Severe proteinuria, sustained for 6 months, induces tubular epithelial cell injury and cell infiltration in rats but not progressive interstitial fibrosis

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Abstract

Background. Sustained proteinuria is reported to be very harmful to the tubulointerstitium, leading to severe interstitial injury. However, it remains unclear whether sustained proteinuria itself is responsible for severe interstitial injury because, in the previously reported models, the development of factors other than proteinuria in tubulointerstitial lesions could not be excluded completely.

Methods. After treatment to induce immune tolerance to mouse immunoglobulin, 20 rats were injected with anti-rat slit diaphragm monoclonal antibody (mAb) 5-1-6 twice a week for 6 months and were then sacrificed.

Results. mAb 5-1-6 induced massive proteinuria in 11 rats. In nine rats with mild proteinuria, no histological alteration could be detected with light microscopy and immunofluorescence. In nephrotic rats, light microscopy showed minor glomerular abnormalities, with interstitial oedema, tubular epithelial cell degeneration and interstitial cell infiltration. Immunofluorescence revealed increased expression of vimentin and an increased number of OX1-, OX19- and ED1-positive cells. However, we could not detect any accumulation of type I and IV collagen or laminin in the tubulointerstitial area. RT-PCR showed that the expression of mRNA for type I collagen was not increased, compared with that in control rats.

Conclusions. We succeeded in developing a model of persistent nephrosis without severe glomerular abnormalities, nephrectomy or other manoeuvres known to induce disturbed haemodynamics, using an agent without tubulointerstitial toxicity, and considered it to be suitable for investigating the direct toxicity of proteinuria. In this model, isolated massive proteinuria induced interstitial injury. However, the degree of injury was suggested to be much less than that observed in other previously developed models.

Keywords: monoclonal antibody 5-1-6; sustained proteinuria; tubulointerstitial injury

Introduction

Sustained proteinuria is reported to have pathogenic importance in the progression of chronic renal failure in clinical [1,2] and experimental nephritis [1–6]. There is a clear relationship between the degree of proteinuria and the severity of interstitial cell infiltration in purino- mycin aminonucleoside (PAN) nephritis [3] and protein-overload proteinuria [4]. Interstitial fibrosis is induced by proteinuria in PAN nephrosis [5], protein-overload proteinuria [4] and adriamycin nephropathy [6] within few months. According to these findings, proteinuria itself is assumed to have severe toxicity to induce severe interstitial injury, i.e. interstitial fibrosis, and to result in the progression of chronic renal failure. However, it remains unclear whether sustained proteinuria itself is responsible for severe interstitial injury because, in the previously developed models, the involvement of factors other than proteinuria in tubulointerstitial lesions could not be completely excluded. In order to investigate whether tubulointerstitial injuries would be caused directly by continuous proteinuria, a new animal model of persistent nephrosis needed to be developed.

We have established a monoclonal antibody (mAb), 5-1-6, which binds specifically to the p51 antigen located on the slit diaphragm of rat glomerular epithelial cells and causes severe proteinuria when injected into rats [7]. mAb 5-1-6 does not appear to bind to antigens on tubular epithelial cells or in the interstitial area. The proteinuria induced by mAb 5-1-6 is independent of complement and inflammatory cells [7,8]. Histological alterations can hardly be detected, after a single injection of mAb 5-1-6. Even the foot processes of the podocytes remain well preserved except for focal areas of effacement [7]. Since mAb 5-1-6 can therefore be considered to exert no direct toxicity on the tubulointerstitium and does not cause severe glom-
erular abnormalities, we sought to establish an animal model of persistent nephrosis using multiple injections of mAb 5-1-6.

In the present study, we succeeded in producing a rat model of continuous proteinuria using multiple injections of mAb 5-1-6 after treatment to induce immunotolerance to mouse immunoglobulin (Ig). We then applied this model to investigate the toxic effects of enhanced and sustained excretion of host protein on the tubulointerstitium, without interference caused by other toxic agents. During the 6-month observation period, tubular epithelial cell injury and interstitial mononuclear cell infiltration were observed but interstitial fibrosis was not.

