Studies on Antinephritic Effect of TJ-8014, a New Japanese Herbal Medicine, and Its Mechanisms (1): Effects on Original-Type Anti-GBM Nephritis in Rats and Platelet Aggregation

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Abstract—In this study, we investigated the antinephritic effects of TJ-8014 and crude drugs in TJ-8014, in comparison to dipyridamole, on original-type anti-GBM nephritis in rats. TJ-8014 (2.0 and 3.0 g/kg/day, p.o., for 12 days) markedly inhibited the urinary protein excretion and the elevation of the plasma urea nitrogen (UN). In addition, TJ-8014 was effective in inhibiting the histopathological changes of hypercellularity and adhesion in glomeruli. Although dipyridamole (0.4 g/kg/day, p.o., for 12 days) had no effect on the plasma UN level, it was as effective as TJ-8014 on the other parameters. When each crude drug which constitutes TJ-8014 was given p.o., daily at 0.2 g/kg, only Ho/en was effective in inhibiting the urinary protein excretion as well as histopathological changes. Ginseng radix reduced both the hypercellularity and the adhesion, while Bupleuri radix, Glycyrrhizae radix and Zizyphi fructus reduced only the hypercellularity. TJ-8014 and dipyridamole inhibited the platelet aggregation in normal and nephritic rats. These results indicate that TJ-8014, like dipyridamole, has a beneficial effect on original-type anti-GBM nephritis in rats and the antinephritic action of TJ-8014 may be partly due to the antiplatelet action of this agent.

Although Japanese herbal medicine therapy has been increasing year after year in our country, there have been very few reports as to whether Japanese herbal medicines have a beneficial effect on experimental nephritis. Senaga and Kawashima (1) reported that Chai-Ling-Tang (Sairei-To in Japanese) was effective on nephrotic syndrome in children. Abe et al. (2) demonstrated by electron microscopy that Chai-Ling-Tang remarkably inhibited histological damages in the kidneys of rats with puromycin amionucleoside-induced nephrosis. We have already reported that Chai-Ling-Tang and Xiao-Chai-Hu-Tang (Syo-Saiko-To in Japanese) are effective in reducing proteinuria and in improving histological damages in glomeruli in original-type and crescentic-type anti-glomerular basement membrane (anti-GBM) nephritis in rats (3, 4).

In the present study we first investigated the antinephritic effect of TJ-8014, a new herbal medicine, on original-type anti-GBM nephritis in rats in comparison with that of dipyridamole, an anti-platelet agent, which had been proven to be effective in this model (5). In the second experiment, the effects of crude drugs that constitute TJ-8014 on this nephritis were investigated. In the third experiment, the in vitro and in vivo effects of TJ-8014 on platelet aggregation were examined in order to elucidate the mechanisms of the antinephritic action of this agent.

Materials and Methods

Animals: Male Sprague-Dawley strain SPF rats, weighing approx. 155 g (Shizuoka Agricultural Cooperative Association for
Laboratory Animals, Shizuoka), were used in the experiment. These animals were housed in an air-conditioned room at 23±1°C during the experimental period.

**Drugs:** Drugs used were TJ-8014 (a lyophilized extract; Tsumura Co., Ltd., Tokyo), eight crude drugs which constitute TJ-8014 (lyophilized extracts; Tsumura Co., Ltd., Tokyo) and dipyridamole (Boehringer Ingelheim, West Germany). Compositions of the crude drugs of TJ-8014 are shown in Table 1. These drugs were suspended in 1% gum arabic for the in vivo test.

**Induction of original-type anti-GBM nephritis:** Original-type anti-GBM nephritis was induced in rats by injecting 0.75 ml of rabbit anti-rat GBM serum (anti-GBM serum) into their tail veins, as described previously (6).

**Evaluation of antinephritic effects of drug:** The 24 hr-urine samples after the anti-GBM serum injection were collected, and the rats were then divided into groups of 8 animals, so that the average protein content in the 24 hr-urine samples of each group were at the same level. Test drugs were given to each group p.o. daily in a volume of 1.0 ml/100 g of body weight from the next day of anti-GBM serum injection (the 1st day) to the 12th day. One group of nephritic rats served as the nephritic control and was given p.o. only the vehicle (1% gum arabic). In addition, a non-treated (normal) group of 8 rats was used for comparison with nephritic groups. On the 13th day, blood was drawn, and the kidneys were taken. Evaluation of the antinephritic effect of test drugs was done by comparing biochemical parameters such as urinary protein and plasma urea nitrogen contents and histopathological parameters in the kidneys of the drug-treated group with those of the control group.

