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Treatment with an adenosine uptake inhibitor attenuates glomerulonephritis in mice

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Abstract

This study evaluated the effects of KF24345 (3-[1-(6,7-diethoxy-2-morpholinoquinazolin-4-yl)piperidin-4-yl]-1,6-dimethyl-2,4(1*H*,3*H*)-quinazolinedione hydrochloride), a novel adenosine uptake inhibitor, on experimental glomerulonephritis induced in mice by two intravenous injections of rabbit anti-mouse glomerular basement membrane antiserum. Mice with glomerulonephritis showed continuous proteinuria and the histological evaluation revealed glomerular and tubular damage at 7 weeks after the first antiserum injection. KF24345 as well as prednisolone and cyclophosphamide significantly inhibited proteinuria and glomerular damage when it was orally administered once a day from 2 to 7 weeks. Prednisolone elevated plasma bilirubin and glutamic–pyruvic transaminase levels, and cyclophosphamide decreased erythrocytes. Moreover, both prednisolone and cyclophosphamide decreased spleen and thymus weights. KF24345 did not show this kind of side effects. These results demonstrate that KF24345 ameliorates glomerulonephritis with minimal side effects in mice, suggesting that the adenosine uptake inhibitor may be useful for the treatment of glomerulonephritis.

Keywords: KF24345; Adenosine; Glomerulonephritis

1. Introduction

Glomerulonephritis is an inflammatory disease of the kidney, characterized by the accumulation of extracellular matrix within the damaged glomeruli, impaired filtration and proteinuria (Bruijn et al., 1988; Stratta et al., 1999). The formation of immune complexes within the glomerulus is a key initiating pathophysiological event in this disease and leukocytes are involved in the glomerular injury (O'Meara and Brady, 1997). Complement system, platelet-activating factor, chemokines and cytokines are also involved in the pathogenesis of glomerulonephritis (Camussi, 1994; O'Meara and Brady, 1997). In addition, initial insult-induced loss of nephron units may lead to the increase of glomerular pressure and the hyperfiltration in remaining nephrons. These events can aggravate glomerulosclerosis and result in further loss of nephron units (Jackson, 1997).

Adenosine, an endogenous purine nucleoside, is released from various tissues and modulates a variety of physiological responses by interacting with specific extracellular receptors (Collis and Hourani, 1993). Four subtypes of adenosine receptors have been identified as A_1, A_{2A}, A_{2B} and A_3 receptors (Fredholm et al., 1994; Olah and Stiles, 1995). Under several adverse conditions, including inflammation and ischemia, the local tissue concentrations of extracellular adenosine are increased due to the release of adenosine itself and/or the increased demand for energy supplied by ATP, which is metabolized extracellularly to AMP and adenosine ultimately (Eigler et al., 1997). This increased adenosine can protect against excessive cellular damage or dysfunction (Ralevic and Burnstock, 1988). In addition, adenosine has been reported to modulate various renal functions as well as other organs or tissues (Jackson, 1997). In the renal microcirculation, adenosine constricts preglomerular vessels via adenosine A_1 receptors, and relaxes postglomerular vessels via adenosine A_2 receptors (Haas and Osswald, 1981; Murray and Churchill, 1985; Agmon et al., 1993), leading to the decreased intraglomerular pressure, which is beneficial for the nephritic glomerulus. On the other hand, adenosine has been shown to

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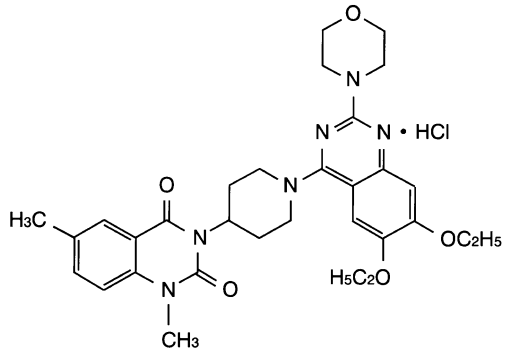

Fig. 1. Chemical structure of KF24345.

exhibit anti-inflammatory effects both in vitro and in vivo (Cronstein, 1995; Cronstein et al., 1995). These observations lead us to a hypothesis that endogenous adenosine could ameliorate the severity of chronic renal failure with inflammation and elevated glomerular pressure, like glomerulonephritis.

