltration in glomeruli in the acute phase of renal inflammation, we compared the number of the infiltrated ED1⁺ and ED3⁺ macrophages, CD4⁺ and CD5⁺ T lymphocytes, and the expressions of IL-2 and IFN- γ at mRNA and protein levels in glomeruli on day 7 after mAb 1-22-3 injection between the HD-GTW group and the Vehicle group. Here, we chose day 7 because the significant accumulation of ED3⁺ macrophages was observed and the notable

Fig. 6. Low-dose effects of GTW on protein expression of IL-2 and IFN- γ in the chronic anti-Thy1.1 GN model. Proteins of IL-2 and IFN- γ were extracted and subjected to semiquantitative western blot analysis by using an anti-IL-2 antibody or anti-IFN- γ antibody. Equal loading of protein per lane was verified by reprobing the blot with anti- β -actin antibody. A) The data show the representative blot of IL-2 (upper), IFN- γ (middle), and β -actin (lower) out of three independent experiments. B) The data show the evaluation of IL-2 or IFN- γ / β -actin ratios in glomeruli. Data are expressed as the mean $\pm$ S.E.M., ** P < 0.01 vs. normal group, # P < 0.05 and ## P < 0.01 vs. the Vehicle group.

change in the expressions of IL-2 and IFN- γ were detected at this time point, based on our previous study (9) and the reports by other investigations (30, 31). In the acute anti-Thy1.1 GN model, our results showed that GTW at the high dose of 100 mg/kg BW daily prevented macrophage activation together with the down-regulation of the mRNA expression for IL-2 and that this had no influence on the accumulation of CD4 $^{+}$ and CD5 $^{+}$ T lymphocytes and the expression of IFN- γ in glomeruli.

Two clinical investigations reported that the activated inflammatory cells including ED3 $^{+}$ macrophage and CD4 $^{+}$ T helper lymphocyte are known to secrete many kinds of potentially injurious inflammatory factors such as IL-2 and IFN- γ in the pathological process of human MsPGN (32, 33). Similarly, in the progressive phase of anti-Thy1.1 GN induced by mAb 1-22-3 injection, CD4 $^{+}$ T helper lymphocyte play a critical role to develop the progression of glomerular inflammation by controlling the activation of macrophage and the expressions of IL-2 and IFN- γ (24, 34). Consequently, to clarify the anti-inflammatory effect of GTW at low dose on the active inflammatory cell in glomeruli in the chronic state of renal inflammation, we repeatedly compared the number of the infiltrated CD4 $^{+}$ and CD5 $^{+}$ T lymphocytes, ED3 $^{+}$ and ED1 $^{+}$ macrophages, and the expressions of IL-2 and IFN- γ at mRNA and protein levels on day 45 after two injections of mAb 1-22-3 between the LD-GTW group and the Vehicle group, individually. In the chronic anti-Thy1.1 GN model, our data shown that GTW at the low dose of 50 mg/kg BW daily also reduced the accumulation of CD4 $^{+}$ T helper lymphocytes and ED3 $^{+}$ macrophages together with the suppression of mRNA and protein expressions for IL-2 and IFN- γ in glomeruli.

Hence, we confirmed that, in comparison with the significant anti-inflammatory effects of GTW at the high dose of 100 mg/kg BW daily in the acute anti-Thy1.1 GN

Table 1. Dose-effects of GTW on glomerular injury in two types of anti-Thy1.1 GN model

