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Protective effects of antithrombin on puromycin aminonucleoside nephrosis in rats

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ABSTRACT

We investigated the effects of antithrombin, a plasma inhibitor of coagulation factors, in rats with puromycin aminonucleoside-induced nephrosis, which is an experimental model of human nephrotic syndrome. Antithrombin (50 or 500IU/kg/i.v.) was administered to rats once a day for 10 days immediately after the injection of puromycin aminonucleoside (50mg/kg/i.v.). Treatment with antithrombin attenuated the puromycin aminonucleoside-induced hematological abnormalities. Puromycin aminonucleoside-induced renal dysfunction and hyperlipidemia were also suppressed. Histopathological examination revealed severe renal damage such as proteinaceous casts in tubuli and tubular expansion in the kidney of control rats, while an improvement of the damage was seen in antithrombin-treated rats. In addition, antithrombin treatment markedly suppressed puromycin aminonucleoside-induced apoptosis of renal tubular epithelial cells. Furthermore, puromycin aminonucleoside-induced increases in renal cytokine content were also decreased. These findings suggest that thrombin plays an important role in the pathogenesis of puromycin aminonucleoside-induced nephrotic syndrome. Treatment with antithrombin may be clinically effective in patients with nephrotic syndrome.

1. Introduction

Nephrotic syndrome is characterized by damage of the kidneys resulting in proteinuria, hypoalbuminemia, hyperlipidemia and edema (Gitlin et al., 1956; Kaysen et al., 1984; Kaysen et al., 1986; Radhakrishnan et al., 1993; Rabelink et al., 1994). Furthermore, abnormalities of the blood coagulation system are also closely related to the development and progression of nephrotic syndrome (Llach, 1985; Citak et al., 2000). Although the physiological and pathophysiological mechanisms involved in coagulation abnormalities in nephrotic syndrome are complex and not fully elucidated, there is evidence that a variety of hemostatic abnormalities, including platelet activation, alteration of fibrinolytic and coagulation factors, are associated with nephrotic syndrome (Rabelink et al., 1994; Sarasin and Schifferli, 1994). In addition, antiplatelet and anticoagulant drugs have been administered for the treatment of the thromboembolic complications of nephrotic syndrome for some patients (Kuhn et al., 1998).

Antithrombin is a 59kDa glycoprotein that belongs to plasma α2 globulin fraction, which is produced in the liver and vascular endothelial cells. Antithrombin plays a central role in regulating the coagulation cascade by inhibiting several coagulation factors, such as thrombin and activated factor X (Rosenberg and Damus, 1973). The inhibitory activity of antithrombin against coagulation factors is low in the absence of heparin. However, antithrombin activity is markedly potentiated by heparin-like glycosaminoglycans on endothelial cells or exogenously administered heparin. These produce a conformational change in the structure of antithrombin and its inhibitory activity is greatly enhanced about 1000-fold (Okajima et al., 1989; Rosenberg, 1989). Antithrombin is currently used to protect thrombus formation in patients with hereditary antithrombin deficiency. Moreover, antithrombin can also be used in the treatment of complex coagulation disorders, sepsis with disseminated intravascular coagulation and acute thromboembolic events with reduced antithrombin activity.

In the present study, we first examined whether treatment with antithrombin would have a protective effect on abnormalities of coagulation and hematological parameters in puromycin aminonucleoside-induced nephrotic syndrome in rats, which are similar to those seen in minimal change of nephrotic syndrome in humans (Shiiki et al., 1998). Next, we investigated if antithrombin treatment could suppress renal dysfunction and hyperlipidemia induced by puromycin aminonucleoside. Finally, we evaluated the effects of antithrombin treatment on renal tissue injury, apoptotic changes of renal tubular epithelial cells and increase of renal cytokine content induced by puromycin aminonucleoside.

2. Materials and methods

2.1. Animals

Male Wistar rats (8weeks old) were purchased from Japan SLC (Shizuoka, Japan). All animals were housed in a light-controlled

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room with a 12-hour light/dark cycle under specific pathogen-free conditions and were allowed to eat a standard laboratory chow (CRF-1, Oriental Yeast, Chiba, Japan), and to drink water *ad libitum*. One week later, rats in generally good conditions with no abnormalities and with weight gain were selected for the study. Animal care and experimental protocols for the experiment were based on the Guidelines for Animal Experiments from Nihon Pharmaceutical Co. Ltd.

2.2. Drugs

Antithrombin was purified by Nihon Pharmaceutical Co. (Tokyo, Japan). Antithrombin containing sodium glutamate, sodium citrate and sodium chloride, was dissolved in sterile water, just prior to its administration. Puromycin aminonucleoside was obtained from Sigma Chemical Co. (St. Louis, MO). Other chemicals were obtained from Katayama Kagaku (Osaka, Japan) and Wako Pure Chemical Industries (Osaka, Japan).