Materials and methods

Animals

Six-week-old female Wistar rats weighing 140–160 g were purchased from Charles River, Japan, Inc. (Atsugi, Japan). All animal experimentation was conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Induction of immunotolerance to mouse γ-globulin (MGG)

To prevent the production of antibodies to injected mAb 5-1-6, immunotolerance to mouse Ig was induced. We prepared MGG from the sera of normal mice by precipitation with 50% saturated ammonium sulfate, followed by dialysis against phosphate-buffered saline (PBS). The MGG was centrifuged at 17,000 g for 30 min and the supernatant, designated soluble MGG (sMGG), was collected. As a preliminary to confirm that a tolerant state was induced in rats by an intravenous injection of 5 mg of sMGG, 2 weeks after this initial injection, we challenged three sMGG-treated and three untreated control rats with 1 mg of MGG administered subcutaneously with Freund’s complete adjuvant. Two weeks after the challenge injection, their sera were sampled and the titre of antibody to mouse Ig was examined by indirect enzyme-linked immunosorbent assay (ELISA) as described in ‘Estimation of serum antibody titre’. The titre of anti-mouse Ig in the sMGG-treated group was less than one-tenth of that in the untreated group, showing that tolerance had been induced successfully. For further experiments, each rat was therefore injected with 5 mg of sMGG to induce immunotolerance.

Preparation of mAb

mAb 5-1-6 was prepared as described previously [7]. The hybridoma was cultured in serum-free medium (protein-free hybridoma medium II, Gibco-BRL, Rockville, MD) and the supernatant was collected. Ammonium sulfate (350 g/l) was added immediately to the supernatant, which was then stored at 4°C. The preparation subsequently was centrifuged at 10,000 r.p.m. for 30 min. The sediment was suspended in PBS, dialysed extensively against PBS and stored at −20°C. Just before injection, the mAb was diluted to 500 µg or 250 µg/ml with PBS. Only traces of proteins other than IgG were detected by silver staining after (SDS–PAGE). As a control, murine IgG1 mAb, RVG1 (against rotavirus, demonstrated not to react with rat kidneys) was used.

Experimental design

For the first time, we used an mAb derived from the supernatant of a culture in serum-free medium. In a preliminary study, we confirmed that the initial injection of 500 µg, followed by the twice weekly injections of 250 µg, was enough to induce sustained proteinuria.

As shown in Figure 1, we first injected 5 mg of sMGG intravenously into 24 rats to induce immunotolerance to mouse IgG. Two weeks later, 500 µg of mAb 5-1-6 was injected into 20 rats. From the next injection onwards, 250 µg mAb was injected twice a week (every Monday and Friday). Four rats were injected with the same dose of mAb RVG1 as negative controls. Twenty-four hour urine was collected on Monday–Tuesday and Thursday–Friday, and sera were sampled every 4 weeks. Twenty-five weeks after the first injection, the rats were killed under anaesthesia with ether. After cardiac puncture for blood sampling, the left renal artery and vein were clamped and the left kidney was removed and weighed. The left kidney was then used for extraction of RNA. The right kidney was perfused with PBS via the heart and was then removed for light, electron and immunofluorescence (IF) microscopy.

Characterization of proteinuria

Twenty-four hour urinary specimens were collected from the rats using metabolic cages. The urine was centrifuged at 1000 g for 10 min, and the protein content was determined by the biuret method using bovine serum albumin (BSA) as the standard.

Urine and serum were sampled at 4, 16 and 25 weeks from rats with massive proteinuria, in order to calculate the selectivity index. The samples were heated for 2 min at 100°C in SDS–PAGE sample buffer (10% sucrose, 6.25 mM Tris·HCl (pH 6.8), 2% SDS, 10 mM dithiothreitol, and 0.0025% bromophenol blue). Urinary protein (about 20 µg) and serum protein (20 µl of serum diluted 1:50) in sample buffer were applied to a 7.5% gel. SDS–PAGE was performed as previously described [7]. The albumin, IgG and other bands were stained with brilliant blue R (Sigma, St Louis, MO) and quantified by densitometry (ATTO, Tokyo, Japan). The amount of protein which was greater than albumin in urinary protein (20 µg) was used. The amount of protein which was greater than albumin in urinary total protein was calculated. From urinary albumin (Ualb), urinary IgG (UIgG), serum albumin (Salb) and serum IgG (SIgG) levels thus measured, the selectivity of urinary protein (selectivity index; SI) was also calculated using the following formula [9]: SI = (UIGG × Salb)/(Ualb × SIgG)