**Urine and blood collections:** The 24 hr-urine samples were obtained by keeping each animal in an individual metabolic cage for 24 hr. At the beginning of the urine collection, each animal received 8 ml of distilled water orally without feeding. The urine was then centrifuged at 3,000 rpm for 10 min at 4°C, and the supernatant was used for the determination of protein. Immediately after the urine collection, each 0.4 ml of blood was drawn from the tail vein of conscious animals with a disposable microsyringe and put into a tube containing 4.5 μmol of EDTA·2Na. The blood was centrifuged at 5,000 rpm at 4°C to obtain plasma for the determination of urea nitrogen.

**Determinations of urinary protein and plasma urea nitrogen (UN) contents:** The urinary protein content was determined by the method of Kingsbury et al. (7) and expressed as mg/24 hr urine. The UN content was determined in accordance with the method of Searcy and Cox (8) and expressed as mg/dl of plasma.

**Assessment of histopathological parameters:** For light microscopic study, kidneys were dehydrated and fixed by immersing the tissues stepwise into low to high concentrations of ethyl alcohol. The tissues were then embedded in paraffin and sectioned into 2–3 μm-thick slices. The sections were stained with hematoxylin and eosin and Masson trichrome. The number of nuclei (hypercellularity) and adhesion to Bowman’s capsule of capillary walls (adhesion) in the glomeruli were observed under light microscopy. For assessing the hypercellularity, the

<table>
<thead>
<tr>
<th>Grude drugs</th>
<th>Contents</th>
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</thead>
<tbody>
<tr>
<td>Bupleuri radix</td>
<td>7 g</td>
</tr>
<tr>
<td>Pinelliae tuber</td>
<td>5 g</td>
</tr>
<tr>
<td>Glycyrrhiza radix</td>
<td>2 g</td>
</tr>
<tr>
<td>Scutellariae radix</td>
<td>3 g</td>
</tr>
<tr>
<td>Ginseng radix</td>
<td>3 g</td>
</tr>
<tr>
<td>Coptidis rhizoma</td>
<td>1 g</td>
</tr>
<tr>
<td>Holen</td>
<td>3 g</td>
</tr>
<tr>
<td>Zizyphi fructus</td>
<td>3 g</td>
</tr>
</tbody>
</table>

The amounts of each crude drug required to prepare 4.5 g of TJ-8014 extract.
number of nuclei was counted and expressed as the mean number per equatorial cross section in 10 glomeruli/animal. For assessing the adhesion, fifty glomeruli per section were observed and the degree of adhesion was scored as 1 (mild), 2 (moderate), and 3 (severe) in accordance with typical examples of the degree of adhesion shown in

![Fig. 1. Typical micrographs of the adhesion of capillary walls to Bowman’s capsule in glomeruli.](image)
Fig. 1. The number of glomeruli corresponding to each score was represented as $n_1$, $n_2$ and $n_3$. The adhesion index (AI) was calculated by the following formula: $AI = 1 \times n_1 + 2 \times n_2 + 3 \times n_3$. All the above experiments were performed "blindly" on a coded section.

The in vitro and in vivo effects of drugs on platelet aggregation: Platelet aggregation was measured with a whole blood aggregometer (Chlonolog Co., Ltd., Tokyo) as reported previously (9). In order to test the in vitro effects of test drugs on platelet aggregation, blood was taken into a disposable syringe containing 0.25 ml of 0.38% sodium citrate to the amount of 2.5 ml from the renal vein of a normal rat under pentobarbital anesthesia (30 mg/kg, i.p.). Five hundred μl of the blood was mixed with 495 μl of the solution of TJ-8014 or dipyridamole dissolved with 0.9% NaCl to various concentrations in cuvettes. After incubating the mixture for 5 min at 37°C, either collagen (5 μg/mL) or arachidonate (160 μM/ml), was added to the incubated mixture, and platelet aggregation was then measured. The platelet aggregation was expressed as resistance rate between two electrodes. To evaluate the in vivo effects of TJ-8014 and dipyridamole, both drugs were given p.o. daily to groups of 5 rats after anti-GBM serum injection. Control group of 5 rats was given p.o. the vehicle (1% gum arabic) instead of test drugs. The animals of drug-treated and control groups were taken blood from the renal vein on the 0, 1st, 5th and 10th days, respectively, after anti-GBM serum injection. The platelet aggregation was measured as done in the in vitro test.