Group	n	Dose (mg/kg)	Total cell numbers of 30 glomerular cross-sections	Score		
				Matrix	α -SMA	Collagen type I
Normal	5	-	50.99 $\pm$ 2.21	0.41 $\pm$ 0.07	0.45 $\pm$ 0.10	0.53 $\pm$ 0.10
Vehicle (acute anti-Thy1.1 GN)	7	-	87.55 $\pm$ 2.31**	2.22 $\pm$ 0.29**	2.30 $\pm$ 0.33**	-
HD-GTW	7	100	70.12 $\pm$ 2.31 [#]	1.83 $\pm$ 0.21 [#]	1.93 $\pm$ 0.28 [#]	-
Vehicle (chronic anti-Thy1.1 GN)	5	-	88.53 $\pm$ 1.39**	2.95 $\pm$ 0.27**	-	2.63 $\pm$ 0.15**
LD-GTW	5	50	61.22 $\pm$ 1.87 [†]	1.89 $\pm$ 0.47 [†]	-	1.94 $\pm$ 0.17 [†]

** P < 0.01 vs. normal group, [#] P < 0.05 vs. the Vehicle group (acute anti-Thy1.1 GN), [†] P < 0.05 vs. the Vehicle group (chronic anti-Thy1.1 GN). Data are expressed as the mean $\pm$ S.E.M.

Fig. 7. High-dose effects of GTW on glomerular injury and proteinuria in the acute anti-Thy1.1 GN model. The top photomicrographs of periodic acid–Schiff (PAS) staining (A – C) and immunofluorescence micrographs of α -smooth muscle action (SMA) (D – F) show that mesangial cell proliferation, severe matrix expansion, and intense α -SMA staining were detected in the Vehicle group (B, E), but ameliorated in the HD-GTW group (C, F), respectively. A and D Findings are those from normal rats (original magnification, 200 $\times$). G) The data show the kinetics of urinary protein excretion during 7 days in the acute anti-Thy1.1 GN model. Data are expressed as the mean $\pm$ S.E.M., ** P < 0.01 vs. normal group and # P < 0.05 vs. the Vehicle group.

Table 2. Dose–effects of GTW on renal function, body weight, and kidney weight in two types of anti-Thy1.1 GN model

Group	n	Dose (mg/kg)	Ser (mg/dl)	BUN (mg/dl)	BW (g)	KW (g)
Normal	5	-	0.31 $\pm$ 0.05	26.22 $\pm$ 1.50	198 $\pm$ 8.37	0.94 $\pm$ 0.09
Vehicle (acute anti-Thy1.1 GN)	7	-	0.29 $\pm$ 0.03	31.69 $\pm$ 3.84*	200.71 $\pm$ 10.18	0.97 $\pm$ 0.07
HD-GTW	7	100	0.30 $\pm$ 0.04	21.84 $\pm$ 3.83 ^{##}	194.29 $\pm$ 9.76	0.93 $\pm$ 0.04
Vehicle (chronic anti-Thy1.1 GN)	5	-	0.74 $\pm$ 0.08	62.02 $\pm$ 16.81	391 $\pm$ 17.10	1.20 $\pm$ 0.14
LD-GTW	5	50	0.58 $\pm$ 0.09 [†]	34.48 $\pm$ 6.95 ^{††}	390 $\pm$ 7.91	1.16 $\pm$ 0.15

* P < 0.05 vs. normal group, ^{##} P < 0.01 vs. the Vehicle group (acute anti-Thy1.1 GN), [†] P < 0.05 and ^{††} P < 0.01 vs. the Vehicle group (chronic anti-Thy1.1 GN). Data are expressed as the mean $\pm$ S.E.M.

model, GTW at the low dose of 50 mg/kg BW daily could prevent the infiltration of the activated macrophages and T lymphocytes and their associated inflammatory cytokine expressions in glomeruli in the chronic

anti-Thy1.1 GN model as well. Here, we need to bring up two additional points. First, in view of the large number of clinical observations in China, the routine effective dose range of GTW in adults can be approximated as

Fig. 8. Low-dose effects of GTW on glomerular injury and proteinuria in the chronic anti-Thy1.1 GN model. The top photomicrographs of periodic acid-Schiff (PAS) staining (A – C) and immunofluorescence micrographs of collagen type I (D – F) show that severe extracellular matrix expansion and intense staining of collagen type I were detected in the Vehicle group (B, E), but improved in the LD-GTW group (C, F), respectively. A and D) Findings from normal rats (original magnification, 200 ×). G) The data show the kinetics of urinary protein excretion during 45 days in the chronic anti-Thy1.1 GN model. Data are expressed as the mean $\pm$ S.E.M., ** P < 0.01 vs. normal group, # P < 0.05 and ### P < 0.01 vs. the Vehicle group.