Laboratory investigations

Serum creatinine, serum blood urea nitrogen (BUN), serum total cholesterol and albumin, serum N-acetyl glucosaminase (NAG) activity and complement (CH50), urinary NAG activity and urinary creatinine levels were measured. From the data on serum creatinine (S), urine creatinine (U), 24 h urine volume (V) and body weight (BW) at sacrifice, the
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<table>
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<th>Experimental design</th>
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<td>sMGG injection</td>
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<td>5-1-6 or RVG1 injection</td>
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<td>blood sampling</td>
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<td>sacrifice</td>
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**Fig. 1.** Experimental protocol. Each rat received 5 mg of sMGG intravenously to induce immunotolerance to mouse Ig 2 weeks before the first injection of mAb 5-1-6 or RVG1. The mAb was injected twice a week (Monday and Friday). Twenty-four hour urine was collected every Monday–Tuesday and Thursday–Friday. Blood was sampled every 4 weeks. Ten 5-1-6-injected rats and four RVG1-injected rats were sacrificed on a Tuesday following 24 h urine sampling, and the 10 remaining 5-1-6-injected rats were sacrificed on a Wednesday. sMGG, soluble mouse γ globulin.

24 h endogenous creatinine clearance (Ccr) was calculated using following formula.

\[
\text{Ccr}(\text{ml/min/m}^2 \text{ BS}) = \frac{U (\text{mg/dl}) \times V (\text{ml}) \times 1}{S (\text{mg/dl})} \times \frac{1}{1440} (\text{min}) \times 1/\text{BS} (\text{m}^2)
\]

\[
\text{BS} = \text{body surface area of rats calculated using the formula.}
\]

\[
\text{BS} (\text{m}^2) = \left(\frac{\text{BW} (\text{g})}{100}\right)^{2/3} \times 10
\]

The occult blood and glucose contents of the urine at sacrifice were examined semiquantitatively with Multistix®M (Bayer-Sankyo Co., Tokyo, Japan).

**Morphological and immunohistological study**

For light microscopy, parts of the kidney were fixed with 10% neutral formalin and embedded in paraffin. Sections (2–3-µm thick) were stained with periodic acid–Schiff (PAS) and periodic acid–methenamin silver (PAM). A semiquantitative morphological study of the glomerular lesions was carried out in a blinded manner, as described by Raj et al. [10]. A total of 50 glomeruli were analysed in each specimen. Severity of glomerular injury was expressed as matrix score.

For IF microscopy, the renal tissues were quick-frozen in n-hexane cooled to −70 °C and 4-µm thick sections were cut with a cryostat. For the indirect method, the sections were incubated with the anti-rat leukocyte common antigen mAb,OX1 (Serotec, Oxford, UK), the anti-rat pan T-cell mAb,OX19 (Serotec), the anti-rat pan macrophage/monocyte mAb,ED1 (Biogene, Sundown, NH), the anti-rat neutrophil mAb,RP3 (kindly provided by Dr Sendo, Yamagata University, Yamagata, Japan), rabbit anti-rat transforming growth factor-β1 (TGF-β1) polyclonal antibody (kindly provided by Dr Muramatsu, Research Center, Mitsubishi Kasei, Yokohama, Japan), rabbit anti-vimentin polyclonal antibody (Bio-science products AG, Emmenbruecke, Switzerland), rabbit anti-rat type-I collagen polyclonal anti-body (Chemicon International Inc., Temecula, CA), rabbit anti-rat type IV collagen polyclonal antibody (LSL, Tokyo, Japan), rabbit anti-laminin polyclonal antibody or anti-z-smooth muscle actin (SMA) mAb (Sigma) at 37 °C for 30 min, then washed with PBS for 10 min at room temperature. The sections first incubated with rabbit polyclonal antibodies were then incubated with fluorescein isothiocyanate (FITC)-conjugated anti-rabbit IgG (Dako, Glostrup, Denmark), while those treated with OX1, OX19, ED1 or RP3 were incubated with FITC-conjugated anti-mouse IgG2a (Southern Biotechnology Associates Inc., Birmingham, AL). For the direct method, the sections were incubated with FITC-conjugated anti-mouse IgG2a (Southern Biotechnology Associates Inc., Birmingham, AL). The occult blood and glucose contents of the urine at sacrifice were examined semiquantitatively with Multistix®M (Bayer-Sankyo Co., Tokyo, Japan).

For electron microscopy, a part of the renal cortex from each rat was cut into small pieces and fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer (PB) for several days at 4 °C. After washing in PB and post-fixing in 1% OsO₄ for 2 h, the fixed material was dehydrated through an ethanol–propylene oxide series and embedded in Araldite M.
Ultrathin sections were prepared, stained with uranyl acetate and lead citrate, and examined under a JEOL 1200EX electron microscope (Tokyo, Japan).

