

# A single simultaneous injection of two monoclonal antibodies causes novel progressive renal lesions with characteristic proteinuria kinetics

Yumiko Fujioka<sup>1</sup>

<sup>1</sup>Department of Health and Nutrition Science, Faculty of Human Health Science, Matsumoto University  
2095-1 Niimura, Matsumoto, Nagano, Japan 390-1295

Key words : Progressive renal lesions, Proteinuria, Monoclonal antibody.

## Abstract

In the clinical setting, we have encountered cases of renal lesions, although once resolved, that progress gradually to end-stage renal disease after an extended latent period. However, appropriate model explaining renal lesions with such an insidious progression is limited. It has been reported that prior minor podocyte injury without proteinuria could exacerbate mesangial alterations and cause irreversible mesangial proliferative glomerulonephritis with persistent proteinuria. To better understand the possible protective relationship of podocytes and mesangial cells against chronic disease progression, we examined the effect of concomitant damage to both cells using anti-Thy-1 monoclonal antibody (mAb) 1-22-3 and anti-nephrin mAb 5-1-6. When injected separately, the mAbs induced reversible renal lesions with transient proteinuria, whereas simultaneous injection evoked immediate massive proteinuria that steadily decreased to low levels until 3 months. The most remarkable feature of this model was that the proteinuria then began to gradually rise until 9 months, at which time electron microscopy revealed effacement of foot processes and accumulation of collagen fibrils in the tubulointerstitium and immunofluorescence showed deposition of collagen type I in glomeruli.

When the proteinuria commenced increasing at 3 months, mesangial proliferation was already evident in light microscopy and collagen deposition was present in glomeruli according to immunofluorescence. These results suggested that simultaneous insult to podocytes and mesangial cells caused novel progressive renal lesions with the characteristic proteinuria kinetics of chronic glomerulonephritis and indicated the possibility of a coordinated protective cellular role. There have been no other models of a single injection causing such progressive renal lesions to date. This novel system may shed light on the mechanism and treatment of gradually deteriorating renal lesions.

## Introduction

In order to identify the mechanisms of development and progression in human chronic renal disease and establish evidence-based therapeutic interventions, it is essential to develop models that mimic the clinical features and abnormal changes during the disease course. Chronic glomerulonephritis usually manifests as proteinuria that subsides to apparently subclinical levels for years or decades while renal dysfunction steadily progresses.

In many chronic glomerulonephritis models, a renal lesion is developed which then worsens over time. To

date, however, no model has been established whereby a subclinical period exists after initiation of the disease that masks gradual deterioration until the end stage of chronic renal failure. In the development of such a construct, earlier models have employed the simple repetition of similar injurious effects or the addition of artificial functional load. One example is using 2 consecutive injections of monoclonal antibody (mAb) 1-22-3 that induces anti-Thy1.1 nephritis to cause mesangial sclerotic changes with persistent proteinuria [1-2]. Another method is by single injection of mAb 5-1-6 in rats to

evoke anti-nephrin nephritis and its associated massive proteinuria [3]. Anti-Thy-1 glomerulonephritis has been widely used for the development of mesangial proliferative glomerulonephritis [4-8]. The binding of mAb 1-22-3 to the limited areas of the mesangial cell surface facing endothelial cells induces severe proteinuria and typical morphological changes, but these conditions are reversible [9]. Anti-nephrin glomerulonephritis is often adopted to elucidate the mechanism of proteinuria [10-11]. Binding specifically to rat nephrin on the slit diaphragm of podocytes (epithelial cell foot processes), mAb 5-1-6 results in severe, but reversible, proteinuria. It has also been reported that prior minor podocyte injury without proteinuria exacerbated mesangial alteration, causing irreversible mesangial proliferative glomerulonephritis with persistent proteinuria [5].

To better understand the possible interdependent protective relationship of podocytes and mesangial cells against chronic renal disease progression, we investigated the effects of damaging both cells simultaneously by a single application of selected mAbs. Such a model could uncover unknown prognostic factors expressed during remission and enable the establishment of therapeutic interventions against chronic progression.