1–2 mg/kg BW daily, which may correspond to the dose range of 50–100 mg/kg BW daily in rats with an average BW of 200 g by taking account of the ratio of 6.25. For this reason, the highest dose of 100 mg/kg BW or the lowest dose of 50 mg/kg BW used in this study corresponds to 120 or 60 mg/day of GTW for an adult GN patient with an average BW of 60 kg. Second, in the chronic anti-Thy1.1 GN model, we, indeed, found that GTW at low dose did affect the number of activated ED3⁺ macrophages and CD4⁺ T lymphocytes in glomeruli. This could be another therapeutic mechanism underlying the beneficial effects of GTW in the chronic course. Unexpectedly, GTW at low dose had little influence on the number of CD5⁺ cells and ED1⁺ cells. The reasons for this result were unclear. A previous study from Kawachi's group, using the same chronic model induced by two injections of mAb 1-22-3 demonstrated that the prominent accumulation of CD5⁺ T lymphocytes and ED1⁺ macrophages in glomeruli was not observed at any time points after the second injection. Instead, the accumulation of activated inflammatory cells, such as CD4⁺ and CD8⁺ cells and ED3⁺ cells in glomeruli was

found (24). Based on the similar observations, we speculated that the activated glomerular inflammatory cells only respond to GTW treatment with low dose. Further detailed analysis of the relationship between the dose-effect of GTW and the activation of infiltrated inflammatory cells in glomeruli in the chronic anti-Thy1.1 GN are needed to address this hypothesis.

To our knowledge, there is a strong causality between the glomerular inflammatory response and mesangial injurious change in the development of anti-Thy1.1 GN induced by mAb 1-22-3 (35). Therefore, we contrasted mesangial morphological change, α -SMA and collagen type I staining score, serum level of creatinine and BUN, and proteinuria after GTW treatment with different doses between the acute and chronic anti-Thy1.1 GN models. The same as our results in the past studies (9, 35), using two types of anti-Thy1.1 GN model, we corroborated again that both high and low doses of GTW could promote the restoration of glomerular injury. Furthermore, it is likely that the suppression of the activated inflammatory cells, such as ED3⁺ macrophages and CD4⁺ T helper lymphocytes, might contribute to the amelioration of

Fig. 9. Dose-effects of GTW on the light microscopic characteristics of liver in two types of anti-Thy1.1 GN model. The photomicrographs of hematoxylin and eosin staining show hepatic cell dissolution and death (C), sinusoid dilation (D), and inflammatory cell infiltration (E) in the rats treated with GTW at high dose. There was no marked structural change in the liver except for inflammatory cell accumulation in the acute anti-Thy1.1 GN rats without GTW treatment (B) or in the chronic anti-Thy1.1 GN rats with GTW treatment at low dose (F). A) Findings from normal rat (original magnification, 400 $\times$).

glomerular lesion through the inhibition of inflammatory cytokine expression in glomeruli. Briefly, these investigations prompted us to believe that the anti-inflammatory effect of GTW *in vivo* does not depend upon the oral over-dose level during the course of the chronic anti-Thy1.1 GN induced by mAb 1-22-3 injections. However, once we compared the actions of GTW at high dose and low dose on hepatic function and liver tissue between the acute and chronic anti-Thy1.1 GN models, to our surprise, the results were wildly divergent.

