Effect of DP-1904, a Thromboxane A₂ Synthase Inhibitor, Administered from the Autologous Phase on Crescentic-Type Anti-GBM Nephritis in Rats

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ABSTRACT—The antinephritic effect of DP-1904 [6-(1-imidazolylmethyl)-5,6,7,8-tetrahydronaphthalene-2-carboxylic acid hydrochloride], a thromboxane (TX) A₂ synthase inhibitor, was compared with that of OKY-046 and azathioprine, using an experimental model of nephritis, crescentic-type anti-glomerular basement membrane (GBM) nephritis. Test drugs were given p.o. once daily from an autologous phase in which proteinuria was already fully developed. DP-1904 (15 and 45 mg/kg per day) and OKY-046 (20 mg/kg per day), another TXA₂ synthase inhibitor, significantly inhibited the development of glomerular alteration as well as the elevation of proteinuria. On the other hand, azathioprine (20 mg/kg per day), an immunosuppressive agent, failed to suppress the proteinuria. A single administration of DP-1904 or OKY-046 inhibited glomerular TXB₂ production and increased glomerular prostaglandin (PG) E₂ and 6-keto PGF₁₀ production in nephritic rats. Both drugs apparently decreased the depositions of both rabbit immunoglobulin (Ig) G and rat IgG on GBM in nephritic rats, but azathioprine inhibited only the deposition of rat IgG. These results suggest that DP-1904 may be an effective agent for the treatment of proliferative glomerulonephritis and its antinephritic effect may be due to the amelioration of abnormal metabolism of arachidonic acid.

Keywords: DP-1904, Thromboxane A₂ synthase inhibitor, Crescentic nephritis, Proteinuria

Recently, it has been demonstrated in experimental animal models that induction of immunological glomerular injury is associated with an increase in production of prostaglandins and thromboxane synthesis in isolated glomeruli (1-3) and renal cortex (4). It had been believed that thromboxane (TX) A₂ mediates the deterioration of renal function due to its vasoconstrictive, platelet pro-aggregatory and chemotactic actions (5), while prostaglandins such as prostaglandin (PG) E₂ and PGI₂ improve renal function due to their vasodilator and antiplatelet actions. On the basis of these findings, we have already investigated the antinephritic effect of DP-1904 [6-(1-imidazolylmethyl)-5,6,7,8-tetrahydronaphthalene-2-carboxylic acid hydrochloride], a new TXA₂ synthase inhibitor (Fig. 1), given from the heterologous phase (the day of injection of the antiserum against glomerular basement membrane (GBM)) to rats with crescentic type anti-GBM nephritis, which resembles rapidly progressive glomerulonephritis in humans. The beneficial effect of DP-1904 found in such experiments may be due to the inhibition of platelet aggregability and the restoration of decreased renal tissue blood flow (6). However, in the clinical situation, therapy for glomerulonephritis is started from the establishment of nephritic symptoms. Therefore, in the present study, we compared the antinephritic effect of DP-1904 administered from the autologous phase of crescentic-type anti-GBM nephritis in rats with those of OKY-046 and azathioprine.

Fig. 1. Chemical structure of DP-1904.
MATERIALS AND METHODS

1. Animals

Male Sprague-Dawley strain SPF rats (Nihon SLC, Shizuoka), weighing approximately 160 g, were used in the experiment. These animals were housed in an air-conditioned room at 22±2°C during the experimental period.

2. Drugs

DP-1904 and OKY-046 were kindly provided from Daiichi Pharmaceutical Co. (Tokyo) and Kissei Pharmaceutical Co. (Nagano), respectively. Azathioprine was purchased from Sigma Chemical Co. (St. Louis, MO, USA). DP-1904 and OKY-046 were dissolved in distilled water, and azathioprine was suspended in 1% gum arabic.