Extracellular adenosine usually disappears very fast in physiological conditions due to rapid uptake into the adjacent cells such as erythrocytes and endothelial cells (Arch and Newsholme, 1978; Moser et al., 1989). Therefore, prevention of adenosine uptake into the cells is assumed to enhance extracellular concentrations of endogenous adenosine, which may act on adenosine receptors on the cell surface. KF24345 (3-[1-(6,7-diethoxy-2-morpholinoquinazolin-4-yl)piperidin-4-yl]-1,6-dimethyl-2,4(1*H*,3*H*)-quinazolinedione hydrochloride) is a newly synthesized, orally active adenosine uptake inhibitor (Fig. 1). The current study was performed to evaluate the effects of KF24345 on the glomerulonephritis induced by rabbit anti-mouse glomerular basement membrane antiserum in mice, as compared with those of prednisolone and cyclophosphamide. Side effects of these drugs during the treatments were also examined and compared.

2. Materials and methods

2.1. Animals and chemicals

Male ddY mice (weighing 20 to 25 g) and New Zealand White rabbits (weighing 2 to 3 kg) were purchased from Japan SLC (Hamamatsu, Japan). The animals were given access to food and water ad libitum, and were maintained on 12-h light/dark cycle at 22–23 °C. The protocols of all the experimental procedures were approved by the Animal Care and Use Committee of Kyowa Hakko Kogyo (Shizuoka, Japan).

Complete Freund's adjuvant was purchased from IATRON Laboratories, (Tokyo, Japan). Rabbit immunoglobulinG (IgG) was purchased from Sigma (St. Louis, MO, USA). Prednisolone and cyclophosphamide were purchased from Nacalai Tesque (Kyoto, Japan). KF24345 (3-[1-(6,7-diethoxy-2-morpholinoquinazolin-4-yl)piperidin-4-yl]-1,6-dimethyl-2,4(1*H*,3*H*)-quinazolinedione hydrochloride) was synthesized at Pharmaceutical Research Institute of Kyowa Hakko Kogyo. All other chemicals and solvents were used in their analytical pure form.

KF24345, prednisolone and cyclophosphamide were suspended in 0.5% (w/v) methyl cellulose and were orally administered to mice at a volume of 10 ml/kg once a day.

2.2. Preparation of anti-mouse glomerular basement membrane antiserum

Glomerular basement membrane was prepared from ddY mouse kidneys according to the method described previously (Spiro, 1967). In brief, thin kidney slices of mice were forced through 150-mesh stainless sieves, and the material passed through the sieves was collected in phosphate buffered saline (pH 7.4) without Ca^{2+} and Mg^{2+}. After centrifugation (400 × *g*, 4 °C, 10 min), the precipitate was obtained as glomerular basement membrane-rich sediment.

Antiserum against the basement membrane suspension was raised in rabbits as previously described (Nagai et al., 1982). Rabbits were immunized by 4 intramuscular injections of 1 ml of the emulsion containing 10% mouse glomerular basement membrane-rich fractions and complete Freund's adjuvant at weekly intervals. One week after the last injection, antiserum was obtained from the rabbits. Serum was heated to 56 °C for 30 min to inactivate complement and stored at −70 °C.

2.3. Induction of glomerulonephritis in mice

Experimental glomerulonephritis was induced in mice by the previously described method (Wakayama et al., 2000) with minor modifications as noted below.

Mice were sensitized by an intraperitoneal injection of 250 μg rabbit IgG emulsified with 250 μl of complete Freund's adjuvant, and 50 μl/mouse of rabbit anti-mouse glomerular basement membrane antiserum was intravenously injected at 4 and 11 days after the sensitization. Normal mice received saline instead of the anti-glomerular basement membrane antiserum.

2.4. Experimental design, therapeutic treatments with KF24345, prednisolone and cyclophosphamide

In the previous study, KF24345 inhibited [^{3}H]adenosine uptake into washed erythrocytes of mouse in vitro (IC_{50} = 130 nM; Noji et al., 2002). In an ex vivo study, KF24345 also inhibited the [^{3}H]adenosine uptake into the blood cells sampled from mice in a dose-dependent manner (Noji et al., 2002). At 3 and 10 mg/kg, about 50% inhibition and almost a complete inhibition were achieved, respectively, lasting at least up to 10 h after the oral administration. Thus, we employed the dosages of 3 and 10 mg/kg of KF24345 in the present study.

Experimental groups were as follows: normal mice (group 1), vehicle treatment (group 2; control), KF24345 at 3 mg/kg treatment (group 3), KF24345 at 10 mg/kg treatment (group 4), prednisolone at 0.3 mg/kg treatment (group 5), prednisolone at 3 mg/kg treatment (group 6), cyclophosphamide at 10 mg/kg treatment (group 7) and cyclophosphamide at 30 mg/kg treatment (group 8). Each group consisted of 7–10 animals.

