Kidney International, Vol. 62 (2002), pp. 1628–1637

Urinary free fatty acids bound to albumin aggravate tubulointerstitial damage

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Urinary free fatty acids bound to albumin aggravate tubulointerstitial damage.

Background. Evidence indicates that urinary protein is associated with tubulointerstitial damage and thus it is an aggravating factor for chronic renal disease. As free fatty acids (FFAs) are bound to serum albumin, we hypothesized that FFAs were overloaded to the proximal tubule in massive proteinuria and thus caused tubulointerstitial damage. To test this hypothesis, massive proteinuria was provoked in mice and the renal damage examined.

Methods. Mice were intraperitoneally injected with bovine serum albumin (BSA) replete with FFAs (r-BSA group, $N = 10$), FFA-depleted BSA (d-BSA group, $N = 10$), or saline (saline group, $N = 9$) for 14 days.

Results. The kidneys of the r-BSA group showed severe tubulointerstitial damage and those of the d-BSA group showed mild tubulointerstitial damage. Urinary excretion of both total protein and mouse albumin were significantly higher in the r-BSA group than in the d-BSA group. To examine the proximal tubular uptake of albumin, the BSA content in the cultured mouse proximal tubules was measured by ELISA after 90 minutes of incubation with each BSA. In terms of the BSA content in the proximal tubules, there was no significant difference between the r-BSA and the d-BSA groups. These results indicate that r-BSA and d-BSA were similarly reabsorbed into the proximal tubule and that r-BSA causes severe tubulointerstitial damage.

Conclusions. It is the FFAs bound to albumin, rather than albumin itself, which cause severe tubulointerstitial damage by being reabsorbed into the proximal tubule. To our knowledge, this is the first in vivo observation in which FFAs have caused severe tubulointerstitial injury.

The progressive nature of glomerular disease is significantly dependent on tubulointerstitial involvement [1]. Recent studies have shown that urinary protein has renal toxicity and plays a contributory role in the progression of renal damage by causing tubulointerstitial disease [2–6]. However, to date the mechanism by which proteinuria causes tubulointerstitial disease is unknown.

Free fatty acids (FFAs; long chain fatty acids) are bound to albumin [7], filtered through glomeruli and reabsorbed into the proximal tubules. FFAs bound to albumin, therefore, might play a role in the generation of tubulointerstitial disease. In massive proteinuria, FFAs are overloaded to the proximal tubule; these possibly induce inflammatory (including macrophage chemotactic) factors [8, 9], which in turn cause tubulointerstitial damage. FFAs were reported to be overloaded to the proximal tubule not only in massive proteinuria, but also in other stresses that progress renal diseases such as ischemia [10–12] and toxic insults [13].

We hypothesized that FFAs overloaded to the proximal tubule aggravate urinary protein-related tubulointerstitial damage. To test this hypothesis, massive proteinuria was artificially induced in mice by intraperitoneal administration of albumin [14–17] to examine the effects of FFA-repleted bovine serum albumin (r-BSA) and FFA-depleted BSA (d-BSA) on tubulointerstitial damage.

METHODS

Animals

Twelve-week-old female inbred Balb/c mice ($N = 29$) were purchased from the Shizuoka Laboratory Animal Center [Shizuoka, Japan; body weight (BW) 20 ± 0.1 g, mean ± SE]. They were maintained on a standard diet and given free access to tap water. All experimental procedures were conducted in accordance with the guidelines for the care and use of laboratory animals approved by the University of Tokyo.

Experimental design

The mice were divided into three groups: the r-BSA group ($N = 10$) received a once daily intraperitoneal in-

Key words: proteinuria, chronic renal disease, serum albumin, progressive renal disease, long chain fatty acids, tubulointerstitial disease.

Received for publication November 8, 2001
and in revised form May 17, 2002
Accepted for publication June 10, 2002

jection of lipid-repleted BSA (250 mg/mouse, dissolved in 1 mL saline; BSA fraction V, Catalog No. A-4503; Sigma Chemical Co., St. Louis, MO, USA); the d-BSA group ($N = 10$) received lipid-depleted BSA (prepared from BSA fraction V, Catalog No. A-6003; Sigma); the saline group ($N = 9$) received an equivalent volume of sterile normal saline as control. The r-BSA used in this study had the same catalog number as that which Eddy et al used to establish a protein overload model in rats [14]. The d-BSA we used in this study was de-fatted pure albumin derived from the r-BSA used. Mice were individually housed in metabolic cages with free access to tap water; urine was collected on days 0, 4, 7 and 14. The urinary sediment was removed via centrifugation (12,000 rpm for 5 min). Groups of animals were sacrificed on day 7 ($N = 5$ in the r-BSA group, $N = 5$ in the d-BSA group, and $N = 5$ in the saline group), and on day 14 ($N = 5$ in the r-BSA group, $N = 5$ in the d-BSA group, and $N = 4$ in the saline group). Under intraperitoneal anesthesia (pentobarbital, 75 μg/g BW), the polyethylene catheter (PE-10; INTRAMEDIC Polyethylene Tubing; Becton Dickinson, Cockeysville, MD, USA) was inserted retrogradely into the abdominal aorta. The kidneys were fixed with 10% formalin (Wako Pure Chemical Industries, Ltd., Osaka, Japan) by perfusion and then by immersion for 24 hours. The right kidney was divided into two for light microscopy analysis and electron microscopy analysis. Body weight was recorded at the start of the experiment and prior to sacrifice. Plasma was obtained by exsanguination through the abdominal great vein.

Determination of long chain fatty acids in BSA

Long chain fatty acids in the BSA were quantified by gas-chromatography at the SRL Co. Research Service (Tokyo, Japan).

Measurement of the concentration of endotoxin in BSA

The concentration of endotoxin in the BSA was measured by chromogenic limulus test (SRL Co. Research Service).

Measurement of mean blood pressure

The mean blood pressure (MBP) was measured by a programmed sphygmomanometer (BP-200; Softron Co., Ltd., Tokyo, Japan) using the tail-cuff method with conscious mice [18].

Serum biochemistry

Serum urea nitrogen (SUN), creatinine (S_{Cr}), total cholesterol (Tchol) and triglyceride (Tg) values were measured by the enzymatic method and total protein (TP) measured by the Biuret method at the SRL Co. Research Service.