2.3. Experimental design

Twenty four rats (9weeks old) were separated into four groups: 1) normal rats (Normal; n = 6), 2) puromycin aminonucleoside-injected rats treated with vehicle (Control; n = 6), 3) puromycin aminonucleoside-injected rats treated with antithrombin at a dose of 50IU/kg/i.v. (n = 6), and 4) puromycin aminonucleoside-injected rats treated with antithrombin at a dose of 500IU/kg/i.v. (n = 6). In total 18 rats were administered 50mg/kg/i.v. of puromycin aminonucleoside as a single dose. Antithrombin or its vehicle (10mL/kg) was slowly administered via the tail vein, immediately after the injection of puromycin aminonucleoside, and then once a day for 10 days. On Days 5 and 10 of treatment, the rats were individually housed in metabolic cages for 24h, and the 24-hour urine was collected. Moreover, on Day 10 blood samples were withdrawn from the abdominal aorta into plastic syringes containing 1/9 volume of 3.8% sodium citrate or EDTA-2K at the end of the urine collection period. The plasma fraction was then separated by centrifugation. These samples were used to measure urinary and blood parameters. In different animals, the kidneys were excised on Days 5 and 10 of treatment to examine renal histology, TUNEL-positive cells and cytokine content.

2.4. Blood coagulation measurements

Prothrombin time, activated partial thromboplastin time, fibrinogen, and antithrombin activity were determined using an automatic blood coagulation system (CA530, Sysmex, Kobe, Japan).

2.5. Blood cell counts

White blood cell and platelet counts were determined using a multi-automatic blood cell counter for animals (MICROS abc LC-152, Horiba, Tokyo, Japan). Neutrophil counts were determined using blood smears stained with Wright-Giemsa stain according to standard procedures. Evaluations were made in a blinded manner.

2.6. Biochemical evaluation

Urinary protein and urinary *N*-acetyl-β-D-glucosaminidase were measured by the sulphosalicylic method and colorimetric method, respectively. Plasma albumin was measured by the bromocresol green method. Triglycerides, total cholesterol, low density lipoprotein cholesterol and high density lipoprotein cholesterol were measured by enzymatic methods. Very low density lipoprotein cholesterol was calculated using Friedewald's (1972) equation.

2.7. Histological studies

Kidneys were fixed in 10% phosphate-buffered formalin (Wako, Tokyo, Japan), after which the kidneys were chopped into small pieces, embedded in paraffin wax, cut into 3-μm sections, and stained with hematoxylin–eosin stain. The tissues were processed for light microscopic observation (80 magnification), according to standard procedures. Histopathological changes were analyzed for proteinaceous casts in tubuli and tubular expansion. Proteinaceous casts in tubuli and tubular expansion were graded as follows: no damage (− or 0), mild (± or 1), moderate (+ or 2), severe (++ or 3) and very severe (+++ or 4). Evaluations were made in a blinded manner.

2.8. Identification of apoptosis (TUNEL)

Fragmentated DNA in apoptotic cells were nick end-labeled using an in situ apoptosis detection kit according to the manufacturer's instructions (Promega, Madison, WI). Formalin-fixed and 5-μm thick paraffin-embedded sections were deparafinized and dehydrated. Nuclear proteins were stripped from DNA through incubation in proteinase K, and endogenous peroxidase was blocked with H_2O_2. Those sections were incubated in a buffer containing TdT and digoxigenin-labeled dUTP, followed by digoxigenin conjugated peroxidase. Diaminobenzidine was used as the chromogen. TUNEL-positive cells were microscopically observed under 320 magnification and counted. Evaluations were made in a blinded manner.

2.9. Renal cytokine content

Excised renal tissues were weighed and homogenized in a lysis buffer (Bio-Rad, Hercules, CA) on ice. The homogenates were centrifuged at 4500g for 4min at 4°C. The cytokine concentration in the supernatant was measured using a Bio-Plex rat cytokine 9-plex panel (Bio-Rad) according to the manufacturer's instructions. Acquired data were analyzed using the Bio-Plex suspension array system (Luminex 100 system). The renal cytokine content is expressed in pg/g weight tissue.

2.10. Statistical analysis

Values are expressed as the mean ± S.E.M. All statistical differences among multiple groups were tested by one-way analysis of variance followed by Fischer's least-significant-differences method, and histological data were analyzed using the Kruskal–Wallis nonparametric method combined with the Steel-type multiple comparison method using the SAS program (Version 8.02; SAS

Table 1
Effects of antithrombin (50 and 500 IU/kg, i.v.) on prothrombin time, activated partial thromboplastin time, fibrinogen, antithrombin activity, platelets, white blood cells and neutrophils at Day 10 after puromycin aminonucleoside injection

Parameter	Day 0	Day 10		
	Normal	Control	50 IU/kg	500 IU/kg
	(n=6)	(n=6)	(n=6)	(n=6)
Prothrombin time (s)	17.4±0.1	16.1±0.1[b]	15.5±0.4	16.4±0.2
Activated partial thromboplastin time (s)	18.1±0.8	15.9±0.5[a]	14.5±0.6	16.7±0.6
Fibrinogen (mg/dl)	178.9±7.7	485.4±29.9[b]	399.6±53.0	234.0±25.5[d]
Antithrombin activity (%)	138.7±1.8	110.7±4.2[a]	111.6±14.2	130.0±7.8
Platelets (×10^4/μl)	68.2±1.9	90.8±5.7[b]	81.3±1.0[c]	80.5±1.9[c]
White blood cells (×10^2/μl)	41.2±2.0	49.0±5.5	41.5±2.1	43.0±1.9
Neutrophils (×10^2/μl)	6.6±0.9	9.1±1.6	9.2±1.7	5.0±0.6[c]

Values are the mean±S.E.M. [a]P<0.05, [b]P<0.01, compared with Normal rats. [c]P<0.05, [d]P<0.01, compared with Control rats.