Two weeks after the first anti-glomerular basement membrane antiserum injection, mice were assigned to one of the seven groups (group 2 to 8) so that initial urinary protein excretion in each group would be almost the same. After the assignment, therapeutic treatments with drugs were started. Mice were orally treated with KF24345 (3 or 10 mg/kg), prednisolone (0.3 or 3 mg/kg), cyclophosphamide (10 or 30 mg/kg) or the vehicle (0.5% methyl cellulose) once a day from 2 to 7 weeks after the first anti-glomerular basement membrane antiserum injection.

On the final day of the experiment, under anesthesia with ether, blood samples anticoagulated with heparin were collected from the abdominal vein, and the kidney, spleen and thymus were quickly removed.

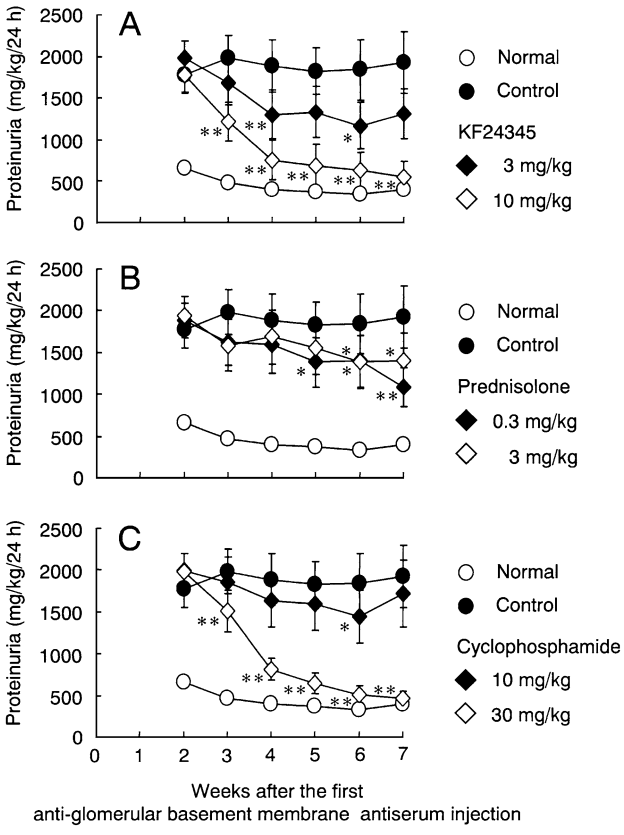

Fig. 2. Therapeutic effects of KF24345 (A), prednisolone (B) and cyclophosphamide (C) on proteinuria in mice with anti-glomerular basement membrane antiserum-induced glomerulonephritis. Drugs were orally administered once a day from 2 to 7 weeks after the first anti-glomerular basement membrane antiserum injection. Values are means ± S.E. of 7–10 animals. $*P<0.05$, $**P<0.01$, significantly different from the vehicle-treated control group.

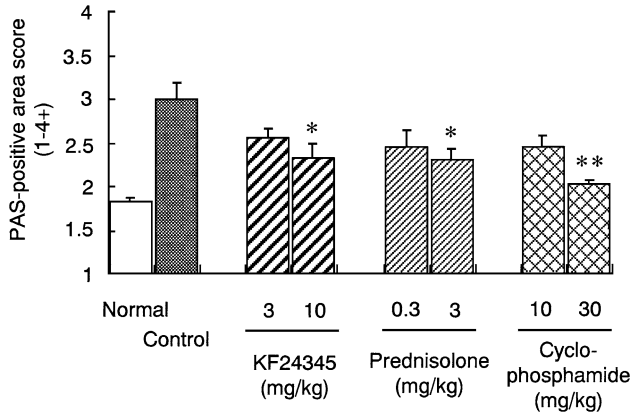

Fig. 3. Therapeutic effects of KF24345, prednisolone and cyclophosphamide on the PAS-positive area in glomerulus in mice with anti-glomerular basement membrane antiserum-induced glomerulonephritis. Drugs were orally administered once a day from 2 to 7 weeks after the first anti-glomerular basement membrane antiserum injection, and PAS-positive area was histopathologically evaluated in arbitrary units of the glomerulus. Values represent means ± S.E. of the indices. Each group contains 7–10 animals. $*P<0.05$, $**P<0.01$, significantly different from the vehicle-treated control group.

2.5. Functional assessment of glomerular injury

Between 2 and 7 weeks after the first anti-glomerular basement membrane antiserum injection, mice were housed individually in cages without food for 24 h to collect urine once a week. At the beginning and 12 h after the start of the urine collection, each mouse was orally administered 50 ml/kg body weight of distilled water. The urine was then centrifuged at 800 × *g* for 5 min (at 4 °C), and the supernatant was used for the determination of protein. The urinary protein concentrations were measured with an autoanalyzer (AU-510; Olympus, Tokyo, Japan). The 24-h urinary protein excretion, as a sign of glomerular protein leakage, was calculated from the 24-h urine volume and the urinary protein concentration, and adjusted with the body weight.