Estimation of plasma anti-BSA antibody

The presence of anti-BSA antibody was estimated by enzyme-linked immunosorbent assay (ELISA). A 96-well ELISA plate (Nalge Nunc International Co., Roskilde, Denmark) coated with r-BSA or d-BSA was incubated with test plasma that was diluted from 1:250 to 1:8000. After being washed extensively with phosphate-buffered saline (PBS) containing 0.05% Tween-20 (Wako), the plates were incubated first with biotinylated anti-mouse IgG using a labeled streptavidin biotin (LSAB) Kit (Dako Japan Co., Ltd., Kyoto, Japan), and second, with peroxidase-conjugated avidin-biotin complexes (Vecta Stain Elite ABC-Peroxidase Kit; Vector Laboratories, Burlingame, CA, USA). The rabbit anti-BSA antibody (Sigma) diluted from 1:8000 to 1:256,000 was used as a positive control and the mouse normal serum diluted from 1:250 to 1:8000 as a negative control. The dilution ratio showing OD = 1 at 450 nm was determined for the test plasma, the positive control and the negative control using a spectrophotometer (MicroReader 4™, Hyperion, Inc., Miami, FL, USA). Quantitative evaluation of the estimation of plasma anti-BSA antibody was performed by the following numerical formula neglecting the difference in ELISA plates:

$$\text{Antibody titer} = \frac{\text{(Dilution rate of test plasma/ Dilution rate of negative control)}}{\text{(Dilution rate of positive control/ Dilution rate of negative control)}} \quad \text{(Eq. 1)}$$

Urinary biochemistry

Total urinary protein was measured using the Lowry assay (Bio-Rad Laboratories, Richmond, CA, USA). Levels of urinary mouse albumin were quantified using a commercially available mouse albumin ELISA Kit (Exocell Inc., Philadelphia, PA, USA) and a spectrophotometer (MicroReader 4™); levels of urinary creatinine were quantified using a commercially available creatinine ELISA Kit (Exocell Inc.) and a spectrophotometer (MicroReader 4™). Urinary protein and urinary mouse albumin were expressed as ratios, either urinary protein/urinary creatinine (mg), or urinary mouse albumin/urinary creatinine (mg).

Renal histological and morphometric analysis

The kidneys for light microscopy analysis were dehydrated and embedded into paraffin. One-micrometer-thick serial sections were obtained for conventional histological study, that is, hematoxylin and eosin (H&E) staining or periodic acid-Schiff staining (PAS), and immunohistochemistry.

The PAS-stained tissue sections were used for evaluation of tubulointerstitial injury. The tubulointerstitial injury was categorized either as tubular dilatation, tubular atrophy or tubular hyaline cast formation. Under ×200 magnification, 20 to 30 non-overlapping fields from the cortical region were selected and the area with tubulointerstitial damage and all the cortical areas were measured, using a computer-aided video manipulator (Hamamatsu-Photomics Co., Hamamatsu, Japan). The tubulointerstitial damage was defined as the area ratio of the area with tubulointerstitial damage to entire cortical area. Glomerulosclerosis was indicated by the disappearance of cellular elements from the capillary tuft, capillary loop collapse, or the folding of the glomerular basement membrane with an accumulation of amorphous material. The grade of sclerosis in each glomerulus was defined as: 0 = no sclerosis; I = 1% to 25% glomerular area affected by sclerosis; II = 26% to 50% glomerular area affected; III = 51% to 75% glomerular area affected; IV = 76% to 100% glomerular area affected. The glomerulosclerosis score for each animal was calculated as [(1 × the number of grade I glomeruli, %) + (2 × one of grade II glomeruli, %) + (3 × one of grade III glomeruli, %) + (4 × one of grade IV glomeruli, %)] [19]. Seventy-six to 185 glomeruli were examined for each animal. One observer who was blinded to the source for each section performed these histological evaluations.

Ultrastructural studies

Pieces of renal cortex fixed with 10% formalin by perfusion were processed for electron microscopy. Following 2.0% paraformaldehyde (TAAB, Berks, UK)/2.5% glutaraldehyde fixation (TAAB), the tissues were postfixed in 1% osmium tetroxide (Wako), dehydrated in graded acetone (Wako), and embedded in an Epon 812 mixture (TAAB). Sections were cut at 60 nm, stained with uranyl acetate (Wako) and lead citrate (Wako), and examined with an electron microscope (H7000; Hitachi Ltd., Ibaragi, Japan).

Immunohistochemical examination

Immunohistochemistry was performed using a monoclonal antibody against mouse macrophages (rat anti-mouse F4/80 antibody; Medical and Biological Laboratories Co., Ltd., Nagoya, Japan) based on the LSAB Kit (Dako Co.). The renal sections were incubated for 20 minutes at 37°C in 0.1% trypsin (Wako) and then incubated in a 3% hydrogen peroxide solution for five minutes at room temperature to inactivate the endogenous peroxidase activity. The first antibody was applied at a dilution of 1:10 in the blocking solution of the LSAB Kit and incubated for 30 minutes at room temperature. The sections were incubated with the second antibody (biotinylated rabbit anti rat antibody, 1:400, E468; Dako A/S) for 30 minutes at room temperature and then incubated with horseradish peroxidase-labeled streptavidin solution (Dako Co.) for 10 minutes at room temperature. The slides were rinsed in Tris-buffered saline with 0.1% Tween 20 (TBST) after each incubation step. Peroxidase was visualized by 3,3′-diaminobenzidine tetrahydrochloride (0.2 mg/mL; Sigma) as chromogen with 0.014% hydrogen peroxide as substrate. The slides were washed in tap water and counterstained with hematoxylin, dehydrated and mounted. The negative control by omission of the primary antibody showed no staining. The degree of macrophage infiltration in the cortical interstitium was measured as the average number of F4/80 positive cells per field at ×200 magnification.

Measurement of proximal tubule absorption of BSA

The mProx 24 cells were originally isolated from mouse proximal tubules using microdissection methods (Patent Number: WO0073791) and were maintained in Dulbecco's modified Eagle's medium (DMEM; Sigma) containing 10% fetal bovine serum (FBS; Gibco BRL, Rockville, MD, USA).

Intracellular protein from cultured proximal tubules was extracted following a modified procedure of Kees-Folts, Sadow and Schreiner [9]. Proximal tubules were changed to serum-free K-1 medium (Gibco BRL) 24 hours before the experiments. These tubules were cultured in K-1 medium (Gibco BRL) with 30 mg/mL of r-BSA or d-BSA at 37°C for 90 minutes ($N = 6$). Medium K-1 consisted of a 50:50 mixture of DMEM and Hamm's F12 media, and to this 15 mmol/L Hepes, 13.4 mmol/L sodium bicarbonate, 5 μg/mL insulin, 5 μg/mL transferrin, 5 ng/mL selenous acid (ITS Premix; Sigma); 0.05 μmol/L hydrocortisone (Sigma); 10 ng/mL epidermal growth factor (Sigma) were added. In order to evaluate non-specific uptake, some proximal tubules were incubated as mentioned above but at 4°C on ice ($N = 6$). The tubules were washed with cold Hanks' balanced salt solution (HBSS; Gibco BRL) and 0.01 mol/L PBS (pH 9.4) three times, on ice. After washing, the tubules were scraped and lysed via sonication.

Intracellular BSA was measured using a commercially available BSA ELISA Kit (Yagai Co., Yamagata, Japan) and a spectrophotometer (MicroReader 4™); total protein was measured using the Bio-Rad protein assay (Bio-Rad). Intracellular BSA content was expressed as the ratio of BSA concentration/total protein concentration.