## Materials and Methods

### Animals

All experiments were performed using specific pathogen-free female Wistar rats purchased from Charles River Japan (Atsugi, Japan). All animal experiments conformed to the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

### Experimental protocol

#### Experiment 1: Long-term observation of novel progressive renal lesions

Seven rats received a single injection of 1 ml phosphate-buffered saline (PBS) containing 1 mg mAb 1-22-3 and 10 mg mAb 5-1-6 intravenously through the tail. The preparation of mAbs 1-22-3 and

5-1-6 and induction of anti-Thy1.1 nephritis and anti-nephrin nephritis have been described previously [1, 2]. Twenty-four-hour urine samples were collected from rats housed in metabolic cages after disease initiation every other day for the first 2 weeks and every other week thereafter until 9 months for measuring the amount of proteinuria excretion. All rats were anesthetized at 9 months and blood was drawn by cardiac puncture. The right kidney was removed, weighed, and sectioned for light microscopic assessment.

#### Experiment 2: Comparison of novel progressive renal lesions with anti-Thy1.1 nephritis and anti-nephrin nephritis

To compare the characteristics of our novel progressive renal lesion model with those of anti-Thy1.1 nephritis, anti-nephrin nephritis, and a control, 26 rats were divided into 3 disease groups of 7 rats each and a control group (n=5). One disease group received a single injection of 1 ml PBS containing 1 mg mAb 1-22-3 and 10 mg mAb 5-1-6 intravenously through the tail (Group 2-1). The second group was injected with 1 ml PBS containing 1 mg mAb 1-22-3 only to induce Thy1.1 nephritis (Group 2-2). The third group received an injection of 1 ml PBS containing 10 mg mAb 5-1-6 only to cause anti-nephrin nephritis (Group 2-3). The control group was injected with 1 ml PBS (Control) as shown in Figure 1. Twenty-four-hour urine samples were collected and the amount of proteinuria excretion was measured as described in Experiment 1. At 9 months, all rats were anesthetized, weighed, and drained of blood by cardiac puncture. The right kidney was harvested, weighed, and sectioned for assessment by immunofluorescence and electron microscopy.

#### Experiment 3: Early findings of novel progressive renal lesions in the remission stage

A total of 26 rats were divided into 3 disease groups of 7 animals and a control group (n=5) as described in Experiment 2. The groups were respectively injected with mAbs 1-22-3 and 5-1-6 (Group 3-1), mAb 1-22-3 only (Group 3-2), mAb 5-1-6 only

(Group 3-3), or PBS (Control). At 3 months, all rats were anesthetized and their blood drained by cardiac puncture. Kidneys were removed and sectioned for evaluation of glomerular injury by light and immunofluorescence microscopy.

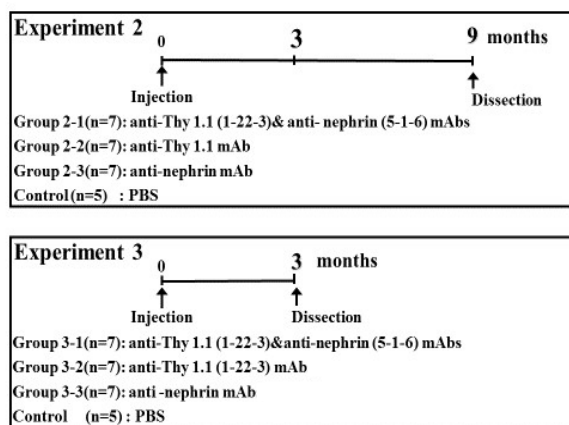


Fig. 1. Protocols for Experiments 2 and 3.

### Laboratory investigation

Proteinuria concentration was determined by a colorimetric assay (BioRad, Oakland, CA, USA) using bovine serum albumin as a standard.

Blood urea nitrogen (BUN), serum creatinine (SCr), urinary creatinine (UCr), and serum albumin levels were measured using standard methods. By means of UCr, 24-hour urine volume (UV), SCr, and body weight (BW), creatinine clearance (CCr) values were calculated using the following formula:  $CCr \text{ (ml/min/100 g BW)} = UCr \text{ (mg/dl)} \times UV \text{ (ml)} / SCr \text{ (mg/dl)} / 1440 \text{ (min)} / BW \text{ (g)} \times 100$ .

### Light microscopy

For light microscopic analysis, isolated kidney samples were fixed with Carnoy solution, embedded in paraffin, cut into 3  $\mu\text{m}$  sections, and stained with periodic acid-Schiff (PAS) and periodic acid-methenamine silver (PAM).

