Effects of FK506 on experimental membranous glomerulonephritis induced by cationized bovine serum albumin in rats

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Abstract

Study suggests that FK506 may be useful in patients with intractable nephrotic syndrome such as MN.

Background. There have been no reports on the effect of FK506, a new immunosuppressive agent, on experimental membranous glomerulonephritis (MN) induced by an exogenous antigen. Therefore we investigated the effects of FK506 on MN induced by cationized bovine serum albumin (C-BSA) in rats.

Methods. Two weeks after the rats were immunized with 1 mg of C-BSA and Freund’s complete adjuvant, they received intravenous injections of 3 mg/kg of C-BSA 3 times weekly for 4 weeks. Rats were divided into four groups: group A (n = 5) received intramuscular injections of 3 mg/kg of FK506 daily for 5 days beginning 2 days before the first immunization; group B (n = 4) received 1 mg/kg of FK506 daily for 2 weeks beginning 2 weeks after the first immunization; and group C (n = 4) received 1 mg/kg of FK506 daily for 2 weeks beginning 4 weeks after the first immunization. Group D (n = 5) received no FK506 and served as the control group. Rats were sacrificed 8 weeks after the first immunization.

Results. Administration of FK506 in the preimmunization stage almost completely suppressed the development of MN in group A. Histological findings in groups B and C were similar to those in group D, the control group. However, urinary protein excretion was significantly lower in group B (24 ± 46 mg/day) and C (220 ± 44 mg/day) than in group D (330 ± 61 mg/day). Expression of intracellular adhesion molecule-1 in glomeruli and the number of leukocyte function-associated molecules-1 were significantly decreased in groups A, B, and C compared with the control group. Administration of FK506 was not associated with any significant side-effects or histological abnormalities. The whole-blood trough levels of FK506 in groups B and C were 9.1 ng/ml and 9.2 ng/ml respectively.

Conclusions. The efficacy of FK506 was significantly increased when the drug was administered in the early phase of immunization in this model. The present study suggests that FK506 may be useful in patients with intractable nephrotic syndrome such as MN.

Key words: cationized BSA; exogenous antigen; experimental membranous glomerulonephritis; FK506; proteinuria

Introduction

FK506 is a new immunosuppressive agent with a molecular action similar to cyclosporin A (CsA). These immunosuppressive agents inhibit the activation of several transcription factors such as nuclear factor (NF) of activated T cells (NF-AT) and NF-κ B, which are critical for T-cell activation [1]. FK506 is a more potent inhibitor of antigen-driven T-cell activation, cytokine production, and lymphocyte proliferation in vitro than CsA [2]. The systemic level of FK506 required to induce and maintain immune suppression is approximately 100-fold below the corresponding blood levels of CsA [3]. FK506 was recently approved in the USA for the treatment and prevention of graft-versus-host rejection after solid-organ and bone-marrow transplantation [4]. However, data on the use of FK506 to treat human glomerulonephritis is limited [5,6]. Studies have shown that FK506 is effective in animal models of spontaneous lupus nephritis [7,8] and nephritis induced by endogenous antigens such as Masugi nephritis [9,10] and Heymann nephritis [10,11]. However, there have been no reports on the effect of FK506 on experimental membranous glomerulonephritis (MN) induced by an exogenous antigen. We investigated the effects of FK506 on MN induced by cationized bovine serum albumin (C-BSA) in rats.

Subjects and methods

Preparation of antigen

To prepare C-BSA, crystallized unmodified BSA (Sigma Chemical Co, St Louis, MO, USA) was chemically cationized according to Border’s method [12].
**Immunosuppressive agent**

FK506 was kindly provided by Fujisawa Pharmaceutical Co. (Osaka, Japan) and was dissolved in saline for injections.