3. Experimental protocols

3.1. Protocol for evaluation of antinephritic effect of DP-1904: Crescentic-type anti-GBM nephritis was induced in rats by injecting 6.5 mg rabbit gamma-globulin in 0.25 ml of Freund complete adjuvant into the hind foot pads, following the injection of 0.6 ml of rabbit anti-rat GBM serum into the tail vein, in accordance with the method reported previously (7). On the 20th day after the anti-GBM serum injection, urine samples were collected for 24 hr. The animals were then divided into 5 groups (n=8), so that the average protein content in the 24-hr urine samples of each group was at a similar level. Each of the five groups was given 5, 15 or 45 mg/kg of DP-1904 (anti-GBM+DP-1904), 20 mg/kg of OKY-046 (anti-GBM+OKY-046) or 20 mg/kg of azathioprine (anti-GBM+azathioprine) in a volume of 1 ml per 100 g of body weight, orally, daily from the 21st day after the anti-GBM serum injection to the 51st day. The remaining group was given orally distilled water instead of test drugs and served as the anti-GBM nephritic group (anti-GBM). In addition, a control group (n = 8) that did not receive the anti-GBM serum was used for comparison with the nephritic group.

3.2. Protocol for evaluation of antinephritic mechanisms of DP-1904: Crescentic-type anti-GBM nephritis was induced by the same method as indicated in section 3.1.

To investigate the effect of a single p.o. administration of test drugs on glomerular TXB2 (the stable metabolite of TXA2), PGE2 and 6-keto PGFIα (the stable metabolite of PGI2) production on the 21st and 51st days, we used 7 groups of 4 rats each: control group, anti-GBM nephritic group, anti-GBM+DP-1904 group (5, 15 and 45 mg/kg), anti-GBM+OKY-046 group (20 mg/kg) and anti-GBM+azathioprine group (20 mg/kg). The control rats were given p.o. distilled water. These animals were killed at 4 hr following the administration of each test drug. Glomeruli were then isolated from the kidneys.

4. Urine and blood collections

The 24-hr urine samples were obtained by keeping each animal in an individual metabolic cage for 24 hr without food and water at various intervals after the induction of nephritis. At the beginning of the urine collection, each animal received 8 ml of distilled water orally. The urine was then centrifuged at 810 × g for 15 min at 4°C, and the supernatant was used for the determination of protein.

Blood samples were also obtained at various intervals. Each 0.4 ml of blood was drawn from the tail vein of a conscious animal with a heparinized microsyringe. The blood was centrifuged at 2,250 × g for 20 min at 4°C to obtain the plasma for the determination of cholesterol and antibody titer against rabbit gamma-globulin. Immediately after the last collection of urine samples, blood was also taken from the renal vein under pentobarbital anesthesia for the same measurements.

5. Measurement of urinary protein, plasma creatinine and antibody titer

The urinary protein content was determined by the method of Kingsbury et al. (8) and expressed as mg/24-hr urine. The plasma creatinine content was determined with a commercial assay kit (CRE-EN; Kainos, Inc., Tokyo) and expressed as mg/dl per 100 g body weight. The plasma antibody titer against rabbit gamma-globulin was determined by indirect hemagglutination using sensitized sheep red blood cells (9) and expressed as the loge of the hemagglutination titer.

6. Assessment of histopathological parameters in the glomeruli

For light microscopic study, the kidneys were fixed in alcohol, and the tissues embedded in paraffin were then cut into 2- to 3-μm thick sections. The sections were stained with hematoxylin and eosin or Masson's trichrome. To assess the glomerular hypercellularity, the number of nuclei was counted and expressed as the number per equatorial cross section in ten glomeruli per animal. Crescent formation, adhesion of Bowman's capsule to capillary walls (adhesion) and fibrinoid necrosis in the glomeruli were evaluated by a different person who did not know the identify of the sections. For assessing each of the histopathological parameters, the degrees of crescent formation, adhesion and fibrinoid necrosis were scored as 1 (mild), 2 (moderate) and 3 (severe) (10). The number of the glomeruli corresponding to each score is shown as n1, n2 and n3. A crescent formation index (CI), an adhesion index (AI) and a fibrinoid necrosis index (FI) were calculated from the following formula:
Cl, Al and FI = $1 \times n_1 + 2 \times n_2 + 3 \times n_3$.