### Immunofluorescence microscopy

For immunofluorescence evaluation, renal tissue was quickly frozen in n-hexane and cooled to  $-70^{\circ}\text{C}$ . Sections of 4  $\mu\text{m}$  thickness were cut with a cryotome (SHANDON, Life Sciences International Japan, Tokyo, Japan) and incubated with anti-rat collagen

type I antibody (Chemicon, Temecula, CA, USA).

### Electron microscopy

Electron microscopy of kidney samples was performed as described previously [3].

### Statistical analysis

Data are presented as the mean where applicable. One-way analysis of variance and the Tukey-Kramer test were employed to compare continuous variables of serum biochemistry. One-way analysis of variance and Dunnett's test were adopted to compare continuous variables of proteinuria. All statistical analyses were carried out using SPSS version 25 software (IBM, Tokyo, Japan). A p-value of  $<0.05$  was considered statistically significant.

### Results

#### Long-term observation of novel progressive renal lesions

As shown in Figure 2, simultaneous injection of mAbs 1-22-3 and 5-1-6 immediately induced massive proteinuria on day 1 (371 mg/day), which decreased steadily and remained low from day 10 (50 mg/day) until 2-3 months. The proteinuria then began rising gradually until the study end point at 9 months (283 mg/day). Two animals died after 3 months due to proteinuria of over 400 mg/day.

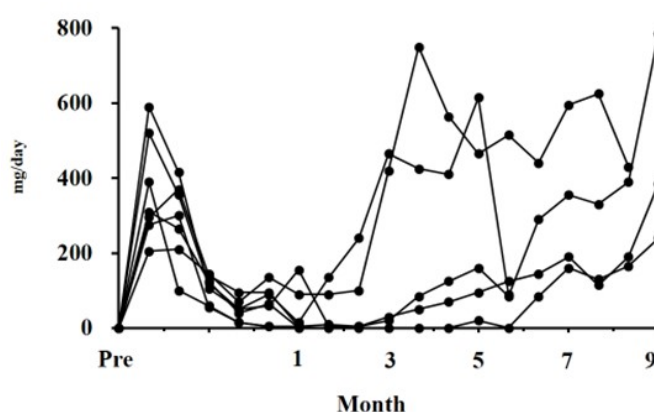


Fig. 2. Characteristic proteinuria kinetics in Experiment 1. Proteinuria immediately peaked and then remained low until 3 months.

Glomerular sclerosis and crescent-like lesions were observed microscopically and tubular atrophy and interstitial fibrosis with inflammatory cell infiltration were evident (Fig. 3).

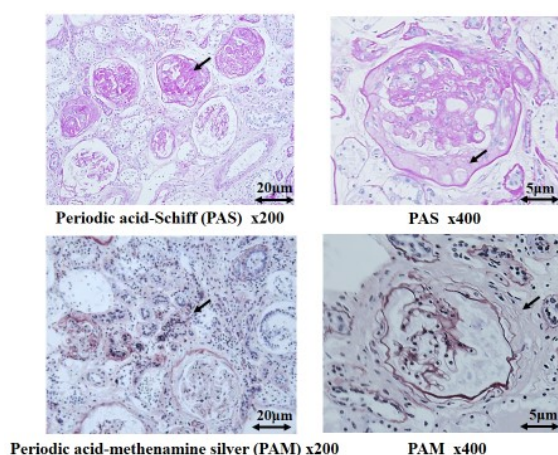


Fig. 3. Representative light microscopic findings in Experiment 1. Glomerular sclerosis (upper left, arrow), crescent-like lesions (upper right, arrow), and tubular atrophy and interstitial fibrosis (bottom, arrows) were visible.

#### Comparison of novel progressive renal lesions with anti-Thy1.1 nephritis and anti-nephrin nephritis

Simultaneous injection of the 2 mAbs caused significantly more proteinuria on day 1 versus the other groups (Group 2-1: 454.2 mg/day, Group 2-2: 105.1 mg/day, Group 2-3: 21.7 mg/day, Control: 4.9 mg/day) (Fig. 4). The proteinuria had reduced markedly by day 35 (Group 2-1: 7.6 mg/day, Group 2-2: 5.0 mg/day, Group 2-3: 6.7 mg/day, Control: 5.8 mg/day) and remained low until approximately 3 months. Proteinuria values then began gradually increasing until the study end point at 9 months (Group 2-1: 132.2 mg/day, Group 2-2: 51.0 mg/day, Group 2-3: 44.0 mg/day, Control: 15.5 mg/day).