**Experimental design**

Six-week-old female Wistar rats (Doken Co., Shimodate, Ibaraki, Japan) weighing 150 g were immunized with 1 mg of C-BSA and Freund's complete adjuvant. Two weeks later, rats were injected intravenously with 3 mg/kg of C-BSA 3 times weekly every other day for 4 weeks. Rats were divided into four groups: group A (n = 5) received intramuscular injections of 3 mg/kg of FK506 daily for 5 days beginning 2 days before the first immunization; group B (n = 4) received daily injections of 1 mg/kg of FK506 for 2 weeks beginning 2 weeks after the first immunization; group C (n = 4) received daily injections of 1 mg/kg of FK506 for 2 weeks beginning 4 weeks after the first immunization, and group D (n = 5) served as the disease control group and received no FK506. All rats were sacrificed 8 weeks after the first immunization.

**Measurements of urinary protein and serum parameters**

Urine samples were collected weekly. Blood samples were collected every 2 weeks. At sacrifice, rats were anaesthetized and blood was obtained via cardiac puncture. The animals were then immediately killed and the kidneys were removed.

The daily urinary protein excretion was measured using the Kingsburg-Clark method. Serum levels of creatinine, total cholesterol, and albumin were measured with an autoanalyzer.

**Histological examination**

Kidney specimens were routinely processed for light, immunofluorescence, and electron microscopy as described previously [13]. For light microscopy, sections fixed in 10% buffered formalin were stained with haematoxylin-eosin (H&E), periodic acid–Schiff (PAS), periodic acid–Schiff methenamine silver (PAM), and Masson's trichrome. Frozen sections for immunofluorescence microscopy were fixed with FITC-labelled rabbit anti-rat IgG and C3 (Organon Teknika Corp., West Chester, PA, USA). Tissues for electron microscopy were fixed immediately in 3% glutaraldehyde. Ultra-thin sections were stained with uranyl acetate and lead citrate.

**Polyethyleneimine (PEI) staining**

To identify anionic sites in the glomerular basement membrane (GBM), specimens were subjected to the PEI (MW: 1800) immersion method of Schurer et al. [14]. The number of PEI particles per 1 μm of GBM was determined at 10 GBM sites. The average of the 10 determinations is the PEI score. A 2000-line/mm diffraction grating (Okenshoji, Tokyo, Japan) was used for calibration.

**Immunohistochemistry**

Indirect immunofluorescence or avidin–biotin complex (ABC) method was performed on frozen sections. The following primary antibodies were used: monoclonal mouse anti-rat intracellular adhesion molecule-1 (ICAM-1) [15], leukocyte functioning-associated molecule-1 (LFA-1) [16], and polyclonal rabbit anti-mouse tumour necrosis factor-α (TNF-α) [17] purchased from Genzyme, Cambridge, MA, USA. For the indirect immunofluorescence method, the secondary antibodies were FITC-conjugated anti-mouse IgG and goat anti-rabbit IgG (Organon Teknika Corp.). These secondary antibodies were absorbed with rat serum before use to avoid non-specific cross-reaction between rat IgG and the antibodies. For the ABC method, the secondary antibodies were peroxidase-conjugated horse anti-mouse IgG and goat anti-rabbit IgG (Funakoshi Inc., Tokyo, Japan). The ABC method was performed according to the manufacturer's instructions except for absorption of antibodies with rat serum.

**Evaluation of immunofluorescence and immunohistochemistry**

The sections were examined without prior knowledge by two of us (MK and KM). The intensity of ICAM-1 was graded as none (−), trace (+), mild (1+), moderate (2+), or severe (3+), and semiquantitatively scored as follows: (−) 0, (+) 0.5 point, (1+) 1 point, (2+) 2 points, and (3+) 3 points. Cells positive for LFA-1 were counted in 10 randomly selected glomeruli. The data are expressed as the number of positive cells per glomerulus.