The index of glomerular lesions (IGL) was calculated for evaluating the degree of glomerular lesions as follows:

$$IGL = \frac{(3 \times CI) + (2 \times Al) + (1 \times FI)}{(3+2+1) \times 50}$$

We gave 3, 2 and 1 points to CI, Al and FI, respectively, because we consider that CI, Al and FI are associated with the glomerular lesion in this order; and the “50” shows the number of glomeruli.

7. Immunoperoxidase studies

An indirect immunoperoxidase technique employing avidin-biotin peroxidase kits (Vecta Stain ABC Kit; Vector Institution, Burlingam, CA, USA) was used throughout the study (11). In brief, the paraffin sections, prepared by the same method as described in section 3.1., were subsequently incubated with 1- to 5-fold diluted normal rabbit serum, an appropriate dilution of the individual mouse monoclonal antibody for rabbit immunoglobulin (Ig) G or rat IgG (Cappel Laboratories, Inc., West Chester, PA, USA), affinity-purified rabbit anti-mouse peroxidase conjugated immunoglobulin also at the appropriate dilution, and finally with diamino-benzidine tetrahydrochloride (0.5 mg/ml in PBS plus 0.01% H$_2$O$_2$) for 3–5 min. Under a light microscope, we selected the glomeruli that were equatorially sectioned and had the vascular pole.

The intraglomerular rabbit IgG or rat IgG depositions were measured by an image-analyzer (Imageanalyzer VI; Toyobo, Tokyo) and expressed as $\times 10^{-3}$ mm$^3$/glomerular cross section (GCS).

8. Effects of a single administration of DP-1904 on glomerular TXB$_2$, PGE$_2$ and 6-keto PGF$_{1\alpha}$ production in nephritic rats

The glomeruli were isolated by the differential sieving technique of Zoja et al. (12) from kidneys perfused with Krebs-Ringer phosphate-buffered saline (pH 7.2) obtained from the normal and nephritic rats that were treated as described in section 3.2. The isolated glomeruli (the purity was more than 80% when observed under a light microscope) were then incubated in Krebs-Ringer phosphate-buffered saline at 37°C for 30 min. The incubation mixture was then centrifuged, and the supernatant was frozen at $-70°C$ for the determination of TXB$_2$, PGE$_2$ and 6-keto PGF$_{1\alpha}$. The amounts of TXB$_2$, PGE$_2$ and 6-keto PGF$_{1\alpha}$ in the glomeruli were determined by radioimmunoassay (New England Nuclear, Boston MA, USA). The protein content in the glomeruli was assayed by a colorimetric method (Bio-Rad, Richmond, CA, USA).

9. Statistical analyses

The results obtained are expressed as the means S.D. The data were analyzed by one-way analysis of variance (ANOVA) or the Kruskal-Wallis test. To determine the significance of differences among the groups, Tukey’s test or Ryan’s procedure was used. Inhibitory percentage was calculated as follows:

$$\text{Inhibitory percentage (\%)} = \frac{(\text{anti-GBM} - \text{test drugs})}{(\text{anti-GBM} - \text{control})} \times 100$$

Fig. 2. Effects of DP-1904 administered from the autologous phase on urinary protein excretion and plasma creatinine content of crescentic-type anti-glomerular basement membrane nephritic rats. ○, □: control; ●, ■: anti-GBM; △, ▪: anti-GBM+DP-1904 (5 mg/kg); ◆, ●: anti-GBM+DP-1904 (15 mg/kg); ○, ◆: anti-GBM+DP-1904 (45 mg/kg); ♦, ▲: anti-GBM+OKY-046 (20 mg/kg); □, ▼: anti-GBM+azathioprine (20 mg/kg). Each plot or each column denotes the mean with S.D. for 8 rats. *P<0.05 versus control. **P<0.01 versus anti-GBM.
RESULTS