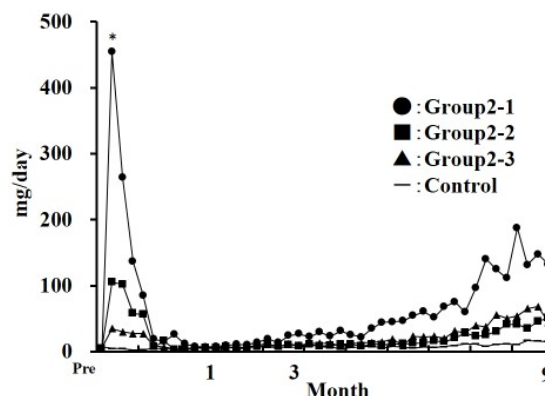


Fig. 4. Comparison of proteinuria kinetics in Experiment 2. \*:  $p < 0.05$  for Group 2-1 compared with Control. Proteinuria increased gradually from month 3 until month 9.

Group 2-1: anti-Thy1.1, anti-nephrin model

Group 2-2: anti-Thy1.1 model

Group 2-3: anti-nephrin model

Serological analysis revealed that SCr was increased in Group 2-1 compared with the other groups. BUN, UV, and serum albumin tended to be higher in Group 2-1, while UCr and Ccr tended to be lower (Table 1).

As shown in Table 2, the kidney weight and kidney weight/body weight ratio in Group 2-1 tended to be lower than in other groups.

The deposition of collagen type I in glomeruli was increased only for Group 2-1 in immunofluorescence microscopy (Fig. 5).

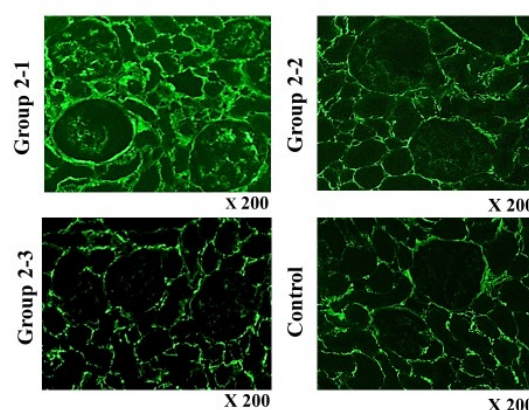


Fig. 5. Representative immunofluorescence findings. Deposition of collagen type I was increased in the glomeruli of Group 2-1.



Table 1. Serum biochemistry and renal function in Experiment 2.

	BUN (mg/dl)	SCr (mg/dl)	UCr (mg/dl)	CCr (ml/min/100gBw)	UV (ml)	Albumin (g/dl)
Group 2-1	18.5±2.4	0.41±0.04	88.91±47.47	0.49±0.12	16.81±7.09	4.20±0.26
Group 2-2	16.3±1.9	0.36±0.07	108.37±51.64	0.57±0.15	14.45±7.73	4.06±0.29
Group 2-3	18.0±3.0	0.40±0.05	116.68±54.68	0.52±0.12	13.40±5.95	4.15±0.27
Control	16.8±2.1	0.35±0.08	102.07±45.47	0.61±0.16	15.67±8.63	4.02±0.34
P value	ns	ns	ns	ns	ns	ns

Table 2. Kidney and body weight in Experiment 2.

	Kidney weight (g)	Body weight (g)	Kidney weight / body weight ratio (%)
Group 2-1	1.48±0.19	443±21	0.34±0.05
Group 2-2	1.53±0.14	445±27	0.35±0.03
Group 2-3	1.54±0.21	451±21	0.34±0.06
Control	1.55±0.13	442±31	0.35±0.03
P value	ns	ns	ns

Data are expressed as the mean value ± standard deviation. Group 2-1: anti-Thy1.1, anti-nephrin model, Group 2-2: anti-Thy1.1 model, Group 2-3: anti-nephrin model, ns: no significance.