Statistical analysis: The data represent the mean±S.D., and the results were statistically evaluated by analysis of variance, Student's t-test and Mann-Whitney's U-test.

Results

1. Effects of TJ-8014 and dipyridamole on original-type anti-GBM nephritis in rats

Body weight and urine volume (data not shown): The average body weight of all groups at the onset of the experiment was 155±4.3 g. On the 12th day when the effects of test drugs were evaluated, the body weight of the normal group increased by 32 g, whereas that of the control group with nephritis lost 6 g (P<0.05). The body weight of TJ-8014 (0.5, 2.0 and 3.0 g/kg/day) and dipyridamole (0.2 and 0.4 g/kg/day)-treated groups lost weight slightly after treatment for 12 days. However, no significant difference was observed between each drug-treated group and the control group.

On the other hand, the urine volume of normal and control groups on the 12th day was 15.5±1.8 ml and 20.8±3.7 ml, respectively. No significant difference was seen between the normal and control groups and between each drug-treated group and the control group.

Urinary protein excretion and plasma UN content (Fig. 2): TJ-8014 at 2.0 and 3.0 g/kg/day inhibited the urinary protein excretion by 35% and 42%, respectively. Dipyridamole at 0.4 g/kg/day also inhibited the protein excretion by 32%. TJ-8014 at 0.5, 2.0 and 3.0 g/kg/day inhibited the elevation of the plasma UN level by 50%–80%. However, the UN level was little affected by dipyridamole (0.4 g/kg/day).

Histopathological parameters in glomeruli (Fig. 3): TJ-8014 at 0.5, 2.0 and 3.0 g/kg/day dose-dependently reduced the hypercellularity by 30%, 53% and 63%, respectively, and the adhesion index by 35%, 58% and 70%, respectively. Dipyridamole at 0.2 g/kg/day showed a 50% inhibition of the adhesion index.

2. Effects of each crude drug which constitutes TJ-8014 on original-type anti-GBM nephritis

Urinary protein excretion and plasma UN content (Table 2): Of the eight crude drugs of TJ-8014, only Holen (0.2 g/kg/day) significantly inhibited the urinary protein excretion by 24% and only Zizyphi fructus (0.2 g/kg/day) was significantly effective in inhibiting the elevation of the plasma UN level.

Histopathological parameters in glomeruli (Table 2): Ginseng radix, Bupleuri radix, Glycyrrhizae radix, Holen and Zizyphi fructus (0.2 g/kg/day) significantly reduced the hypercellularity in glomeruli by 35%–70%. Ginseng radix and Holen significantly reduced the adhesion index by 28% and 65%, respectively.

2. Effects of TJ-8014 and dipyridamole on
Antinephritic Effect of TJ-8014

Fig. 2. Effects of TJ-8014 and dipyridamole on urinary protein excretion and plasma urea nitrogen content in original-type anti-GBM nephritis in rats. Test drugs were given orally, daily during the period from the next day (the 1st day) of anti-GBM serum injection to the 12th day. Each column denotes the mean±S.D. of 8 rats. The number in parentheses indicates a percent inhibition which is derived from the following formula:

\[
\text{C-T} \times 100 \quad (\text{C: Control, T: Test drug, N: Normal})
\]

* and ** indicate a significant difference from the control at P<0.05 and 0.01, respectively.

Fig. 3. Effects of TJ-8014 and dipyridamole on histopathological parameters in glomeruli in original-type anti-GBM nephritis in rats. The value for the adhesion of the normal group is 0. The number in parentheses indicates the percent inhibition, which is derived from the following formula:

\[
\frac{\text{C-T}}{\text{C-N}} \times 100 \quad (\text{C: Control, T: Test drug, N: Normal})
\]

*, ** and *** indicate a significant difference from the control at P<0.05, 0.01 and 0.001, respectively. For other references, see the legend to Fig. 2.