1. Antinephritic effects of DP-1904 on crescentic-type anti-GBM nephritis

1.1. Urinary protein excretion and plasma creatinine content (Fig. 2): When test drugs were given from the 21st day after the anti-GBM serum, DP-1904 at 15 and 45 mg/kg significantly suppressed the urinary protein excretion by 45% and 54%, respectively, through the 31st to 50th days. In addition, by the 51st day, this agent at the 2 doses mentioned had inhibited the increase of plasma creatinine content returning it to almost the normal level. OKY-046 at 20 mg/kg also inhibited the urinary protein excretion by 35%, and the plasma creatinine content was almost at the normal level on the 51st day. On the other hand, azathioprine at 20 mg/kg showed only a tendency to diminish both the urinary protein excretion and the plasma creatinine content.

1.2. Histopathological parameters in the glomeruli (Table 1 and Figs. 3 and 4): Regarding histopathological alteration of the glomeruli, DP-1904 at 5, 15 and 45 mg/kg had dose-dependently reduced the glomerular hypercellularity by 37% to 77% (Table 1). In addition, DP-1904 at the 3 doses reduced the IGL by 48% to 74% at the 51st day (Fig. 3). OKY-046 (20 mg/kg) also reduced it by 50%. On the other hand, azathioprine (20 mg/kg) slightly reduced it by 20% at the 51st day. Representative micrographs of the glomeruli from drug-treated and untreated anti-GBM nephritic rats are given in Fig. 4.

1.3. Effects on plasma antibody titer against rabbit gamma-globulin: At the 51st day, the elevation of the antibody titer due to nephritis was significantly inhibited by DP-1904 (15 and 45 mg/kg) (P <0.05 vs anti-GBM).

Table 1. Effect of DP-1904 administered from the autologous phase on glomerular hypercellularity in crescentic-type anti-GBM nephritis in rats

<table>
<thead>
<tr>
<th>Treatment</th>
<th>N</th>
<th>Hypercellularity (cells/GCS)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>8</td>
<td>51.0±0.6</td>
</tr>
<tr>
<td>Anti-GBM</td>
<td>8</td>
<td>60.5±0.7a</td>
</tr>
<tr>
<td>+ DP-1904 5 mg/kg</td>
<td>8</td>
<td>57.1±1.0b</td>
</tr>
<tr>
<td>+ DP-1904 15 mg/kg</td>
<td>8</td>
<td>54.6±0.3b</td>
</tr>
<tr>
<td>+ DP-1904 45 mg/kg</td>
<td>8</td>
<td>53.2±0.7b</td>
</tr>
<tr>
<td>+ OKY-046 20 mg/kg</td>
<td>8</td>
<td>56.3±0.1b</td>
</tr>
<tr>
<td>+ Azathioprine 20 mg/kg</td>
<td>8</td>
<td>58.1±1.4b</td>
</tr>
</tbody>
</table>

Test drugs were given p.o., daily to rats from the 21st day after injection of anti-GBM serum to the 51st day; and both kidneys were taken on the 51st day for light microscopic study. Values indicate the means ± S.D., and N indicates the number of rats used. GCS stands for glomerular cross-section. * indicates a significant difference from the control at P <0.01; † indicates a significant difference from the anti-GBM at P <0.05.

Azathioprine (20 mg/kg) markedly suppressed the antibody titer (P <0.01 vs anti-GBM). The antibody titer at the 51st day was as follows: anti-GBM, 6.4±0.9; anti-GBM + DP-1904, 5 mg/kg, 6.0±1.5; 15 mg/kg, 5.4±0.9; 45 mg/kg, 5.3±1.0; anti-GBM + OKY-046, 6.1±1.2; anti-GBM + azathioprine, 3.9±0.7.

1.4. Effects on the deposition of rabbit IgG and rat IgG in the glomeruli (Fig. 5): In the section of anti-GBM nephritic rats, a linear deposition of rabbit IgG (hetero-antibody) and rat IgG (auto-antibody) was observed along the GBM. DP-1904 (15 and 45 mg/kg) and OKY-046 (20 mg/kg) apparently reduced the depositions of both rabbit IgG and rat IgG. On the other hand, azathioprine (20 mg/kg) markedly reduced only the deposition of rat IgG.