**Measurement of serum levels of anti-C-BSA antibody**

The serum level of antigen-specific IgG was measured by ELISA. Briefly, flat-bottomed microtitre plates were coated overnight with 0.5 μg/well of C-BSA in 0.02 M sodium carbonate buffer, pH 9.8. After the wells were washed five times with phosphate-buffered saline (PBS) containing 0.05% Tween 20 (PBS/T), blocking was performed using 1% Block Ace solution (Yamanouchi Pharmaceutical Co., Tokyo, Japan), and the plates were incubated at room temperature for 1 h. The wells were then washed as before and serum samples diluted in 1% Block Ace solution were added in duplicate or triplicate, plates were then incubated at 37 °C for 1 h. After wells were washed, 50 μl of peroxidase-conjugated goat anti-rat IgG (× 10000) in 1% Block Ace solution was added to the wells. After plates were incubated for an additional hour and washed, 100 μl of an o-phenylenediamine substrate solution was added. The reaction was stopped by the addition of 50 μl of stopping buffer (2 mM NaNO₃) and the absorbance was determined at 450 nm. Data were expressed as OD 450 units.

**Trough levels of FK506 in whole blood**

We measured the trough levels of FK506 in whole blood from groups B and C using a two-step enzyme immunonassay (EIA) [18]. Briefly, blood samples were obtained in groups B and C 24 h after the last FK506 injection. Heparinized whole blood (0.5 ml) was extracted by dichloromethane. Samples were processed by two-step EIA in a flat-bottom microtitre plate. The detection limit was 0.5 ng/ml.

**Statistical analysis**

Data are expressed as the mean ± standard deviation. Data were analysed using the unpaired t-test or the Mann–Whitney U test. A level of P < 0.05 was considered statistically significant.
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Results

Urine protein excretion

Urinary protein excretion was less than 10 mg/day in all rats in the preimmunization phase and at 1 and 2 weeks after immunization (Figure 1), and showed no significant change throughout the experimental period in group A. Urinary protein excretion in group B was similar to that in group A and was significantly lower than in group D by 5 weeks. Urinary protein excretion gradually increased in group D. At 8 weeks, urinary protein excretion was significantly lower in group C than in group D (Figure 1, Table 1).

Serum parameters

There was no significant difference in the serum creatinine level at 8 weeks among groups (Table 1). The serum level of albumin in group A tended to be higher than in groups B, C, and D. The level of total cholesterol was significantly lower in groups A, B, and C than in group D.

Histological findings

There were no significant differences in light microscopic findings among groups. Immunofluorescence microscopy showed marked rat IgG and C3 staining in the peripheral capillary walls and to some extent in the mesangial area in groups B, C, and D in inverse order of intensity (Table 2). Weak staining was observed in group A.

Transmission electron microscopy showed no electron-dense deposits (EDDs) in the glomerular basement membrane or the mesangial area in group A (Figure 2). Patchy subepithelial EDDs were observed in group B (MN stage I, based on Ehrenreich and Churg's classification [19]). A patchy and diffuse

![Graph showing urinary protein excretion over weeks.](image)

Fig. 1. Urinary protein excretion.

Table 1. Serum and urinary measurements at sacrifice

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>UP (mg/day)</th>
<th>s-Cre (mg/dl)</th>
<th>Albumin (g/dl)</th>
<th>T. chol (mg/dl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>5</td>
<td>6 ± 4**</td>
<td>0.6 ± 0.1</td>
<td>5.1 ± 0.2</td>
<td>79.5 ± 8.3**</td>
</tr>
<tr>
<td>B</td>
<td>4</td>
<td>24 ± 46**</td>
<td>0.7 ± 0.2</td>
<td>4.6 ± 0.8</td>
<td>72.4 ± 18.8**</td>
</tr>
<tr>
<td>C</td>
<td>4</td>
<td>220 ± 44**</td>
<td>0.6 ± 0.1</td>
<td>4.4 ± 0.5</td>
<td>221.7 ± 18.7*</td>
</tr>
<tr>
<td>D</td>
<td>5</td>
<td>330 ± 61</td>
<td>0.6 ± 0.0</td>
<td>3.9 ± 0.2*</td>
<td>385.7 ± 59.4</td>
</tr>
</tbody>
</table>

UP, urinary protein excretion; s-Cre, serum creatinine; T. chol, total cholesterol.