platelet aggregation

The in vitro assay (data not shown): The IC50 value (concentration producing 50% inhibition) for TJ-8014 on collagen- and arachidonate-induced platelet aggregation was 575 µg/ml and 551 µg/ml, respectively. The IC50 value for dipyridamole on collagen- and arachidonate-induced platelet aggregation was 380 and 320 µg/ml, respectively. The potency ratio of TJ-8014 to dipyridamole was 0.66 and 0.58, respectively, on collagen- and arachidonate-induced aggregation.
Table 2. Effects of crude drugs that constitute TJ-8014 on urinary protein excretion, plasma urea nitrogen and histopathological parameters in glomeruli in original-type anti-GBM nephritis in rats

<table>
<thead>
<tr>
<th>Groups</th>
<th>Urinary protein (mg/day)</th>
<th>Urea nitrogen (mg/dl)</th>
<th>Hypercellularity (number/section)</th>
<th>Adhesion (index)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>15.2 ± 4.5</td>
<td>18.8 ± 3.9</td>
<td>51.9 ±0.8</td>
<td>0</td>
</tr>
<tr>
<td>Control</td>
<td>241.3±59.4</td>
<td>30.8± 5.4</td>
<td>87.2±7.4</td>
<td>32.1±13.6</td>
</tr>
<tr>
<td><strong>Bupleuri radix</strong></td>
<td>245.8±63.3</td>
<td>31.2± 5.9</td>
<td>69.4±5.0***</td>
<td>29.5±10.3</td>
</tr>
<tr>
<td><strong>Pinelliae tuber</strong></td>
<td>238.3±79.1</td>
<td>34.1±10.0</td>
<td>83.1±6.9</td>
<td>24.9± 8.7</td>
</tr>
<tr>
<td><strong>Glycyrrhizae radix</strong></td>
<td>231.6±69.9</td>
<td>34.5± 4.9</td>
<td>68.2±6.1***</td>
<td>27.6± 8.7</td>
</tr>
</tbody>
</table>

Control                  | 206.5±47.5               | 23.7± 3.1             | 72.4±3.5                          | 26.4± 5.9        |
| **Scutellariae radix**  | 193.7±73.2               | 22.6± 2.7             | 67.5±8.1                          | 25.1± 8.4        |
| **Ginseng radix**       | 160.9±46.9               | 20.4± 2.9             | 57.6±6.9**                        | 18.9± 6.1*       |
| **Coptidis rhizoma**    | 205.3±50.5               | 21.9± 2.3             | 71.0±5.0                          | 25.3± 8.5        |

Control                  | 301.8±67.0               | 22.5± 3.1             | 85.2±3.5                          | 34.4± 9.0        |
| **Holien**              | 232.3±58.0*              | 21.9± 4.4             | 74.7±6.7**                        | 11.9± 6.7***     |
| (24.3)                  |                          |                      | (31.5)                            | (65.0)           |
| **Zizyphi fructus**     | 296.5±47.5               | 19.6± 3.1*            | 74.1±5.2***                       | 27.1± 4.1        |
| (74.2)                  |                          |                      | (33.3)                            |                  |

The value for the adhesion of the normal group is 0. The number in parentheses indicates the percent inhibition, which is derived from the following formula:

\[
\frac{C-T}{C-N} \times 100 \quad (C: \text{Control}, \ T: \text{Test drug}, \ N: \text{Normal})
\]

*, ** and *** indicate a significant difference from the control at \(P<0.05\), \(0.01\) and \(0.001\), respectively. For other references, see the legend to Fig. 2.

Fig. 4. Effects of TJ-8014 and dipyridamole on platelet aggregation in original-type anti-GBM nephritis in rats. Test drugs were given orally, daily from the next day (the 1st day) of anti-GBM serum injection to the 10th day. Each plot denotes the mean±S.D. of 5 rats. The number in parentheses indicates the percent inhibition, which is derived from the following formula:

\[
\frac{C-T}{C-N} \times 100 \quad (C: \text{Control}, \ T: \text{Test drug}, \ N: \text{Normal})
\]

*, ** and *** indicate a significant difference from the normal group at \(P<0.05\), \(0.01\) and \(0.001\), respectively. * and *** indicate a significant difference from the control at \(P<0.05\) and \(0.001\), respectively.
The in vivo assay (Fig. 4): All through the experimental period of the 1st to 10th day, the platelet aggregation of the nephritic group (control) was significantly higher than that of the normal group. TJ-8014 (2.0 g/kg/day) inhibited the increase in platelet aggregation by 77% and 98%, respectively, on the 5th and 10th days. Dipyridamole (0.4 g/kg/day) showed a significant inhibition (62%) only on the increase in the platelet aggregation on the 10th day.