Reverse transcriptase–polymerase chain reaction (RT–PCR)

Total RNA was extracted from the kidney cortex with Trizol (Gibco-BRL) following the standard protocol. The final product was air dried, dissolved in diethyl pyrocarbonate (DEPC)-treated water, and stored at −80°C. First strand complementary DNA (cDNA) was synthesized using the SuperScript Preamplication System (Gibco-BRL), following the standard protocol. Amplification was carried out using a PC-800 programmable temperature control system (Astec, Fukuoka, Japan) through 20–40 cycles of denaturation at 95°C for 30 s, annealing at individual temperatures for 30 s and extension at 72°C for 10 min. The optimal cycle numbers were determined in a preliminary trial to be in the linear phase of amplification. Products were analysed by molecular weight on 1.5% agarose gels, and stained with ethidium bromide. mRNA for collagen type I and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were quantified bydensitometry (ATTO), and collagen type I/GAPDH was calculated. The following rat sequences were used as primers: (i) rat collagen type I (sense 5'-CCACGTAGGTTGCTAAGGT-3', antisense 5'-CCGGTAGGCTAAAATATATAAAGG-3'), (ii) rat GAPDH (sense 5'-GGATGACCTTGCCCACAGC-3', antisense 5'-CTCTACCCACGGGCAGTTCAAAGG-3').

Estimation of serum antibody titre

The rat anti-mouse IgG antibody titre was measured by an indirect ELISA. Microtitre plates were coated with 100 μl of a 50 μg/ml solution of 5-1-6 in 0.05 M Tris–HCl buffer (pH 7.4) for 2 h at 37°C. After extensive washing, the plate was blocked with 0.5% BSA for 2 h at 37°C. The following reagents were added sequentially, washing after each addition: 100 μl of test serum (1:1000 dilution), 100 μl of peroxidase-conjugated anti-rat IgG (1:200000 dilution; Jackson Immunoresearch Laboratories, West Grove, PA) and 200 μl of substrate solution (0.05 M sodium citrate, pH 5.0, containing 0.4 mg/ml o-phenylenediamine and 0.4 mg/ml urea hydrogen peroxide). The reaction was stopped by adding 50 μl of 6M H2SO4 and the absorbance was measured at 490 nm with an ELISA autoreader (Bio-Rad, Boston, MA). The reagent blanks and normal rat serum were used as negative controls. Rat sera which had been sampled 2 weeks after the injection of 1 mg of 5-1-6 with Freund’s complete adjuvant were used as positive controls. A standard curve was drawn from the optical densities (ODs) of the positive control sera diluted 1:100, 1:300, 1:1000, 1:3000, 1:10000, 1:30000, 1:100000 and 1:300000 with 0.05 M Tris–HCl buffer (pH 7.4), and the titre for each test serum was calculated by comparison with the standard curve to determine the percentage titre relative to the positive control sera. The rats used as positive controls were immunized with mouse Ig at the pre-treatment dose for inducing immune complex glomerulonephritis by heterogeneous IgG [11].

Statistical analysis

Statistical analysis was performed using Student’s or Welch’s t-test or Mann–Whitney’s U-test as appropriate. A P value <0.05 was considered significant.

Results

Proteinuria

Large inter-individual variations in the degree of proteinuria were observed in 5-1-6-injected rats (Figure 2A). Generally, heavy proteinuria was observed on Mondays and relatively light proteinuria on Thursdays. The baseline level of proteinuria increased until the second week (Figure 2B). The average degree of proteinuria was calculated from the amount of urinary protein excreted between day 22 and day 169. As shown in Figure 2A, 11 of the 20 rats exhibited massive proteinuria (average level >200 mg/day; group 1) while the remaining nine excreted a relatively small amount of urinary protein (average level <100 mg/day; group 2). More than 100 mg of urinary protein was excreted on day 4 in all rats in group 1, and <50 mg of urinary protein was excreted in all rats in group 2. The four rats injected with RVG1 (control group) did not excrete significantly abnormal levels of urinary protein. The selectivity of urinary protein remained high throughout the study. The selectivity index was between 0.025 and 0.049 in three rats with the heaviest proteinuria. Urinary protein with a molecular weight larger than albumin was >10% of urinary total protein in these rats.

Body weight and kidney weight

Body weight and kidney weight differed significantly between group 1 and group 2 (Table 1). A positive correlation of kidney weight with proteinuria (Figure 3A) was observed.