Estimation of the viability of cultured proximal tubules after the incubation with BSA

We evaluated the viability of cultured proximal tubules after the incubation with r-BSA or d-BSA using the AlamarBlue™ Assay (Trek Diagnostic Systems, Inc., Westlake, OH, USA), in which AlamarBlue™ dye changed from an oxidized form to a reduced form due to the metabolic activity of the cultured cells. The mProx 24 cells were

Table 1. Quantification of fatty acid contents in bovine serum albumin (BSA)

Name	Number of C atoms and double bonds	r-BSA		d-BSA		Standard of human serum	
		μg/mL	%	μg/mL	%	μg/mL	%
Lauric acid	C12:0	>0.1	>0.01	0.3	1.73	>9	>0.35
Myristic acid	C14:0	0.6	0.34	0.2	1.16	5.8–40	0.36–1.42
Palmitic acid	C16:0	24.6	14	2.9	16.76	280–790	19.91–25.14
Palmitoleic acid	C16:1	2.9	1.65	0.5	2.89	16–79	0.98–2.85
Stearic acid	C18:0	57.2	32.5	2.7	15.61	100–250	6.63–9.00
Oleic acid	C18:1	60.2	34.2	7.3	42.2	190–770	13.39–25.11
Linoleic acid	C18:2	23.9	13.6	3.4	19.65	400–950	23.34–36.58
r-Linolenic acid	C18:3	>0.2	>0.01	>0.2	>0.01	1.8–21	0.10–0.81
Linolenic acid	C18:3	>0.5	>0.01	>0.2	>0.01	6.6–37	0.42–1.31
Arachidic acid	C20:0	>0.5	>0.01	>0.5	>0.01	4.4–8.6	0.21–0.41
Eicoenoic acid	C20:1	>0.5	>0.01	>0.5	>0.01	1.5–9.6	0.09–0.35
Eicosadienoic acid	C20:2	>0.5	>0.01	>0.5	>0.01	2.4–7.2	0.15–0.27
Dihomo-r-linolenic acid	C20:3	1.4	0.79	>0.5	>0.01	11–43	0.60–1.83
Arachidonic acid	C20:4	2.4	1.36	>0.5	>0.01	85–210	4.19–9.51
Eicosapentanoic acid	C20:5	>0.5	>0.01	>0.5	>0.01	12–110	0.54–5.20
Behenic acid	C22:0	>1.0	>0.01	>1.0	>0.01	9.7–19	0.43–0.96
Erucic acid	C22:1	1.7	0.96	>1.0	>0.01	2.3>	0.12>
Docosatetranoic acid	C22:4	>1.0	>0.01	>1.0	>0.01	2.2–6.7	0.12–0.27
Docosapentanoic acid	C22:5	>1.0	>0.01	>1.0	>0.01	6.5–20	0.36–0.80
Lignoceric acid	C24:0	>1.0	>0.01	>1.0	>0.01	11–20	0.47–1.01
Docosahexanoic acid	C22:6	>1.0	>0.01	>1.0	>0.01	49–150	2.33–7.34
Nervonic acid	C24:1	>1.0	>0.01	>1.0	>0.01	22–42	0.88–2.32

cultured by the same method mentioned above and incubated in AlamarBlue™-containing K-1 medium with 30 mg/mL of r-BSA or d-BSA at 37°C or on ice for 90 minutes. The final concentration of AlamarBlue™ was 10% vol/vol. The collected supernatant was mixed with methanol (1:1), vortexed and centrifuged (15,000 rpm for 30 min). The absorbance of the supernatant was measured at 595 nm and 570 nm by a spectrophotometer (Micro-Reader 4™) and the percent difference in reduction was estimated according to the numerical formula shown in the manufacturer's manual. The viability of cultured cells was expressed as the ratio of the percent difference in reduction (%)/total protein concentration (mg).

Statistical analysis

All values are expressed as the mean ± SE. Differences among the three groups were analyzed by Scheffe's multiple comparison procedure. A statistical analysis of in vitro experiments was performed by Mann-Whitney's U test used for nonparametric distributions. These statistical analyses were performed using a computer software program for the Macintosh Power Book G3 (Stat View 5.0; SAS Institute Inc., Cary, NC, USA). P values less than 0.05 were considered significant.

RESULTS

Quantification of long chain fatty acid contents in BSA

Fatty acids included in r-BSA and d-BSA were composed of mainly oleic acid (18:1 ω-9), stearic acid (18:0), palmitic acid (16:0) and linoleic acid (18:2 ω-6), and the composition was approximately similar to that of human serum (Table 1). However, the concentration of fatty acids in d-BSA was much lower (about 1/7 to 1/20) than that in r-BSA.

Concentration of endotoxin in BSA

The concentration of endotoxin in r-BSA (250 mg/mL) was 0.01 μg/mL and that in d-BSA (250 mg/mL) was 0.3 μg/mL.

Measurement of mean blood pressure and body weight

The MBP and BW values were similar among the three groups before and during the experiments (Table 2).

Urinary biochemistry

Replete-BSA and d-BSA overloaded animals developed an almost identically high level of urinary protein on day 4 (256 ± 18 and 256 ± 42 mg protein/mg creatinine, respectively; Fig. 1). Thereafter, urinary protein levels differed significantly, the r-BSA group values remaining high (229 ± 22 and 240 ± 20 mg protein/mg creatinine on days 7 and 14, respectively) while the d-BSA group values decreased (118 ± 12 and 89 ± 13 mg protein/mg creatinine on days 7 and 14, respectively; $P < 0.0001$).

Urinary mouse albumin showed the same pattern as the total urinary protein and was at the same level in the r-BSA and the d-BSA groups on day 4 (27 ± 5 and 28 ± 5 mg albumin/mg creatinine, respectively; Fig. 2).

Table 2. Mean blood pressure (MBP) and body weights of mice in the r-BSA, d-BSA and saline groups on days 0, 7 and 14

	Body weight *g*			MBP *mm Hg*		
	day 0	day 7	day 14	day 0	day 7	day 14
r-BSA	19.8 ± 0.2	20.2 ± 0.2	20.4 ± 0.4	87 ± 3	90 ± 1	90 ± 2
d-BSA	19.7 ± 0.1	20.6 ± 0.3	20.7 ± 0.3	87 ± 2	88 ± 2	89 ± 2
Saline	19.8 ± 0.1	20.0 ± 0.0	20.2 ± 0.3	82 ± 4	83 ± 5	83 ± 5

There was no significant difference in the three groups. Data are mean ± SE.