Discussion

In the present study, the original-type anti-GBM nephritis in rats employed for evaluating the antinephritic effect of drugs is characterized by moderate proteinuria and mild proliferation of mesangial cells, and it resembles mild proliferative glomerulonephritis in human (10).

In the first experiment, TJ-8014, a new Japanese herbal medicine, like dipyridamole, an antiplatelet agent, was markedly effective in inhibiting the urinary protein excretion as well as glomerular histopathological changes such as hypercellularity and adhesion to Bowman's capsule of capillary walls.

Although TJ-8014 is a lyophilized extract prepared from eight crude drugs as shown in Table 1, it is unclear which crude drugs have the antinephritic action. Therefore, the next experiment was carried out to evaluate the effect of each crude drug on this nephritis. In this case, all crude drugs were given p.o. at a dose of 0.2 g/kg/day, which is the dose required to show various other pharmacological actions. Of the crude drugs, only Holen was significantly effective in reducing proteinuria. Holen also significantly reduced both the hypercellularity and the adhesion. Ginseng radix was effective on both histopathological parameters. Bupleuri radix and Zizyphi fructus were effective only on the hypercellularity. Therefore, the marked antinephritic effect of TJ-8014 may be due to the synergism of these crude drugs.

Currently, it is generally believed that intraglomerular coagulation and platelet aggregation play an important role in the development and progression of various renal diseases (11–15). Platelets may adhere to collagen exposed by the endothelial denudation on injured glomerular capillary walls and then release ADP and adrenaline from the adhered platelets. This released ADP and adrenaline may then cause platelet aggregation, which leads to the obstruction of the intraglomerular microcirculation and causes the release of vasoactive amines such as serotonin and histamine. Both vasoactive amines may cause the increase in the permeability of the GBM, which leads to the leakage of a large amount of blood albumin into the urine. Moreover, it has been demonstrated that platelet-derived growth factor (PDGF) released from the platelets could cause the proliferation of mesangial cells, which may lead to adhesion of capillary walls to Bowman's capsule and hypercellularity in glomeruli (16). Therefore, in the third experiment, we examined the effect of TJ-8014 on platelet aggregation as a possible mechanism of the antinephritic action of this medicine. In the in vitro assay, the potency ratio of TJ-8014 to dipyridamole was approx. 0.66 times. The platelet aggregation of the nephritic control group significantly elevated from the 1st day after anti-GBM serum injection and was maintained at higher levels than normal levels until the 10th day (the last day of the assay). In the in vivo assay, TJ-8014 (2.0 g/kg/day) significantly inhibited the elevation of the platelet aggregation on the 5th and 10th days, while dipyridamole (0.4 g/kg/day) inhibited it only on the 10th day. Although we did not examine the antiplatelet action of each crude drug which constitutes TJ-8014, it has been indicated that ginsenosides Rg1, Rg2, and Rg3, which are contained in Ginseng radix, and Holen have antiplatelet action in an in vitro assay system (17–19). In the present study, Ginseng radix and Holen also showed effectiveness in this model. Our results and the above findings suggest that TJ-8014 may prevent the urinary protein excretion and glomerular histopathological changes (i.e., adhesion of capillary walls to Bowman's capsule and hypercellularity) by inhibiting the release of vasoactive amines and the PDGF from platelets as a result of the antiplatelet action of this medicine. Therefore, the mechanisms of the antinephritic
action of TJ-8014 may be partly due to the antiplatelet action of this agent.

The detailed mechanisms of the antiplatelet action of TJ-8014 remain unclear. In the preliminary experiment, however, we found that TJ-8014 inhibited the synthesis of thromboxane A2 in isolated glomeruli. This result indicates that TJ-8014 inhibits the platelet aggregation through the inhibition of thromboxane synthesis in glomeruli.

Dipyridamole, an antiplatelet agent, used as a comparative drug, was first applied for the clinical treatment of renal diseases such as in anticoagulant therapy by Kincaid-Smith et al. (20). They reported that dipyridamole in combination with anticoagulants in some cases resulted in a dramatic improvement of renal function and disappearance of proteinuria. We previously reported that trimetazidin, an antiplatelet agent, and Y-19018, a thromboxane synthetase inhibitor, had a beneficial effect on anti-GBM nephritis in rats (21, 22). These findings suggest that drugs which have antplatelet action may be useful for the treatment of nephritis.

References