2.6. Histological assessment of glomerular injury

After being fixed in 10% (v/v) buffered formalin and embedded in paraffin, the kidneys were cut at a thickness of 2 to 3 μm and stained with periodic acid-Schiff (PAS) for light microscopic examinations. Approximately 80 to 120 glomeruli from each section were examined in a blinded fashion for the treatment and the animal identification. The glomerular lesions were evaluated according to the percentage of the glomerular tuft (100%) occupied by the PAS-positive area. Grading for the glomerular lesions was performed as follows—1: less than 25%; 2: 25–50%; 3: 50–75%; and 4: more than 75% glomerular tuft occupied by the PAS-positive area. Results were expressed as the aver-

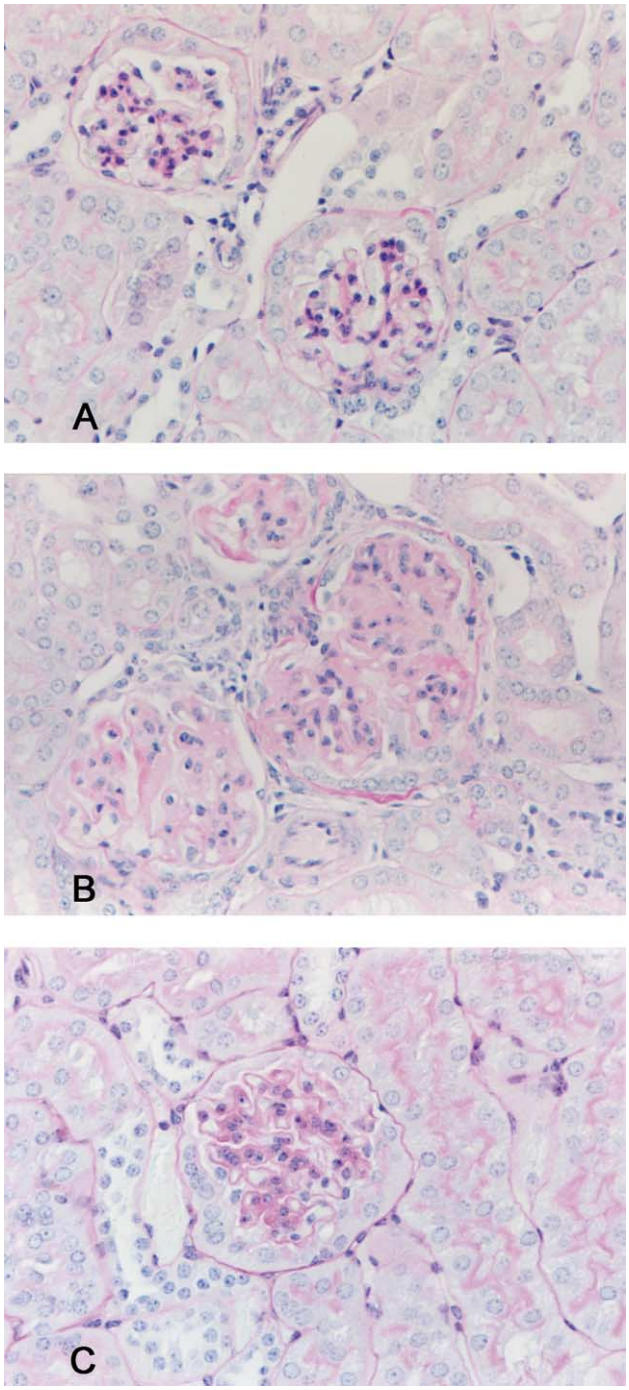

Fig. 4. Photomicrographs of glomeruli from mice of the normal group (A; PAS-positive scoring grade 1), the control group treated with anti-glomerular basement membrane antiserum (B; grade 4) and the KF24345 (10 mg/kg)-treated group (C; grade 2). The mice were examined 7 weeks after the first anti-glomerular basement membrane antiserum injection. Note that attenuation of the PAS-positive area following treatment with KF24345 was observed (PAS stain, magnification × 400).

age score of glomeruli per section. In the normal, control and KF24345 (10 mg/kg)-treated groups, hematoxylin and eosin (H&E) staining was also applied to the other sections from the kidney for the evaluation of histopathological characteristics of the renal lesions, e.g., crescent formation in the glomeruli, protein cast formation and interstitial inflammatory cell infiltration.

2.7. Assessment of side effects after the drug treatment

Erythrocyte counts were determined using a cell counter (Celltacα MEK-6158; Nihon Kohden, Tokyo, Japan). Then blood samples were centrifuged (1200 × *g*, 10 min, 4 °C), and plasma bilirubin and glutamic–pyruvic transaminase were measured with the Olympus autoanalyzer. Spleen and thymus from each mouse were weighed in the groups 1, 2, 4, 6 and 8.