Laboratory investigations

Serum albumin, total cholesterol, CH50 levels and urinary NAG activity differed significantly between group 1 and group 2 (Table 1), whereas BUN and creatinine clearance (Ccr) did not differ significantly (Table 1). Serum albumin (Figure 3B) and total cholesterol (Figure 3C) correlated significantly with the severity of proteinuria. Glucose and occult blood were not detected in the urine of any of the rats at sacrifice.

Titre of anti-mouse IgG antibody

The anti-mouse IgG antibody titre increased from 4 weeks in all groups of rats. The serum titre in group 2 was 2–5 times greater than that in group 1 at all times except on day 0. The serum titres of eight of the rats in group 2 and all of the rats in group 1 was >10% of the values recorded for the positive control.

Morphological and immunohistological study

Light microscopy. No global glomerulosclerosis was observed. Segmental matrix expansion was observed in some glomeruli from rats in all groups but, in each, the area of expansion was <25% of the mesangial area (Figure 4A and B). Capsular adhesions were
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**Fig. 2.** Time course of proteinuria. The time courses of changes in the level of proteinuria for each rat injected with mAb 5-1-6 are shown (A). Large amounts of protein were excreted by 11 rats (group 1) and small amounts by nine rats (group 2). The time course of changes in the average amount of proteinuria for each group is shown (B).

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Group 1 (n=11)</th>
<th>Group 2 (n=9)</th>
<th>Controls (n=4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Proteinuria (mg/24 h)</td>
<td>434.5 ± 133.9*</td>
<td>30.8 ± 19.4*</td>
<td>1.3 ± 0.2</td>
</tr>
<tr>
<td>Body weight (g)</td>
<td>261.4 ± 14.2*</td>
<td>287.1 ± 18.6</td>
<td>296.3 ± 17.0</td>
</tr>
<tr>
<td>Kidney weight (g)</td>
<td>1.367 ± 0.159*</td>
<td>1.041 ± 0.077</td>
<td>1.092 ± 0.078</td>
</tr>
<tr>
<td>Kidney index</td>
<td>0.525 ± 0.067*</td>
<td>0.363 ± 0.021</td>
<td>0.337 ± 0.027</td>
</tr>
<tr>
<td>BUN (mg/dl)</td>
<td>21.8 ± 2.6</td>
<td>21.7 ± 3.8</td>
<td>21.6 ± 4.3</td>
</tr>
<tr>
<td>Total cholesterol (mg/dl)</td>
<td>267.7 ± 74.8*</td>
<td>78.1 ± 13.5*</td>
<td>63.7 ± 6.0</td>
</tr>
<tr>
<td>Urinary NAG activity (mU/24 h)</td>
<td>225.7 ± 140.7*</td>
<td>71.1 ± 38.5</td>
<td>72.5 ± 72.6</td>
</tr>
<tr>
<td>Serum NAG activity (U/l)</td>
<td>9.7 ± 3.0</td>
<td>8.0 ± 1.1</td>
<td>8.4 ± 0.6</td>
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<tr>
<td>Matrix score (glomerulus)</td>
<td>0.149 ± 0.053</td>
<td>0.118 ± 0.033</td>
<td>0.105 ± 0.038</td>
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<tr>
<td>α-SMA score (IF score)</td>
<td>0.026 ± 0.020</td>
<td>0.022 ± 0.034</td>
<td>0.037 ± 0.016</td>
</tr>
<tr>
<td>OX1-positive cells</td>
<td>26.0 ± 11.6*</td>
<td>6.3 ± 3.8</td>
<td>4.1 ± 0.6</td>
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<tr>
<td>ED1-positive cells*</td>
<td>2.3 ± 1.1*</td>
<td>0.9 ± 0.5</td>
<td>0.5 ± 0.2</td>
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<td>OX19-positive cells*</td>
<td>10.1 ± 4.8*</td>
<td>1.5 ± 0.9</td>
<td>1.3 ± 0.5</td>
</tr>
<tr>
<td>RP3-positive cells*</td>
<td>0.28 ± 0.22</td>
<td>0.23 ± 0.16</td>
<td>0.16 ± 0.05</td>
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</table>

Values are mean ± SD.

Kidney weight/body weight × 100.

Number of cells (per high power field in the interstitium).

*P < 0.01 vs group 2; *P < 0.001 vs group 2; †P < 0.05 vs control; ‡P < 0.001 vs control.

Observed in some glomeruli from some rats of group 1, but the renal interstitium of these rats was not particularly damaged. Matrix scores are shown in Table 1.