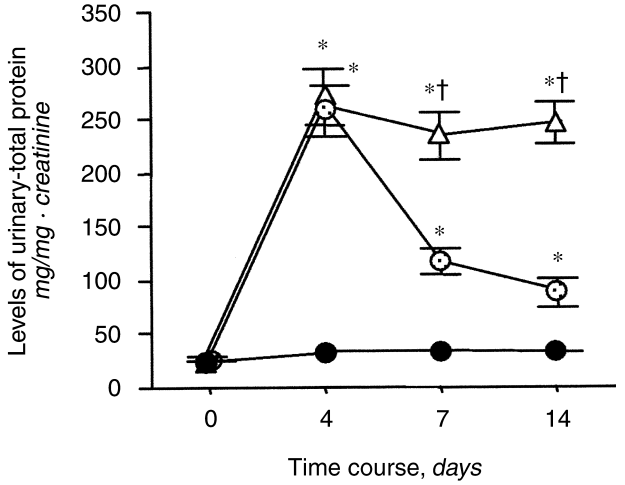

Fig. 1. Levels of urinary total protein expressed as a ratio of urinary total protein/urinary creatinine (mg/mg creatinine). Symbols are: (△) r-BSA group; (☉) d-BSA group; (●) saline group. Data are mean ± SE. $*P < 0.05$ compared to the saline group and $\dagger P < 0.05$ compared to the d-BSA group.

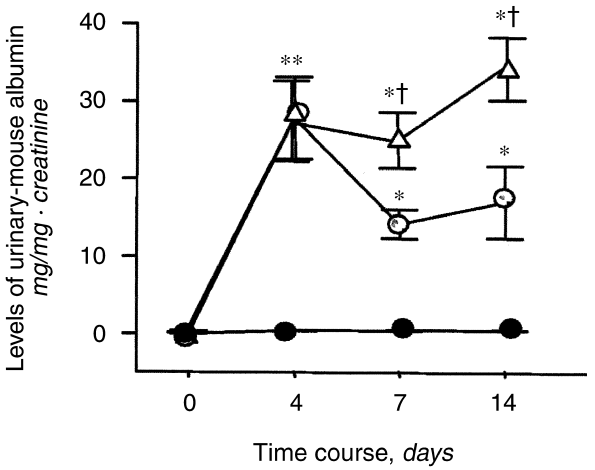

Fig. 2. Levels of urinary mouse albumin expressed as a ratio of urinary mouse albumin/urinary creatinine (mg/mg creatinine). Symbols are: (△) r-BSA group; (☉) d-BSA group; (●) saline group. Data are mean ± SE. $*P < 0.05$ compared to the saline group and $\dagger P < 0.05$ compared to the d-BSA group.

Thereafter, in the r-BSA group the urinary mouse albumin remained at a high level (25 ± 4 and 34 ± 4 mg albumin/mg creatinine on days 7 and 14, respectively). In the d-BSA group, the level of urinary mouse albumin decreased (14 ± 2 and 16 ± 5 mg albumin/mg creatinine on days 7 and 14, respectively). Urinary mouse albumin was significantly higher on days 7 and 14 in the r-BSA group than in the d-BSA group ($P < 0.01$).

Serum biochemistry

Total protein in the r-BSA and the d-BSA groups was significantly higher than that in the saline group on days 7 and 14 (Table 3). Levels of SUN, creatinine and Tg were not significantly different among the three groups. Tchol was significantly higher in the r-BSA group than in the d-BSA group on day 7.

Plasma anti-BSA antibody levels

On day 7, the antibody titer was not significantly different among the three groups (0.14 ± 0.018, 0.122 ± 0.012 and 0.122 ± 0.012 in r-BSA, d-BSA and saline groups, respectively, NS; Fig. 3). In the r-BSA and the d-BSA groups, the antibody titer was elevated on day 14 ($P <$ 0.05 compared to saline group), at which point there was not a significant difference between the two groups (0.243 ± 0.025, 0.201 ± 0.012 and 0.128 ± 0.009 in r-BSA, d-BSA and saline groups, respectively; $P < 0.05$ r-BSA group vs. saline group; $P < 0.05$ d-BSA group vs. saline group and NS r-BSA group vs. d-BSA group).

Histological evaluation

The renal histological damage was evaluated in the three groups by PAS-staining on days 7 and 14. In the r-BSA group, proximal tubular dilation and tubular hyaline cast formation in the interstitium were more prominent than those in the d-BSA group on day 14 (Fig. 4). The tubulointerstitial damage measure was significantly higher in the r-BSA group than in both the d-BSA and the saline groups on day 14 ($P < 0.01$; Fig. 5). The measure of the d-BSA group was not significantly higher than that of the saline group on days 7 and 14. Glomerulosclerosis was not observed on day 7, but mild mesangial expansion was seen on day 14 in both groups, and to a similar extent (0.13 ± 0.08 in r-BSA group and 0.18 ± 0.12 in d-BSA group, NS; data not shown).

Ultrastructural examination of the kidneys of both the r-BSA and the d-BSA groups on day 14 was performed (data not shown). There was no significant difference between the two groups in the number of electron dense deposits in the subepithelial space of the glomerular basement membrane and in the foot process effacement. Also, there was no obvious difference in the intracellular structure of the proximal tubules between the two groups.

Macrophages infiltration into the interstitium

An infiltration of macrophages was observed in the interstitium in the r-BSA and the d-BSA groups when

Table 3. Renal function, serum total protein and serum lipids of mice in the r-BSA, d-BSA and saline groups on days 7 and 14

	SUN *mg/dL*		Creatinine *mg/dL*		Total protein *mg/dL*		Total cholesterol *mg/dL*		Trigylceride *mg/dL*	
	day 7	day 14	day 7	day 14	day 7	day 14	day 7	day 14	day 7	day 14
r-BSA	33 ± 4	79 ± 16	0.2 ± 0.1	0.2 ± 0.0	6.3 ± 0.2[a]	6.1 ± 0.1[a]	88 ± 6[b]	96 ± 9	55 ± 11	54 ± 6
d-BSA	26 ± 4	52 ± 20	0.2 ± 0.0	0.2 ± 0.1	6.4 ± 0.2[a]	6.6 ± 0.5[a]	64 ± 4	71 ± 19	44 ± 9	44 ± 5
Saline	29 ± 2	34 ± 6	0.2 ± 0.0	0.1 ± 0.0	4.7 ± 0.1	4.8 ± 0.1	79 ± 5	85 ± 5	49 ± 7	49 ± 11

[a] $P < 0.05$ compared to the saline group
[b] $P < 0.05$ compared to the d-BSA group