2.8. Statistical analysis

Data were presented as means ± S.E. Statistical significance was examined by the Student's *t*-test when comparing two groups, or by one-way analysis of variance followed by the Dunnett's test for multiple groups. $P < 0.05$ was considered to be statistically significant.

3. Results

3.1. Development of the disease in the control mice with glomerulonephritis

The control mice treated with anti-glomerular basement membrane antiserum showed proteinuria 2 weeks after the first anti-glomerular basement membrane antiserum injection. The proteinuria was observed in all the control mice and continued almost at the same level throughout the experiment (Fig. 2). The light microscopy revealed the expansion of the PAS-positive area in glomerulus in the control mice at the end of the experiment (Figs. 3 and 4; Table 1). In addition, the control mice showed crescent formation in the glomeruli,

Table 1
Quantification of the PAS-positive area in glomerulus in mice with anti-glomerular basement membrane antiserum-induced glomerulonephritis

Treatment	(mg/kg)	PAS-positive area score
Normal	–	1.83 ± 0.05
Control	–	2.99 ± 0.20
KF24345	3	2.55 ± 0.12
	10	2.32 ± 0.18[a]
Prednisolone	0.3	2.44 ± 0.20
	3	2.29 ± 0.13[a]
Cyclophosphamide	10	2.44 ± 0.13
	30	2.03 ± 0.03[b]

Drugs were orally administered once a day from 2 to 7 weeks after the first anti-glomerular basement membrane antiserum injection, and PAS-positive area was histopathologically evaluated. Values represent means ± S.E. of the indices. Each group contains 7–10 animals.

[a] $P < 0.05$, significantly different from the vehicle-treated control group.

[b] $P < 0.01$, significantly different from the vehicle-treated control group.

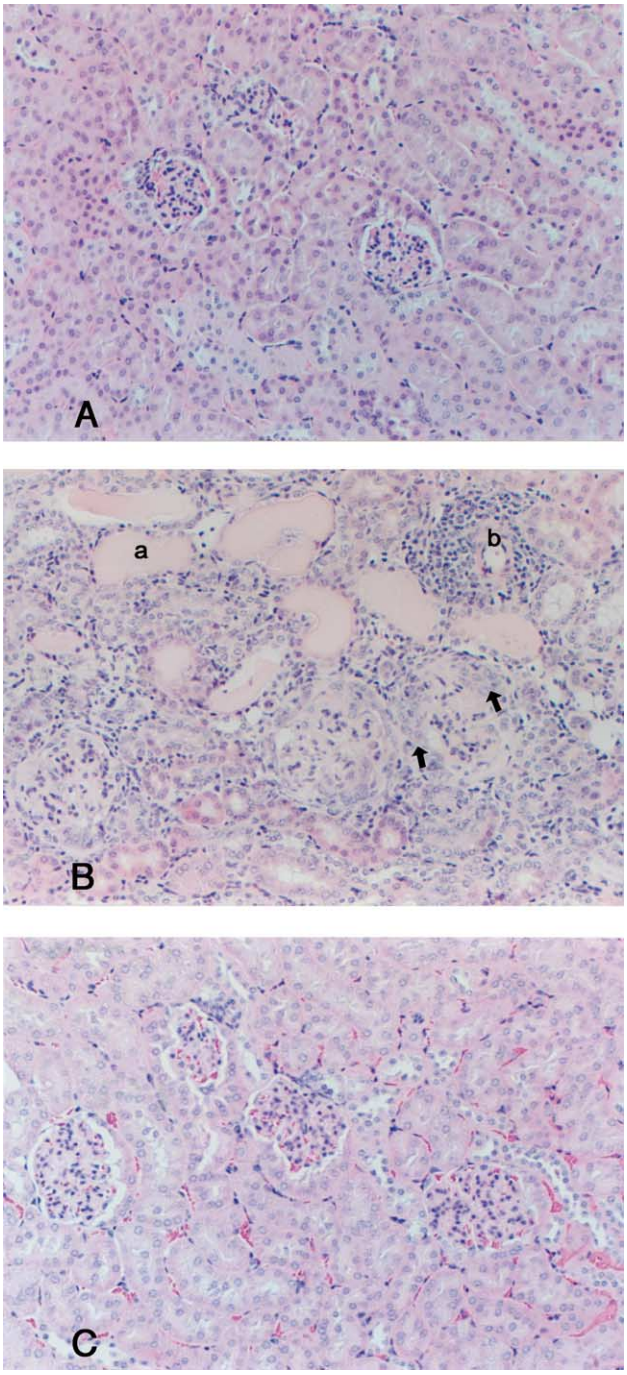

Fig. 5. Photomicrographs of glomeruli and tubules from mice in the normal group (A), the control group treated with anti-glomerular basement membrane antiserum (B) and the KF24345 (10 mg/kg)-treated group (C). The mice were examined 7 weeks after the first anti-glomerular basement membrane antiserum injection. The control mice (B) showed beginning crescent formation in the glomeruli (arrow), protein cast formation (a) and diffuse and perivascular interstitial inflammatory cell infiltration (b). These changes were attenuated in the KF24345-treated mice (H&E stain, magnification × 200).