As shown in Figure 4, interstitial oedema, mononuclear cell infiltration and degeneration of the tubular epithelial cells were observed in the rats of group 1, but no accumulation of PAS- and PAM-positive material was observed in the interstitium. No marked abnormalities were found in group 2 or in the control group.

**Immunofluorescence microscopy.** On direct IF, mouse IgG was detected in the glomeruli from rats in both group 1 and group 2 but was not seen in the tubulointerstitial area (Figure 5A and B). A granular pattern could be seen more clearly in group 1, while a pseudo-linear pattern was observed in group 2. In the control group, neither the glomeruli nor the tubulointerstitial area were stained with anti-mouse IgG. No significant deposits of rat Ig occurred in the glomeruli or the interstitium in any group. Rat C3 was not deposited in the glomeruli in any group, but was observed in the intratubular space in the rats in group 1 (Figure 5C).

On indirect IF, vimentin was expressed only on the glomerular epithelial cells in all rats in the control group and group 2 (Figure 6B) and in rats with relatively low-grade proteinuria in group 1, but was detected on the glomerular epithelial and tubular epithelial cells in rats with relatively high-grade proteinuria in group 1 (Figure 6A). OX19- and ED1-positive cells as well as OX1-positive cells had infiltrated the interstitium of the rats in group 1 (Figure 7A and C). The number of cells was significantly increased in group 1 and related to the severity of proteinuria (Figure 3D, Table 1). No significant accumulation of type I or IV collagen or of laminin could be detected in the areas of the interstitium evaluated (at least 10 mm²) in any of the groups (Figure 8). α-SMA-expressing interstitial cells were observed in some rats with relatively high-grade proteinuria in group 1 (Figure 9A). TGF-β was expressed focally on
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Fig. 3. Correlations between various parameters and the severity of proteinuria. Kidney weight (A) was positively correlated with the severity of proteinuria. Serum albumin (B) was negatively and serum total cholesterol (C) was positively correlated with the severity of proteinuria. The number of OX1-positive cells in the interstitium was related to the severity of proteinuria (D). BUN and Cer were not significantly related to the severity of proteinuria (E and F).

Electron microscopy. No mesangial cell proliferation, matrix expansion nor subepithelial electron-dense deposits were observed in the glomeruli of rats from any of the groups. Partial effacement of the foot processes of the epithelial cells was found occasionally, especially in group 1 (Figure 10).

Expression of mRNA for type I collagen

Expression of mRNA for type I collagen was not increased in group 1 (three rats with the heaviest proteinuria) when compared with the controls after 35 PCR cycles (Figure 11).

Discussion

In the present study, large inter-individual variations in the degree of proteinuria were observed (Figure 2). The inter-individual variation in the amount of urinary protein induced by mAb 5-1-6 has been demonstrated to be larger in Wistar than in Lewis or Brown-Norway rats [12]. This larger variation in Wistar rats is thought to be a result of outbreeding. In the present model, rats with less proteinuria after the first injection continued to excrete less urinary protein even after repeated injections, suggesting that inter-individual variation was derived from their genetic background. Multiple injections are considered to enhance variation. However, in our study, the large variation between individuals was useful in investigating the relationship between histological alterations and the severity of proteinuria.

Previous in vivo studies to evaluate the injurious effects of proteinuria have used models such as PAN nephrosis [3,5], protein-overload proteinuria [4], adriamycin nephropathy [6] or passive Heymann nephritis [13], in which many factors may be involved in the pathogenesis. PAN or adriamycin may be injurious to tubular epithelial cells [3,6]. In the chronic PAN nephritis model in the unilaterally nephrectomized rat, the obliteration of the post-glomerular capillaries induced by glomerulosclerosis may lead to severe interstitial injury following tubulointerstitial ischaemia [14,15]. In addition, the artificial modification of haemodynamics may cause the progression of tubular damage. Haemodynamics are also greatly modified by nephrectomy and hyperalbuminaemia in the protein-overload proteinuria model in unilaterally nephrectom-
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Fig. 4. Light photomicrographs illustrating morphological alterations. The sections were stained with PAS (A, B, C and D) or PAM (E and F). Sections from the kidneys of rats in group 1 (A, C and E) and group 2 (B, D and F) are shown. No global glomerulosclerosis was observed in any of the groups and glomerular alterations were minimal in both groups (A and B). Tubular epithelial cell atrophy, interstitial mononuclear cell infiltration and interstitial oedema were observed in group 1 (C). However, accumulation of extracellular matrix was not observed in both group (C–F). (A, B × 400; C–F × 200).