2. Mechanisms of the antinephritic action of DP-1904 and OKY-046

2.1. Effects of a single administration of DP-1904 on glomerular TXB2, PGE2 and 6-keto PGF1α production (Fig. 6): The effects of DP-1904 and OKY-046 on glomerular TXB2, PGE2 and 6-keto PGF1α production were examined at 4 hr after the single p.o. administration of test drugs to nephritic rats on the 21st and 51st days after anti-GBM serum injection. Glomerular TXB2 production in the anti-GBM group was 2.5-fold higher than that of the control group at the 21st and 51st days. On the other hand, the levels of glomerular PGF2α and 6-keto PGF1α production were similar between the anti-GBM nephritic animals and the control animals. At 4 hr after drug treatment, DP-1904 at 5, 15 and 45 mg/kg dose-dependently inhibited the increment of glomerular TXB2 production by 23% to 85% at the 21st and 51st days. In addition,
DP-1904 at 5, 15 and 45 mg/kg caused increases by 15% to 27%, in PGE$_2$ and increases by 21% to 45%, in 6-keto PGF$_{10}$, OKY-046 (20 mg/kg) also decreased TXB$_2$ production by 36% to 39%, while it resulted in an increase of 12%, in PGE$_2$ and an increase of 26% in 6-keto PGF$_{10}$ at the 51st day. On the other hand, azathioprine (20 mg/kg) had no effects on glomerular PGs production.

**DISCUSSION**

The glomerular injury of anti-GBM nephritis in rats has been demonstrated to consist of a heterologous phase and an autologous phase. Immediately after the injection of anti-GBM serum (rabbit anti-GBM antibody, heteroantibody), the heterologous phase is caused by the binding of the injected rabbit anti-GBM antibody to the glomeruli followed by the activation of complement. Following this, 7 to 10 days later, the autologous phase is in-
started drug administration from the autologous phase. The effects of DP-1904 on anti-GBM nephritis were more potent than those of OKY-046. Recently, it has been reported that glomerular synthesis and/or urinary excretion of vasoconstrictive TXA\textsubscript{2} and vasodilator PGE\textsubscript{2} are significantly increased in various experimental models of renal diseases such as anti-GBM nephritis (1, 3, 13, 14), passive Heymann nephritis (15), adriamycin nephrosis (16), cyclosporin A nephrotoxicity (17) and murine lupus nephritis (4). Furthermore, Lianos et al. reported that in anti-GBM nephritic rats, the increased glomerular TXA\textsubscript{2} synthesis correlates positively with the extent of proteinuria and negatively with the decrease in glomerular filtration rate (1). The origin of increased TXA\textsubscript{2} synthesis is uncertain. In anti-GBM nephritis, the heterologous phase is characterized by intraglomerular activation of complement and platelets and infiltration of neutrophils, while the autologous phase is characterized by infiltration of macrophages/monocytes and proliferation of mesangial cells (18). In a platelet depletion study, Wu et al. demonstrated that platelets and neutrophils are critical to the imbalanced glomerular arachidonic metabolism, especially TXA\textsubscript{2} and leukotriene B\textsubscript{4}, in the heterologous phase of anti-GBM nephritis (19). Mesangial cells in culture may be induced to release prostanooids by a variety of cytokines (20–22). Additionally, interleukin (IL)-1 and tumor necrosis factor (TNF)-\alpha increase cyclooxygenase activity and protein mass in mesangial cells (23). Martin et al. recently demonstrated that IL-1 enhanced the expression of cyclooxygenase 2 mRNA and protein synthesis in mesangial cells (24). Therefore, it is considered in the cases of crescentic-type anti-GBM nephritis that the origin of increased synthesis of TXA\textsubscript{2} in the heterologous phase may be mainly neutrophils and platelets, while that in the autologous phase may originate from mainly macrophages/monocytes and the mesangial cells.