protein cast formation and interstitial inflammatory cell infiltration at the end of the experiment (Fig. 5). Electron microscopic evaluation revealed thickening of glomerular basement membrane, expansion of mesangial area and glomerular epithelial foot process fusion in the control mice (unpublished observation).

3.2. Effects of KF24345, prednisolone and cyclophosphamide on proteinuria in mice with glomerulonephritis

KF24345 at 3 and 10 mg/kg suppressed proteinuria (Fig. 2A). At 10 mg/kg, the proteinuria was clearly and continuously inhibited, and was almost normalized at the end of the experiment. Prednisolone and cyclophosphamide also attenuated proteinuria (Fig. 2B,C). The effect of cyclophosphamide at 30 mg/kg was almost equivalent to that of KF24345 at 10 mg/kg. In addition, the effects of prednisolone were not dose-dependent and relatively weaker than those of KF24345.

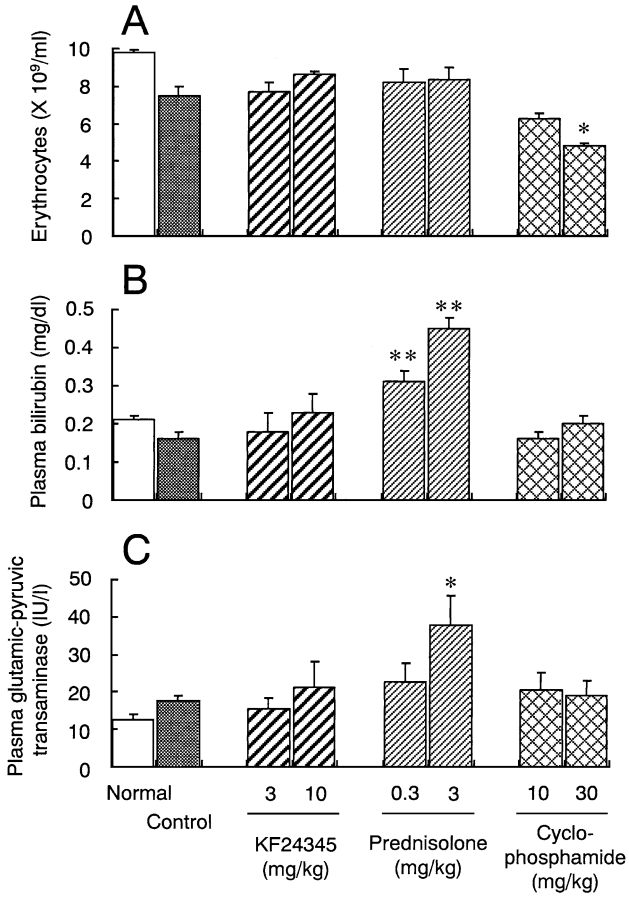

Fig. 6. Influences of 5-week treatments with KF24345, prednisolone and cyclophosphamide on erythrocyte counts in blood (A), plasma bilirubin (B) and glutamic–pyruvic transaminase (C) levels in mice with anti-glomerular basement membrane antiserum-induced glomerulonephritis. Drugs were orally administered once a day from 2 to 7 weeks after the first anti-glomerular basement membrane antiserum injection, and then the parameters were examined. Values are means ± S.E. of 7–10 animals. $*P<0.05$, $**P<0.01$, significantly different from the vehicle-treated control group.

3.3. Effects of KF24345, prednisolone and cyclophosphamide on histological changes in mice with glomerulonephritis

In the KF24345-treated group, the PAS-positive area of glomerulus was less prominent, as compared with that of the control group (Fig. 4). In the mice treated with 10 mg/kg of KF24345 (PAS-positive scoring grade 2.32 ± 0.18), 3 mg/kg of prednisolone (grade 2.29 ± 0.13) and 30 mg/kg of cyclophosphamide (grade 2.03 ± 0.03), the PAS-positive area of glomerulus was significantly lower than that in the control mice (grade 2.99 ± 0.20; Table 1 and Fig. 3). In addition, crescent formation in the glomeruli, protein cast formation and interstitial inflammatory cell infiltration were attenuated in the KF24345 (10 mg/kg)-treated mice (Fig. 5). An appearance of erythrocytes within glomeruli and in peritubular capillaries is also observed in the KF24345 (10 mg/kg)-treated mice (Fig. 5).