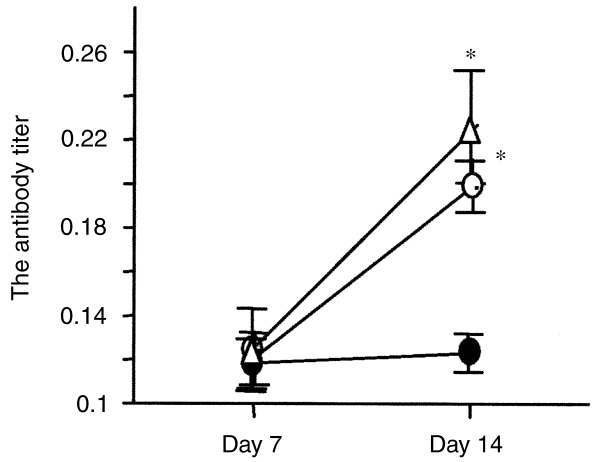

Fig. 3. Estimation of the anti-BSA antibody titer. Symbols are: (△) r-BSA group; (⊙) d-BSA group; (●) saline group. Data are mean ± SE. *$P < 0.05$ compared to the saline group.

first examined on day 7. On day 14, the number of infiltrated macrophages in the r-BSA group was significantly higher than that in the d-BSA group (54.0 ± 12.0, 22.6 ± 2.7 and 0.2 ± 0.1 in r-BSA, d-BSA and saline groups, respectively; $P < 0.05$ r-BSA group vs. d-BSA group; $P < 0.05$ r-BSA group vs. saline group; $P < 0.05$ d-BSA group vs. saline group; Figs. 6 and 7).

Cultured proximal tubular absorption of BSA

Bovine serum albumin content was not significantly different between proximal tubules cultured with r-BSA and those cultured with d-BSA (246.5 ± 64.8 ng albumin/mg protein r-BSA and 494.9 ± 140.9 ng albumin/mg protein d-BSA; $N = 6$; NS; Fig. 8). Approximately 83% of r-BSA uptake and 75% of d-BSA uptake was blocked by cold incubation, indicating that the majority of cellular albumin uptake was specific. No BSA was detected in control preparations that had not been exposed to BSA.

Viability of cultured proximal tubules after the incubation with BSA

The viability of cultured proximal tubules at 37°C was not significantly different between proximal tubules cultured with r-BSA and those cultured with d-BSA (32.5 ± 2.0 and 28.6 ± 1.5 %/mg protein in r-BSA and d-BSA, respectively; NS; Fig. 9). It was significantly inhibited by cold incubation in both r-BSA and d-BSA (16.4 ± 2.1 and 18.6 ± 1.8 %/mg protein in r-BSA and d-BSA, respectively; NS).

DISCUSSION

This study demonstrates that intraperitoneal administration of albumin replete with FFAs causes more severe tubulointerstitial damage than that of pure albumin. Growing evidence has shown that urinary protein is an independent factor for the genesis of tubulointerstitial damage and thus the progression of renal disease [2–6]. The present study shows that FFAs may be responsible for one mechanism leading to tubulointerstitial damage seen in massive proteinuria.

Tubulointerstitial injury is advanced by interstitial inflammation, which is characterized by an interstitial infiltrate of macrophages [14]. Semiquantitative histological analysis showed that the r-BSA group developed more significantly severe tubulointerstitial damage than both the d-BSA and the saline groups on day 14, as the number of infiltrated macrophages was much larger in the r-BSA group than that in the d-BSA group. This result suggests that FFAs bound to albumin caused more severe tubulointerstitial injury than albumin itself.

Concerning the possibility that interstitial injury seen in this model might be cast nephropathy-type injury, conventional histological study (PAS staining) and immunohistochemistry for BSA and mouse macrophages were performed using serial sections (data not shown). Bowman space, injured proximal tubules and a part of casts were stained by anti-BSA antibody in both the r-BSA and the d-BSA groups. Many macrophages did not always infiltrate around proximal tubules with BSA-positive casts. We observed BSA-negative cast formation and macrophage infiltration remarkably around proximal tubules without casts in the r-BSA group. Therefore, we conclude that interstitial injury of protein overload model is not cast nephropathy type injury.

The isoelectric point of r-BSA was reported to be 4.7 to 4.9 and that of d-BSA was 5.3 according to the data sheet of Sigma Chemical Company. We consider that the