When the effects of a single administration of DP-1904 or OKY-046 on the synthesis of glomerular prostanooids were examined using the isolated glomeruli that had been under nephritic conditions, glomerular PGE\textsubscript{2} and 6-keto PGF\textsubscript{1\alpha} productions were similarly increased by both drugs. Accordingly, it is suggested that the antinephritic effect of DP-1904 and OKY-046 may be attributed to the suppression of excessive glomerular TXA\textsubscript{2} synthesis or the increase in the ratio of PGE\textsubscript{2} and PGI\textsubscript{2} to TXA\textsubscript{2} produced by both drugs in the autologous phase as well as in the heterologous phase (6). Although the precise mechanism by which DP-1904 and OKY-046 increase the PGE\textsubscript{2} and PGI\textsubscript{2} production remains unclear, it seems reasonable to consider that the inhibition by TXA\textsubscript{2} synthetase inhibitors of TXA\textsubscript{2} synthesis in arachidonic metabolism promotes the transformation of PG endo-peroxides (PGG\textsubscript{2} and PGH\textsubscript{2}) to PGE\textsubscript{2} and PGI\textsubscript{2}. This observation illustrates the more potent effect of DP-1904 in comparison to OKY-046.

In the present experiment, DP-1904 and OKY-046 apparently reduced the depositions of both rabbit IgG and rat IgG on GBM of anti-GBM nephritic rats. On the other hand, azathioprine markedly inhibited the antibody titer and reduced only the deposition of rat IgG. The decrease in glomerular rabbit IgG deposition by DP-1904 and OKY-046 may be due to the elevation in the ability of both drugs to eliminate glomerular fixed hetero antibody. Regarding these results, we demonstrated that TXA synthetase inhibitors were able to elevate eliminating hetero-substances in the glomeruli (unpublished data). Furthermore, the reduction of antibody titer by a long-term administration of DP-1904 may be partially due to the increase of the immunosuppressive eicosanoid PGE\textsubscript{2}. PGE\textsubscript{2} has been shown to down-regulate T lymphocyte function (25) likely through its ability to decrease IL-1 and IL-2 production (26) and its capacity to decrease class II expression on antigen-presenting cells such as macrophages (27). Moreover, PGE\textsubscript{2} has also been demonstrated to possess, in addition to immunosuppressive effects, the capacity to enhance certain aspects of the immune response such as IgG and IgE production (28). Hattori et al. reported that azathioprine administered from the heterologous phase had a antinephritic effect on rat crescentic-type anti-GBM nephritis (29). In the present study, DP-1904 and OKY-046, TXA\textsubscript{2} synthase inhibitors, administered from the autologous phase was effective against proteinuria, but azathioprine was not. Azathioprine apparently reduced the deposition of rat IgG in the glomeruli according to the suppression of auto-antibody formation. Azathioprine had no effects on glomerular PGs production and glomerular rabbit IgG deposition, although TXA\textsubscript{2} synthase inhibitors increased glomerular PGE\textsubscript{2} and PGI\textsubscript{2} synthesis and diminished glomerular rabbit IgG deposition. These results suggest that, because TXA\textsubscript{2} synthase inhibitors inhibit platelet aggregability and can restore decreased renal tissue blood flow by ameliorating the abnormal production of arachidonic acid metabolites (6) in addition to the decreasing effect of glomerular rabbit IgG; These data establish that TXA\textsubscript{2} synthase inhibitors may be more useful than immunosuppressive agents such as azathioprine for the treatment of glomerulonephritis.

DP-1904 administered from the autologous phase has a more potent antinephritic action than OKY-046 on rat crescentic-type anti-GBM nephritis. OKY-046 has been reported to be clinically effective for the treatment of chronic glomerulonephritis accompanied by a nephrotic syndrome (30). Therefore, DP-1904 is expected to have a clinical effect on this type of chronic glomerulonephritis.
REFERENCES