3.4. Effects of KF24345, prednisolone and cyclophosphamide on erythrocyte counts, plasma bilirubin and glutamic–pyruvic transaminase levels in mice with glomerulonephritis

In the mice treated with 30 mg/kg of cyclophosphamide, erythrocyte counts were significantly lower than that in the control mice (Fig. 6). On the other hand, the treatment with 3 mg/kg of prednisolone caused remarkable elevations of plasma bilirubin and glutamic–pyruvic transaminase levels. In addition, plasma bilirubin was also higher even in the mice treated with 0.3 mg/kg of prednisolone (Fig. 6). The mice treated with KF24345 did not show any of these changes (Fig. 6).

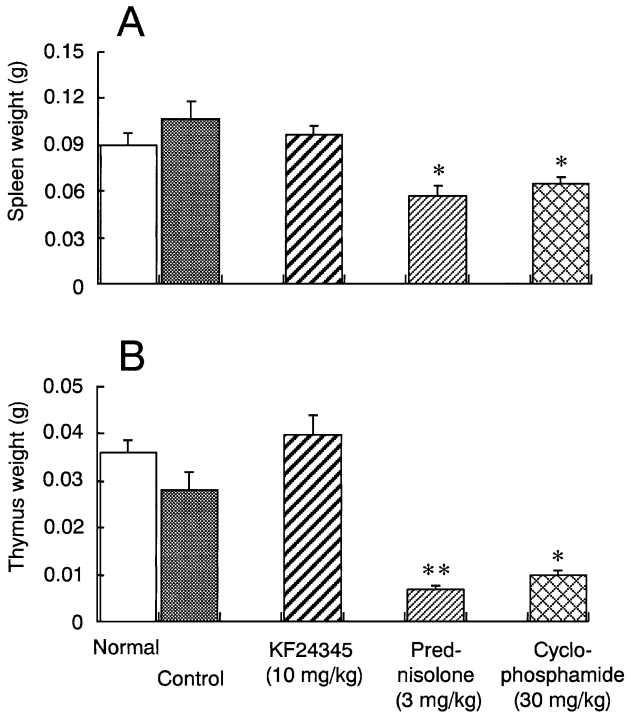

Fig. 7. Influences of 5-week treatments with KF24345, prednisolone and cyclophosphamide on spleen (A) and thymus (B) weights in mice with anti-glomerular basement membrane antiserum-induced glomerulonephritis. Drugs were orally administered once a day from 2 to 7 weeks after the first anti-glomerular basement membrane antiserum injection, and then the parameters were examined. Values are means ± S.E. of 7–10 animals. * $P<0.05$, ** $P<0.01$, significantly different from the vehicle-treated control group.

3.5. Effects of KF24345, prednisolone and cyclophosphamide on spleen and thymus weights in mice with glomerulonephritis

Prednisolone at a dose of 3 mg/kg, and cyclophosphamide at a dose of 30 mg/kg caused significant reductions in spleen and thymus weights (Fig. 7). The treatment with 10 mg/kg of KF24345 did not affect the weights of these organs (Fig. 7). There were no changes in body weights in the control or all drug-treatment groups.

4. Discussion

The present study demonstrated that the adenosine uptake inhibitor KF24345, which is assumed to increase endogenous adenosine, ameliorated the severity of experimental glomerulonephritis in mice. The protective effects of KF24345 were almost equal to, or stronger than those of prednisolone and cyclophosphamide. The side effects of KF24345 were minimal compared with those of prednisolone and cyclophosphamide. These are the first demonstrations in which the adenosine uptake inhibitor exhibits protective effects against glomerulonephritis with minimal side effects in vivo.

In this study, KF24345 as well as prednisolone and cyclophosphamide inhibited proteinuria, and ameliorated glomerular injuries. The anti-nephritic effects of KF24345 at a dose of 10 mg/kg were more prominent than those of prednisolone at 3 mg/kg, and almost equal to those of cyclophosphamide at 30 mg/kg. However, 3 mg/kg of prednisolone elevated the plasma bilirubin and glutamic–pyruvic transaminase levels, and the decreased spleen and thymus weights 5 weeks after oral administration, suggesting an injury of bile duct or liver and the damage of the immune system. In addition, 30 mg/kg of cyclophosphamide decreased spleen and thymus weights, suggesting the damage of the immune system. Erythrocyte counts were also decreased in this cyclophosphamide-treated group, suggesting a hematopoietic system injury. These adverse events were not observed in the KF24345-treated groups. The beneficial effect of KF24345 with minimal systemic side effects may be relevant to the action mechanisms that KF24345 could enhance adenosine only endogenously. Taken together, KF24345 was shown to exhibit anti-nephritic effects with minimal side effects and could be useful for the treatment of glomerulonephritis.