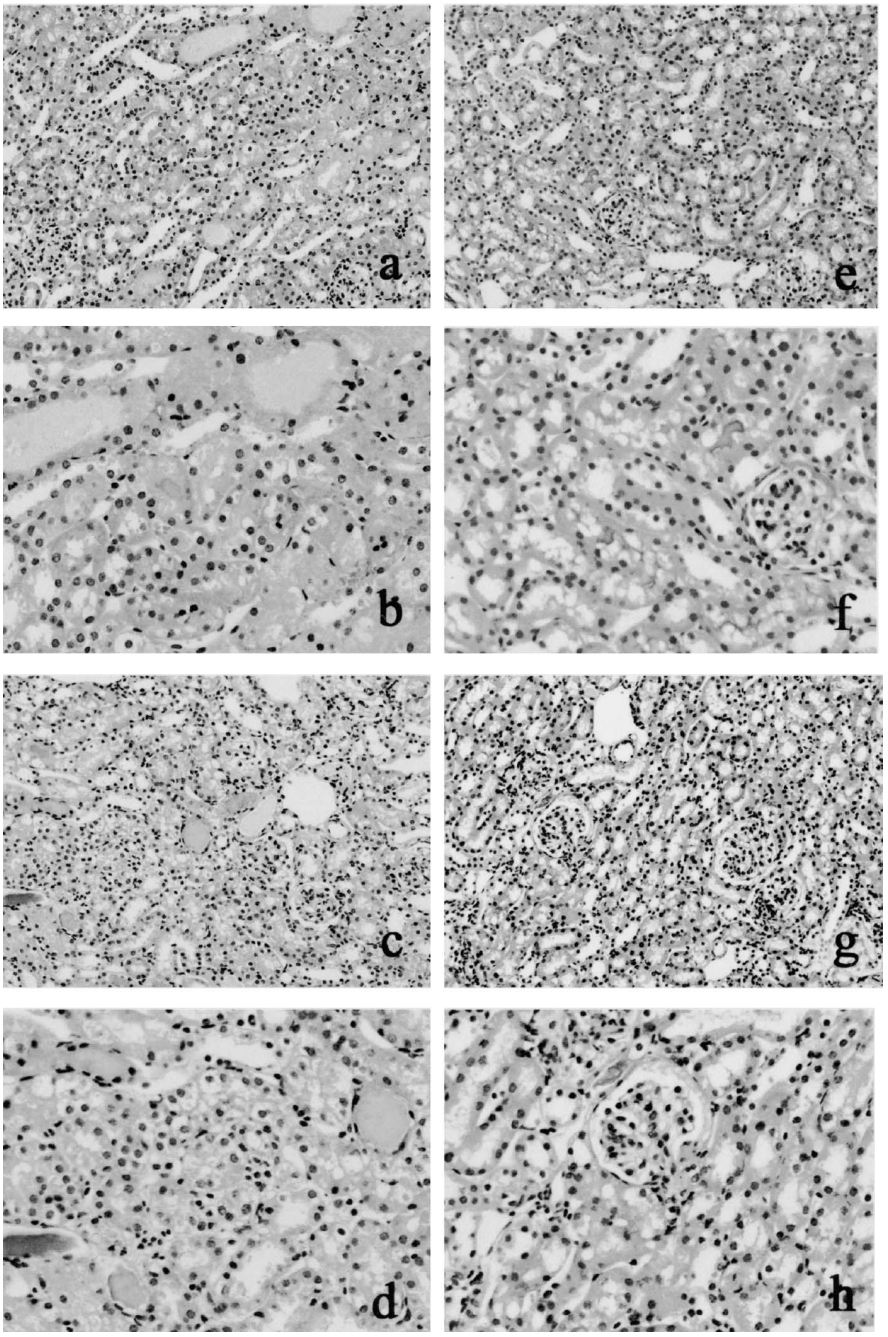

Fig. 4. Histologic evaluation of tubulointerstitial change in mice with the BSA-overload proteinuria (days 7 and 14). Representative photomicrographs of hematoxylin and eosin stained sections from mice in the r-BSA group on day 7 (*a* ×100, *b* ×400); on day 14 (*c* ×100, *d* ×400); in the d-BSA group on day 7 (*e* ×100, *f* ×400); and on day 14 (*g* ×100, *h* ×400).

difference in them was so small that we cannot explain the difference in urinary protein by the difference in isoelectric points.

Endotoxin of r-BSA was lower than that of d-BSA. It was reported that 0.5 μg/mL of endotoxin or more was necessary to release MCP-1 from the proximal tubule [20]; however, the concentration of endotoxin in the r-BSA or d-BSA used in our experiment was much less than that concentration. Moreover, we confirmed that the tubulointerstitial damage was provoked by endotoxin free r-BSA (BSA fraction V, Catalog No. A-9430; Sigma) like r-BSA used in our study (data not shown). Thus, we conclude that there is little possibility that endotoxin contained in the BSA exerted effects on our results.

Levels of serum total protein on days 7 and 14 and levels of urinary protein on day 4 in the r-BSA and the d-BSA groups were similar. This indicates that the amount of BSA absorbed to the systemic circulation from the peritoneal cavity, filtered through glomeruli

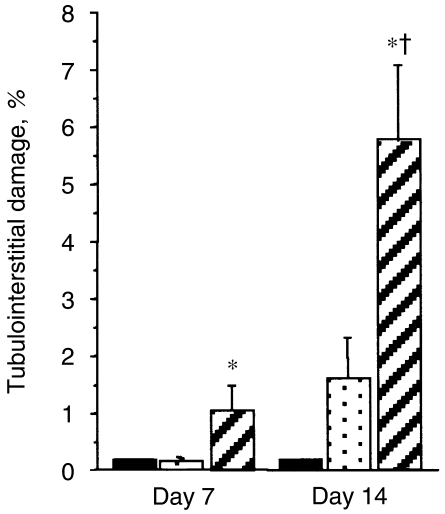

Fig. 5. Tubulointerstitial change was semiquantified as described in Methods section. Symbols are: (▨) r-BSA group; (▩) d-BSA group; (■) saline group. Data are mean ± SE. *$P < 0.05$ compared to the saline group and †$P < 0.05$ compared to the d-BSA group.

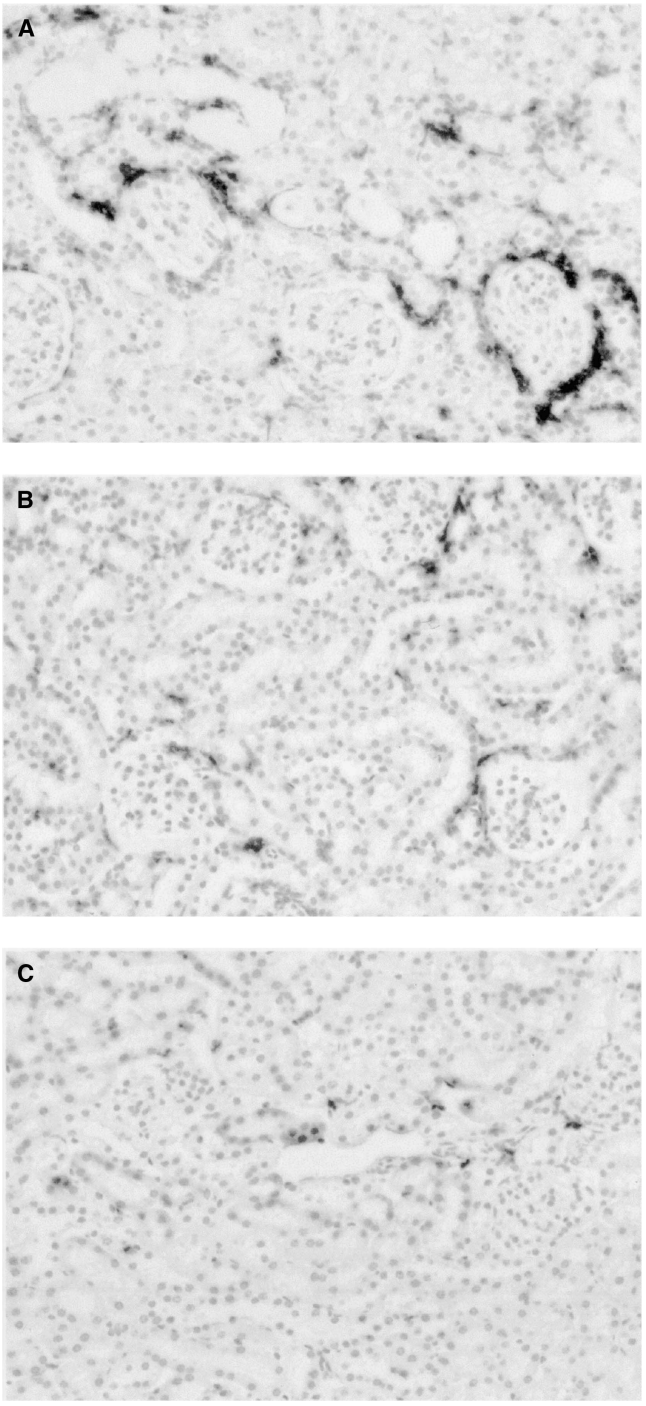

Fig. 6. Renal F4/80 immunostaining in the kidneys of the r-BSA group (*A*), the d-BSA group (*B*), and the saline group (*C*) on day 14 (×200). F4/80 was expressed in the interstitium in the r-BSA group and the d-BSA group.

and loaded to the proximal tubule was equivalent in the both groups. We showed that the proximal tubular uptake of r-BSA and d-BSA and the cellular viability after the incubation with them were not significantly different in the cultured cells. Moreover, in the immunohistochemistry for r-BSA or d-BSA, r-BSA or d-BSA-overloaded cells showed endolysosomal staining similarly (data not shown). Urinary excretion of both total protein and that of mouse albumin on days 7 and 14 were significantly higher in the r-BSA group than in the d-BSA group. This suggests that at the early stage of the experiment, the amount of albumin loaded to the proximal tubules was equivalent in the two groups and that at the late stage of the experiment, the reabsorption of albumin was decreased in the r-BSA group. We conclude that FFAs bound to albumin damage the proximal tubular function more than pure albumin.