ized rats. The main urinary protein component in this model is heterologous albumin [4]. In passive Heymann nephritis, the anti-Fx1A antibodies filtered into the urine might bind to the antigen in the brush border and be harmful to tubular epithelial cells. Therefore, the possibility that factors other than proteinuria contribute to progressive tubulointerstitial injury cannot be ruled out for any of these models, and it remains unclear whether proteinuria alone harms the tubulointerstitium, promoting severe interstitial
Fig. 5. Direct immunofluorescent photomicrographs illustrating the deposition of mouse IgG and rat C3. The sections were stained with anti-mouse IgG (A and B) or anti-rat C3 (C and D). Sections from kidneys of rats in group 1 (A and C) and group 2 (B and D) are shown. A fine granular staining pattern was observed in group 1 (A). Rat C3 was not deposited in the glomeruli in any group but was observed in the intratubular space in the rats in group 1 (C and D). (A and B × 400; C and D × 200).

Fig. 6. Indirect immunofluorescent photomicrographs (× 200) illustrating the expression of vimentin. In some rats with severe proteinuria in group 1, vimentin was expressed on the glomeruli, peritubular capillaries and tubular epithelial cells (A). In group 2, it was expressed on the glomeruli and peritubular capillaries but not on the tubular epithelial cells (B).
Severe proteinuria induces interstitial injury and the progression of chronic renal failure. Taking all these factors into consideration, it is evident that an animal model of persistent nephrosis, which involves no severe glomerular abnormalities or artificially induced haemodynamic alterations, and can be induced by an agent which is not directly toxic to the tubulointerstitium, needs to be developed in order to investigate whether tubulointerstitial injuries would be caused directly by continuous proteinuria.

In this study, no abnormal findings were detected in either group 2 or the control group (Figures 4–9, Table 1). This suggests that 5-1-6 has no direct toxic effect on the tubulointerstitium. We prepared mAb 5-1-6 from the supernatant of a culture in serum-free medium and induced immunotolerance to mouse Ig for this study. These procedures were considered to prevent immune complex formation leading to glomerulonephritis or interstitial damage. In group 1, massive proteinuria, hypoalbuminaemia and hypercholesterolaemia were observed (Figure 3C and D, Table 1), and almost all of the urinary proteins were derived from host serum proteins (the average amount of urinary protein excreted was about 3 g/week and the mAb dose was 500 μg/week in group 1). Therefore, this model was similar to the nephrotic state in humans. In addition, severe glomerular injury was not induced (Figure 4, Table 1) and the animals were not manipulated with nephrectomy in the present model. Thus, we achieved our aim of developing a model of persistent nephrosis with no manoeuvres known to induce artificial haemodynamics and no severe glomerular abnormalities using an agent without direct tubular toxicity.

Using the model established in the present study, we examined the relationships between severity of proteinuria and several other factors. Interstitial oedema, cell infiltration and degeneration of the tubular epithelial cells were observed in the interstitium in nephrotic rats (Figure 4C). The kidney weight was positively correlated with the severity of proteinuria (Figure 3B), and the increase in kidney weight might have been derived mainly from the interstitial oedema. No systemic oedema, massive ascites or pleural effusion was observed at sacrifice. The expression of vimentin indi-
Fig. 8. Indirect immunofluorescent photomicrographs (×200) illustrating the extracellular matrix of the interstitium. The sections were stained with anti-type I collagen (A and B), anti-type IV collagen (C and D) or anti-laminin (E and F). Sections from kidneys of rats in group 1 (A, C and E) and group 2 (B, D and F). No significant accumulation of type I or IV collagen or of laminin could be detected.

cated that tubular epithelial cell injury had occurred in group 1 (Figure 6A). Similar findings have been made in PAN nephritis [3] and protein-overload proteinuria [4,16]. The number of OX1-, OX19- and ED1-positive cells that had infiltrated into the interstitium (Figure 3H, Table 1) was related to the severity of proteinuria. These inflammatory cell infiltrations are considered to be mediated by the release of chemotactants, including MCP-1, RANTES, lipid-derived factors, etc. and by the up-regulation of adhesion
Severe proteinuria induces interstitial injury

Fig. 9. Indirect immunofluorescent photomicrographs (×200) illustrating the expression of α-SMA and TGF-β. The sections were stained with anti-α-SMA (A and B) or anti-TGF-β (C and D). Sections from kidneys of rats in group 1 (A and C) and group 2 (B and D) are shown. No expression of α-SMA was detected in the glomeruli of either group (A and B). α-SMA-expressing interstitial cells were seen in some rats with severe proteinuria in group 1 (A). TGF-β was expressed focally on the tubular epithelial cells of some rats with severe proteinuria in group 1 (C). It was also expressed in some glomeruli from both groups.