The experimental glomerulonephritis reported in mice is mainly caused by nephrotoxic anti-glomerular basement

membrane antiserum or soluble antigen–antibody complexes. From previous reports, the experimental glomerulonephritis employed in this study is assumed to be the mixed type of both diseases induced by nephrotoxic serum and immune complexes (Nagai et al., 1982). Glomerular injury in mice is directly induced by autologous immune deposits or planting antigen on the glomerular basement membrane of mice previously sensitized to this antigen, and soluble antigen–antibody complexes can also cause and aggravate glomerular damage. Protein cast formation and interstitial inflammatory cell infiltration seem to be caused secondarily after the chronic glomerular injury. In the present study, KF24345 attenuated glomerular injury, and this attunuation of glomerular injury probably resulted in attenuation of the protein cast formation and the interstitial inflammatory cell infiltration. Further examination is needed to clarify the precise mechanism of onset and progression of the disease used in this study.

Under several adverse conditions, the production and the release of adenosine are enhanced due to the increased demand for the energy supplied by ATP, which is metabolized to AMP and adenosine (Cronstein, 1995; Cronstein et al., 1995; Eigler et al., 1997). Indeed, the activity of 5′ -nucleotidase, which metabolizes AMP to adenosine, is reported to be elevated in the damaged organs and tissues (Johnson et al., 1999). Accordingly, inhibition of adenosine uptake into the adjacent cells by KF24345 could inhibit the disappearance of increased extracellular adenosine, and enhance the protective effects of endogenous adenosine against the tissue and organ damage. Indeed, as is the case with KF24345, 2chloroadenosine, an adenosine agonist, is known to abolish proteinuria in rats with glomerulonephritis (Poelstra et al., 1992, 1993). These observations suggest that endogenous adenosine, which is increased under the adverse condition such as glomerulonephritis, could play a role in retarding the progress of nephritis. It is thus likely that the adenosine uptake inhibitor potentiates such beneficial effect of endogenous adenosine, leading to the amelioration of the nephritis.

As the mode of action for the anti-nephritic effects of KF24345, we assume two possible mechanisms; one is the anti-inflammatory effect and the other is the renal hemodynamic effect, both of which are mediated via enhanced endogenous adenosine. First, the anti-inflammatory effects of adenosine may be involved in the anti-nephritic effects of KF24345. In various in vitro studies, adenosine has been shown to suppress neutrophil functions such as oxygen radical production, adhesion and phagocytosis, and macrophage functions including tumor necrosis factor-α production (Cronstein, 1997; Rosengren and Firestein, 1997). In addition, in vivo studies have shown that adenosine exerts anti-inflammatory effects in various experimental models (Schrier et al., 1990; Le Vraux et al., 1993). These anti-inflammatory effects of adenosine seem to play roles in the anti-nephritic effects by KF24345.

In addition to the anti-inflammatory effects of adenosine, the renal hemodynamic effects of adenosine may also be relevant to the anti-nephritis effects of KF24345. Enhanced adenosine could reduce glomerular pressure via its three possible actions as follows: (i) in the renal microcirculation, adenosine directly constricts preglomerular vessels via activation of adenosine A_1 receptors and relaxes postglomerular vessels via activation of adenosine A_2 receptors (Haas and Osswald, 1981; Murray and Churchill, 1985; Agmon et al., 1993), resulting in the decline of intraglomerular pressure; (ii) adenosine has been reported to mediate tubuloglomerular feedback system via adenosine A_1 receptors, leading to reduction of single nephron glomerular filtration rate (Osswald et al., 1980); (iii) adenosine attenuates renin release from juxtaglomerular cells via adenosine A_1 receptors (Osswald et al., 1978; Churchill and Bidani, 1987). The resultant inhibition of renin–angiotensin system, via reducing the postglomerular vasoconstrictor angiotensin II, could lead to the decreased glomerular pressure. In addition, inhibition of angiotensin II may retard the development of renal fibrosis since angiotensin II has been reported to contribute to renal fibrosis (Ghiggeri et al., 2000; Guo et al., 2001). These three actions of adenosine are beneficial for the treatment of nephritis and may contribute to the anti-nephritic effects of KF24345.

In conclusion, we have shown that KF24345 ameliorates the severity of glomerulonephritis in mice. Increased endogenous adenosine, which could suppress inflammation and decrease the glomerular pressure, seems to be involved in the anti-nephritic effects of KF24345. The present data suggest that the adenosine uptake inhibitor may be useful in the treatment of glomerulonephritis.

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