*P < 0.05, **P < 0.01 vs group D.
Table 2. Histological and PEI findings

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>IgG score ± SD</th>
<th>C3 score ± SD</th>
<th>Stage of MN</th>
<th>PEI score ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>5</td>
<td>0.6 ± 0.3*</td>
<td>0.5 ± 0.6*</td>
<td>0</td>
<td>32.5 ± 1.1*</td>
</tr>
<tr>
<td>B</td>
<td>4</td>
<td>2.6 ± 0.4</td>
<td>1.2 ± 0.4</td>
<td>I</td>
<td>29.4 ± 4.6*</td>
</tr>
<tr>
<td>C</td>
<td>4</td>
<td>2.9 ± 0.1</td>
<td>1.4 ± 0.5</td>
<td>I–II</td>
<td>18.1 ± 1.6</td>
</tr>
<tr>
<td>D</td>
<td>5</td>
<td>3.0 ± 0.0</td>
<td>1.7 ± 0.3</td>
<td>I–II</td>
<td>20.1 ± 5.5</td>
</tr>
</tbody>
</table>

PEI score, number of PEI particles per 1 μm of glomerular basement membrane.

*P < 0.05 vs group D.

distribution of subepithelial EDDs was observed in groups C and D (MN stage I to II).

**PEI staining of the GBM**

The PEI score was similar in groups C and D, which was significantly lower than in groups A and B (Table 2), indicating that the anionic sites of the GBM were relatively well preserved in groups A and B (Figure 3).

***Immunohistochemical findings***

No positive staining for ICAM-1 was observed in glomeruli from animals in group A. Glomerular expression of ICAM-1 was observed mainly in the capillary walls and faintly in the mesangial areas in groups B and C (Figure 4). The ICAM-1 and LFA-1 scores were significantly higher in group D than in groups A, B, and C (Table 3).

No peripheral capillary staining for TNF-α was observed in any animal. Some of the glomeruli in each group showed intraglomerular cells and subepithelial cells that were positive for TNF-α.

***Serum anti C-BSA antibody titre***

The anti C-BSA titres were significantly lower in group A than group D, although titres in group A were not completely negative between 2 and 8 weeks after immunization (Figure 5). The titres in groups C and D increased rapidly by 2 weeks after immunization and remained elevated throughout the experiment. The titres in group B were similar to those in groups C and D, except for a significant decline in group B at 6 weeks.

![Fig. 2 A–D. Electron microscopy of glomerular capillary walls. (A) The structure of glomerular capillary walls was normal in group A (×13 000). (B) Scattered electron-dense deposits (EDDs) were observed in subepithelial sites in group B, but the foot processes were preserved (×6500). (C) Subepithelial EDDs showed a partly patchy and partly diffuse pattern of distribution in group C. Diffuse fusion of the foot processes was observed (×4700). (D) The pattern of EDDs in group D resembled that in group C (×6300).](image-url)
Fig. 3 A–D. Electron microscopic findings using PEI staining. (A, B) PEI particles were observed in the lamina rara externa and interna at relatively regular intervals in groups A and B. (C, D) The number of PEI particles was decreased and intervals were irregular in groups C and D (×38,000).
Table 3. Immunohistochemical findings

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>ICAM-1 score</th>
<th>LFA-1 score</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>5</td>
<td>0.0 ± 0.0*</td>
<td>0.4 ± 0.3*</td>
</tr>
<tr>
<td>B</td>
<td>4</td>
<td>0.8 ± 0.3**</td>
<td>0.7 ± 0.4*</td>
</tr>
<tr>
<td>C</td>
<td>4</td>
<td>0.5 ± 0.3**</td>
<td>0.8 ± 0.3*</td>
</tr>
<tr>
<td>D</td>
<td>5</td>
<td>1.7 ± 0.1</td>
<td>1.5 ± 0.4</td>
</tr>
</tbody>
</table>

ICAM-1 score, the average of glomerular intensity per glomerulus; LFA-1 score, the number of intraglomerular positive cells per glomerulus.