The reason why albumin binding to FFAs caused more severe tubulointerstitial damage than pure albumin should be clarified. In the proximal tubules, FFAs play an essential role as an energy source, as membrane components and as precursors of lipid mediators. When an uncontrolled influx of FFAs into the proximal tubules occurs in massive proteinuria, the proximal tubules may keep the intracellular level of FFAs low by incorporating them into a variety of complex lipids such as triglycerides, diglycerides and phospholipids [8]. This adaptation may be deleterious to the proximal tubular cells because of changes to membrane architecture, which is a pathological effect of the complex lipids listed earlier [21]. Kees-Folts et al reported that some lipid chemoattractants were generated in the proximal tubules and released into the interstitium more by the tubular metabolism of FFAs bound to albumin than by that of albumin itself [9]. These biologically active lipid factors were shown to have a significant role in renal disease [22].

Thomas et al suggested that cultured proximal tubules overloaded with FFAs bound to albumin contained more

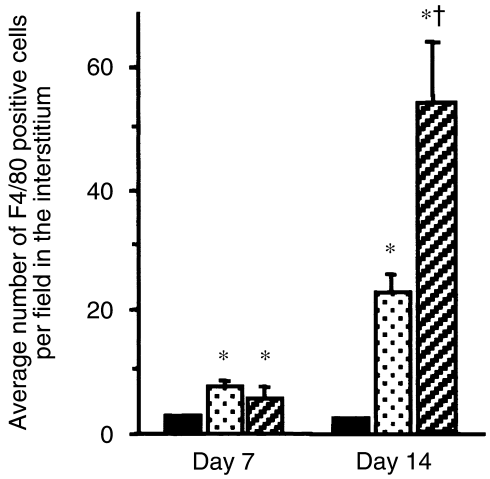

Fig. 7. Average number of F4/80 positive cells infiltrated in the interstitium per field. Symbols are: (▨) r-BSA group; (▩) d-BSA group; (■) saline group. Data are mean ± SE. $*P < 0.05$ compared to the saline group and $\dagger P < 0.05$ compared to the d-BSA group.

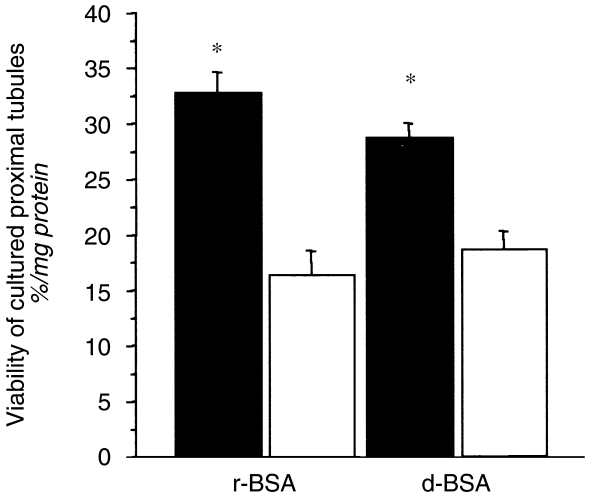

Fig. 9. Viability of cultured proximal tubules after the incubation with BSA. Data are mean ± SE. The bar graph showed the viability of cultured cells that was expressed as the ratio of the percent difference in reduction (%)/total protein concentration (mg) at (■) 37°C and (□) 4°C. $*P < 0.05$ compared to the 4°C incubation; $N = 6$.

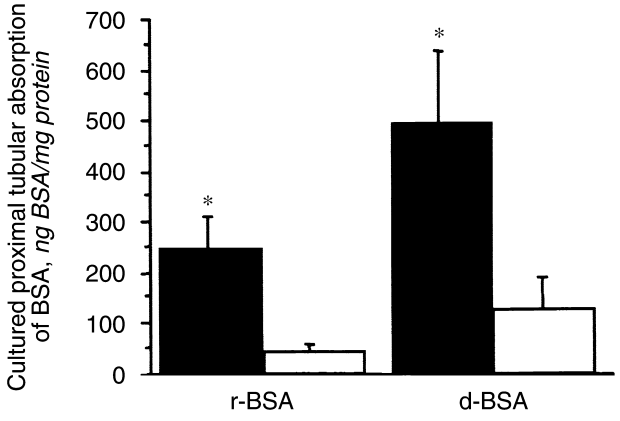

Fig. 8. Cultured proximal tubular absorption of BSA. Data are mean ± SE. The bar graph showed the intracellular BSA concentration/total protein concentration at (■) 37°C and (□) 4°C. $*P < 0.05$ compared to the 4°C incubation; $N = 6$.

intracellular oil red O-positive lipid droplets than those tubules overloaded with albumin itself [21]. However, we did not observe any foam cells in conventional staining, accumulation of lipid droplets in the proximal tubules in oil red staining, nor obvious difference in the intracellular structure of the proximal tubules between the r-BSA and the d-BSA groups in electron microscopy (data not shown). It is unclear why lipid overload to the proximal tubules was not histologically observed. However, histological sensitivity to detect lipid overload might be lower in vivo than in vitro.

In minimal change nephrotic syndrome (MCNS), the tubulointerstitial damage is nil or mild despite massive proteinuria. The short duration of heavy proteinuria with steroid therapy is a possible explanation for its benign outcome. In cases of MCNS, Ghiggeri et al have shown that urinary albumin has a markedly lower fatty acid content than serum albumin [23], which is another possible explanation for the absence or mildness of tubulointerstitial damage in this disease.

Although our study shows an important role for FFAs in the development of tubulointerstitial damage in proteinuria, nevertheless, mild tubulointerstitial damage still developed in mice injected with pure albumin. This change might be mediated by the release of cytokines such as monocyte chemoattractant protein-1 (MCP-1) [20, 24] or regulated upon activation, normal T cell expressed and secreted (RANTES) [25] or by direct injury to tubules caused by exposure to high protein concentration in the urine. As FFAs are bound to albumin with a t1/2 measured in milliseconds [26], d-BSA administered intraperitoneally might bind to FFAs before it reaches the urinary space, and a relatively low amount of FFAs might be loaded to the proximal tubules in the d-BSA group.

Serum total cholesterol in the r-BSA group remained higher than that in the d-BSA group on day 7 ($P < 0.05$) and day 14. This suggested that intraperitoneally injected FFAs in the r-BSA group were utilized for the synthesis of total cholesterol in the liver. As hypercholesterolemia was reported to cause interstitial inflammation and fibrosis [27], we could not exclude the possibility that higher total cholesterol in the r-BSA group might play a certain role in the development of tubulointerstitial damage. Thus, we induced hypercholesterolemia (300 to 320 mg/dL) in mice with a high fat diet. These hypercholesterolemic mice did not develop tubulointerstitial injury, which indicated that total cholesterol level in the

protein overload model did not influence the progression of tubulointerstitial injury (data not shown).