Fig. 10. Electron micrograph (×10000) illustrating glomerular alterations in group 1. No subepithelial electron-dense deposits were observed. Partial effacement of the foot processes of the epithelial cells was found occasionally.

Fig. 11. RT–PCR analysis of type I collagen transcripts in the renal cortex. RT–PCR amplification of type I collagen transcripts was performed using total RNA extracted from the renal cortex of rats in group 1 and the control group, as described in Materials and methods. A comparative analysis of type I collagen transcripts in three rats with the heaviest proteinuria in group 1 and 3 controls after 35 cycles is shown. GAPDH was used to verify the RNA quality. Expression of mRNA for type I collagen was not increased in group 1 (lanes 4–6) when compared with the controls (lanes 1–3) after 35 PCR cycles.

molecules expression, as suggested by Eddy et al. [17]. In some rats with relatively high-grade proteinuria in group 1, the expression of TGF-β on the tubular epithelial cells and the number of interstitial α-SMA-
expressing cells were increased (Figure 9A and C). Sustained proteinuria was suggested to induce these tubulointerstitial alterations dose dependently. However, renal function, which was evaluated by BUN and creatinine clearance, was not related to the severity of proteinuria (Figure 3E and F).

Progressive interstitial fibrosis was reported to be induced by sustained proteinuria in chronic PAN [5] and protein-overload proteinuria [4]. Jones et al. reported that in six unilaterally nephrectomized and PAN-injected rats which secreted daily urinary albumin of 130–150 mg/100 g BW, interstitial fibrosis and increase of mRNA for collagen type I were induced by 6 weeks [5]. Although proteinuria was not examined selectivity, the amount of urinary daily total protein was estimated to be 200–230 mg/100 g BW (if protein selectivity was entirely lost, content of urinary protein was equal to that of serum protein; albumin is about 65% of total protein; protein whose molecular weight is higher than albumin is about 30%). In the present model, the average amount of proteinuria was 208 mg/100 g BW in six rats with the heaviest proteinuria, and protein whose molecular weight was greater than that of albumin was >10% of total urinary protein in three rats with the heaviest proteinuria. Our observation period, 25 weeks, was 4-fold longer than the 6 weeks in their model. Thus, the tubulointerstitium in the present model is suggested to be exposed to 4-fold higher amounts of total protein and 1.3-fold more protein which was larger than albumin, even if the proteinuria in chronic PAN nephritis would be estimated at maximum. In their model, increases of matrix proteins in the interstitium were assessed semiquantitatively, and a relative intensity score (1 represented a normal scoring pattern; 2, slightly increased ECM staining; and 3, markedly increased ECM staining) was calculated [5]. The scores for collagen types I, III and IV, fibronectin and laminin ranged from 1.8 to 2.3 at 6 weeks [5]. In our model, according to the calculation method described by Jones et al. [5], the relative intensity scores for collagen types I and IV and laminin remained close to 1. Furthermore, interstitial fibrosis was reported to be induced within 3 weeks in the rat model of protein-overload proteinuria. The average amount of urinary protein was about 80 mg/100 g BW in this model. Thus, tubular cells in our model are considered to be totally exposed to a greater amount of urinary protein than those in previously applied models. The possibility that the interstitial fibrosis would be induced also in the present model by exposure to protein for a longer period could not be ruled out because interstitial cell infiltration, interstitial oedema, tubular epithelial cell injury and an increase of TGF-β and α-SMA staining were observed in the present model. However, the tubulointerstitial injury evidently was mild. The reason why the lesser degree of interstitial injury was induced in this study was considered to be that tubulotoxic and fibrogenic factors other than proteinuria were excluded. In order to induce rapidly progressive interstitial fibrosis, tubulotoxic or fibrogenic factors other than proteinuria may be essential.

In conclusion, we developed a novel rat model of nephrosis, which involves no manoeuvres known to induce artificial haemodynamics and no severe glomerular abnormalities. Isolated proteinuria induced the tubulointerstitial lesions, such as oedema, tubular epithelial cell injury and cell infiltrations, but the degree of the injury was much less than that in other previously applied models.

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