*P < 0.05, **P < 0.01 vs group D.

Trough levels of FK506

The whole-blood trough levels of FK506 24 h after the last injection of FK506 was 9.1 ± 1.1 ng/ml and 9.2 ± 0.8 ng/ml in groups B and C respectively.

Discussion

The induction of MN by C-BSA was almost completely suppressed when FK506 was administered during the preimmunization stage in group A. No significant glomerular lesions or specific antibody responses were observed, presumably because FK506 prevented the activation of T-helper cells during the immunization stage.

When FK506 was administered at 2 weeks or 4 weeks after immunization with C-BSA, histological findings resembled those in the control group, although urinary protein excretion was significantly suppressed in the FK506-treated rats. At the time of sacrifice, urinary protein excretion was significantly lower in groups B and C than in group D. The number of animals included in each group was relatively small in the present study, but the staging of membranous nephropathy and proteinuria level in each group was the same as in preliminary experiments (data not shown) which preceded this study. Urinary protein excretion tends to be negatively correlated with the PEI staining in all groups, indicating that FK506 protected the anionic charge of glomerular capillary walls, resulting in decreased urinary protein excretion. There was no significant difference in the anti C-BSA antibody titre among groups B, C, and D, except at 6 weeks. These findings suggest that the dose and duration of FK506 treatment in these phases did not result in profound suppression of the B-cell system.

Except in group A, no clear modification of serum C-BSA antibody titre was observed in the other three groups and the IgG and C3 scores in groups B and C were not statistically different from those in group D.
These results suggest that FK506 does not significantly suppress antibody formation in the stages of MN of groups B and C and that the proteinuria decreasing effect of FK506 is due to other factors rather than the B-cell system suppression. A number of factors are involved in the pathogenesis of experimental MN, including complement, active oxygens, and cytokines [10,20]. Adhesion molecules are critical factors in the immune response [21]. Kawasaki et al. [22] reported that the injection of antibodies directed against ICAM-1 and LFA-1 prevented the development of rat experimental crescentic glomerulonephritis. In the present studies, ICAM-1 was not expressed in group A rats and ICAM-1 expression in the capillary and mesangial areas was weaker in groups B and C than in group D. The number of intraglomerular cells positive for LFA-1 was significantly higher in group D than in the other groups. We suggest that FK506 may have suppressed the expression of ICAM-1 and LFA-1 in this model, although a relationship between proteinuria and ICAM-1 and LFA-1 between groups A, B and C did not exist. TNF-α can induce or exacerbate proteinuria [23,24] and is expressed in human MN [25]. However, in the present study, no positive staining for TNF-α was observed in glomerular capillaries in any group.

The whole blood trough levels of FK506 were 9.1 ng/ml in group B and 9.2 ng/ml in group C, which is compatible with the clinically recommended whole-blood trough level for treatment of rejection in kidney transplantation; 5–15 ng/ml to obtain optimal efficacy with minimal toxicity [26,27]. FK506 was not associated with histological abnormalities and no marked exacerbation of renal function was observed. FK506 was administered for only a short duration and the dose was not high as whole-blood trough levels indicated. The renal vasoconstriction induced by FK506 results from the increased production of the vasoconstrictors endothelin and thromboxane A2 [28]. We cannot exclude the possibility that the decrease of proteinuria in groups B and C could be partially due to a decrease in the glomerular filtration rate because we did not measure this parameter.

We observed that FK506 had a beneficial effect on proteinuria even when MN was established. In the present study, however, we only investigated its effect in the early phase of membranous nephropathy. Further analysis of the effect of FK506 in the middle and later phases of glomerulonephritis is needed to confirm whether FK506 is useful in these phases or not.

In conclusion, the efficacy of FK506 was significantly increased when the drug was administered in the early phase of immunization in this model. The present study suggests that FK506 may be useful in patients with intractable nephrotic syndrome such as MN.

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