As the overload proteinuria was induced by the administration of a heterologous protein (BSA), immunologic mechanisms might play a role in the genesis of tubulointerstitial injury. The present study showed that the circulation of the anti-BSA antibody was raised similarly on day 14 in the both groups. The score of glomerulosclerosis in conventional staining and the foot process effacement in electron microscopy were similar in the r-BSA and the d-BSA groups on day 14. Thus, the glomerular damage could not have been responsible for the tubulointerstitial damage. We also found a few subepithelial electron dense deposits in electron microscopy and the deposition of mouse IgG and C3 in the glomeruli in immunohistochemistry (data not shown), but the degree of them was comparable in both groups. Therefore, we believe that an immunologic response does not affect the progression of tubulointerstitial injury in these experiments.

In summary, this study confirms and extends prior observations on the role of FFAs in the progression of tubulointerstitial damage, specifically that FFAs bound to albumin are significantly more responsible for progressing tubulointerstitial damage than albumin itself.

ACKNOWLEDGMENTS

This study was partly supported by Grant-in-Aid for Scientific Research (c) 13671099, the Ministry for Education, Science and Culture (K. Kimura). We thank Ms. Sanae Ogawa for assistance with the laboratory technique. We also thank Drs. Toshihide Mimura, Masaomi Nangaku, Shoko Tateishi, Shigeyoshi Oba and Satoru Fukuda for their technical support.

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REFERENCES

1. Risdon RA, Sloper JC, De Wardener HE: Relationship between renal function and histological changes found in renal-biopsy specimens from patients with persistent glomerular nephritis. *Lancet* 2:363–366, 1968
2. Eddy AA, McCulloch L, Liu E, Adams J: A relationship between proteinuria and acute tubulointerstitial disease in rats with experimental nephrotic syndrome. *Am J Pathol* 138:1111–1123, 1991
3. Eddy AA: Experimental insights into the tubulointerstitial disease accompanying primary glomerular lesions. *J Am Soc Nephrol* 5:1273–1287, 1994
4. Peterson JC, Adler S, Burkart JM, *et al*: Blood pressure control, proteinuria, and the progression of renal disease. The Modification of Diet in Renal Disease Study. *Ann Intern Med* 123:754–762, 1995
5. Remuzzi G, Ruggenenti P, Benigni A: Understanding the nature of renal disease progression. *Kidney Int* 51:2–15, 1997
6. Remuzzi G, Bertani T: Pathophysiology of progressive nephropathies. *N Engl J Med* 339:1448–1456, 1998
7. Hamilton JA, Era S, Bhamidipati SP, Reed RG: Locations of the three primary binding sites for long-chain fatty acids on bovine serum albumin. *Proc Natl Acad Sci USA* 88:2051–2054, 1991
8. Thomas ME, Schreiner GF: Contribution of proteinuria to progressive renal injury: Consequences of tubular uptake of fatty acid bearing albumin. *Am J Nephrol* 13:385–398, 1993
9. Kees-Folts D, Sadow JL, Schreiner GF: Tubular catabolism of albumin is associated with the release of an inflammatory lipid. *Kidney Int* 45:1697–1709, 1994
10. Humes HD, Nguyen VD, Cieslinski DA, Messana JM: The role of free fatty acids in hypoxia-induced injury to renal proximal tubule cells. *Am J Physiol* 256:F688–F696, 1989
11. Matthys E, Patel Y, Kreisberg J, *et al*: Lipid alterations induced by renal ischemia: Pathogenic factor in membrane damage. *Kidney Int* 26:153–161, 1984
12. Portilla D, Shah SV, Lehman PA, Creer MH: Role of cytosolic calcium-independent plasmalogen-selective phospholipase A2 in hypoxic injury to rabbit proximal tubules. *J Clin Invest* 93:1609–1615, 1994
13. Walker PD, Shah SV: Evidence suggesting a role for hydroxyl radical in gentamicin-induced acute renal failure in rats. *J Clin Invest* 81:334–341, 1988
14. Eddy AA: Interstitial nephritis induced by protein-overload proteinuria. *Am J Pathol* 135:719–733, 1989
15. Eddy AA, Giachelli CM: Renal expression of genes that promote interstitial inflammation and fibrosis in rats with protein-overload proteinuria. *Kidney Int* 47:1546–1557, 1995
16. Eddy AA: Expression of genes that promote renal interstitial fibrosis in rats with proteinuria. *Kidney Int* 49(Suppl 54):S49–S54, 1996
17. Eddy AA, Kim H, Lopez-Guisa J, *et al*: Interstitial fibrosis in mice with overload proteinuria: Deficiency of TIMP-1 is not protective. *Kidney Int* 58:618–628, 2000
18. Hisada Y, Sugaya T, Yamanouchi M, *et al*: Angiotensin II plays a pathogenic role in immune-mediated renal injury in mice. *J Clin Invest* 103:627–635, 1999
19. Nagase M, Kaname S, Nagase T, *et al*: Expression of LOX-1, an oxidized low-density lipoprotein receptor, in experimental hypertensive glomerulosclerosis. *J Am Soc Nephrol* 11:1826–1836, 2000
20. Wang Y, Chen J, Chen L, *et al*: Induction of monocyte chemoattractant protein-1 in proximal tubule cells by urinary protein. *J Am Soc Nephrol* 8:1537–1545, 1997
21. Thomas ME, Morrison AR, Schreiner GF: Metabolic effects of fatty acid-bearing albumin on a proximal tubule cell line. *Am J Physiol* 268:F1177–F1184, 1995
22. Lindner A, Hinds TR, Joly A, Schreiner GF: Neutral lipid from proteinuric rat urine is a novel inhibitor of the red blood cell calcium pump. *J Am Soc Nephrol* 10:1170–1178, 1999
23. Ghiggeri GM, Ginevri F, Candiano G, *et al*: Characterization of cationic albumin in minimal change nephropathy. *Kidney Int* 32: 547–553, 1987
24. Wang Y, Rangan GK, Tay YC, Harris DC: Induction of monocyte chemoattractant protein-1 by albumin is mediated by nuclear factor kappaB in proximal tubule cells. *J Am Soc Nephrol* 10:1204–1213, 1999
25. Zoja C, Donadelli R, Colleoni S, *et al*: Protein overload stimulates RANTES production by proximal tubular cells depending on NF-kappa B activation. *Kidney Int* 53:1608–1615, 1998
26. Spector AA: Fatty acid binding to plasma albumin. *J Lipid Res* 16:165–179, 1975
27. Eddy AA: Interstitial fibrosis in hypercholesterolemic rats: Role of oxidation, matrix synthesis, and proteolytic cascades. *Kidney Int* 53:1182–1189, 1998