Although GTW is regarded as a promising native agent for the treatment of various CKD on the basis of many clinical and experimental studies in China, some adverse events associated with over-dose, such as hepatic dam-

Fig. 10. Dose-effects of GTW on the electron microscopic characteristics of liver in two types of anti-Thy1.1 GN model. The electron micrographs show lipid droplet deposition, mitochondria dilatation, and glycogen particle accumulation in hepatocytes (C) in the rats treated with GTW at high dose. There was no ultrastructural change in the liver in the acute anti-Thy1.1 GN rats without GTW treatment (B) or in the chronic anti-Thy1.1 GN rats with GTW treatment at low dose (D). A) Findings from normal rat (original magnification, 10000 $\times$).

age, reproductive toxicity, gastrointestinal upset, diarrhea, vomiting, and bellyache, have been reported sequentially. More recently, *Triptolide*, an essential active component of GTW, was found to induce steatosis in hepatocytes and increases in serum AST and ALT if it was taken for a long time or at an over-dose (36, 37). On the other hand, GTW itself at the high oral dose of 105 mg/kg BW daily could cause different hepatotoxic reactions between normal and model rats as well (38). Taken into account these disadvantages, it is crucial to understand the toxicological actions related with over-dose level and time of GTW use.

Finally, to exclude the side effects of GTW at the different doses on hepatic damage in two types of anti-Thy1.1 GN model, we emphatically compared serum ALT and AST and histological and ultrastructural characteristics of liver tissue in the rats treated with GTW by LM and EM examinations, respectively. Our data showed that, GTW treatment at high dose of 100 mg/kg BW daily induced increases in serum AL and AST and hepatic histological and ultrastructural changes including centrilobular cell dissolution and death, inflammatory cell infiltration, lipid droplet deposition, mito-

chondria dilatation, and glycogen particle accumulation in hepatocytes. By comparison, GTW treatment at the low dose of 50 mg/kg BW daily had no obvious unwanted actions on liver tissue except for a few lipid droplets in hepatocytes and inflammatory cell accumulation within the period of 45 days' investigation. These results suggested that in vivo GTW at the high dose of 100 mg/kg BW daily could cause hepatic damage under the renal inflammatory state in rats.

As mentioned above, GTW at oral over-dose induced hepatic toxic reactions both in normal and model rats, and hepatic lipidosis might be part of the mechanism for hepatotoxicity (38). On the other hand, another in vivo report proposed that steatosis in hepatocytes is the principal mechanism of hepatic damage by a 400 μ g/kg BW daily dose of *Triptolide* (36). Nevertheless, we still do not fully understand how hepatic damage was induced by GTW exposure with high dose under the glomerular inflammatory condition recovered by GTW. It is reported that, gap junctional intercellular communication (GJIC) plays a crucial role in hepatic damage by chemical drugs, and injurious signals may pass gap junctions in rat liver in vivo (39, 40). We think that, GTW-induced hepatotoxicity can depend on GJIC function in the liver, and in a non-healthy model rat, hepatocytes damaged by exposure to a high dose of GTW may undergo apoptotic death through some signaling pathway linked to the connexin family. Certainly, the toxic action of GTW in vivo on liver cells requires thorough investigation in the future.

In summary, our results showed that GTW at the low dose of 50 mg/kg BW daily could suppress the activation of glomerular inflammatory cells presumably through the reduction of inflammatory cytokines such as IL-2 and IFN- γ in the chronic anti-Thy1.1 GN model and that this dose does not exert any obvious hepatotoxicity. By contrast, GTW at the high dose of 100 mg/kg BW daily could damage the liver, in spite of the significant beneficial effects of GTW on renal inflammation, in the acute anti-Thy1.1 GN model. In the light of the dose-effects of GTW on glomerular inflammation and hepatic damage in two types of anti-Thy1.1 GN, the optimum dose of GTW in safety and effectiveness in vivo is 50 mg/kg BW daily for model rats, which corresponds to 60 mg/day of GTW for an adult patient with an average BW of 60 kg.

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