As evidenced by electron microscopy, effacement of foot processes was apparent in Group 2-1 only (Fig. 6)

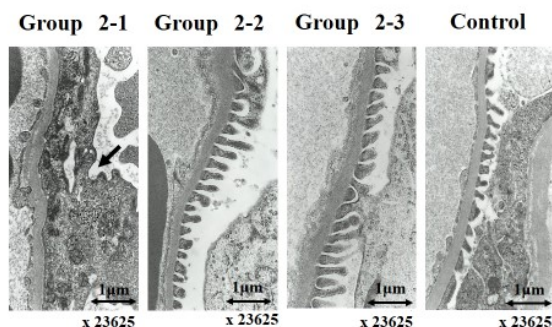


Fig. 6. Representative electron microscopy findings. Effacement of foot processes was detected in Group 2-1 only (arrows).

The accumulation of collagen fibrils in the tubulointerstitium was observed only in Group 2-1 by electron microscopy (Fig. 7).

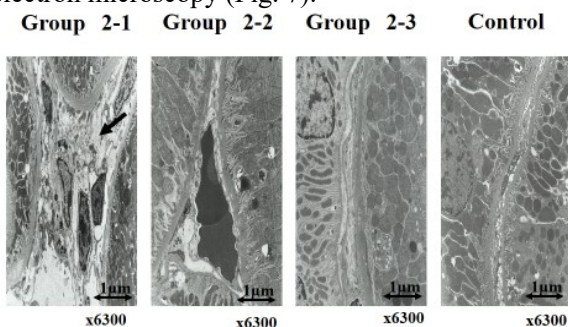


Fig. 7. Representative electron microscopy findings. Accumulation of collagen fibrils in the tubulointerstitium was detected in Group 2-1 only (arrows).

### Early findings of novel progressive renal lesions in the remission stage

Slightly increased mesangial cell proliferation was already detected at month 3 in Group 3-1 by light microscopy (Fig. 8).

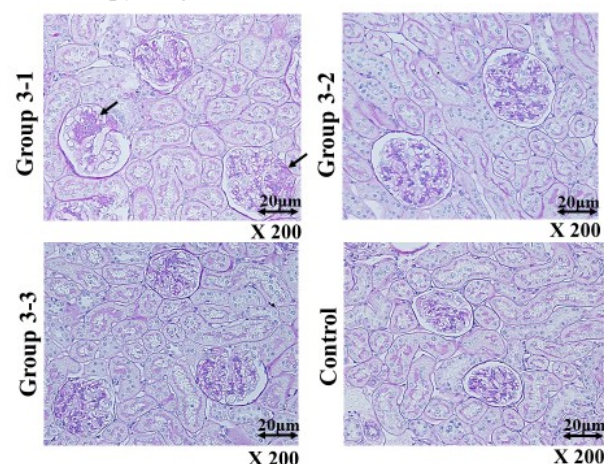


Fig. 8. Representative light microscopic findings. Mesangial cell proliferation was slightly higher in Group 3-1 (arrow).

Deposition of collagen type I in glomeruli had already increased in Group 3-1 in immunofluorescence microscopy (Fig. 9).

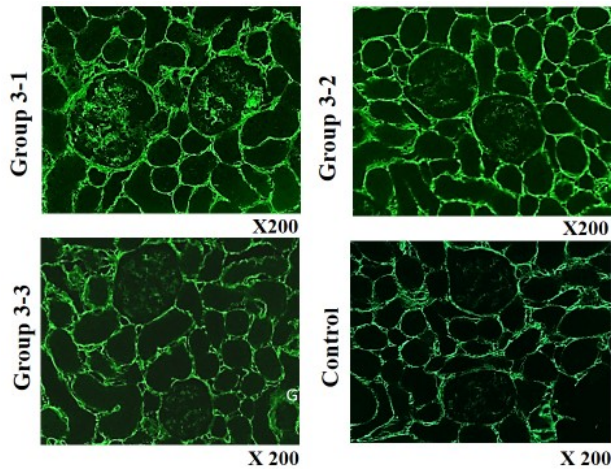


Fig. 9. Representative immunofluorescence findings. Deposition of collagen type I in glomeruli was increased in Group 3-1.

### Discussion

In the present study, a single simultaneous injection of anti-Thy1.1 and anti-rat nephrin mAbs in rats resulted in the development of a novel model of chronic progressive renal disease whereby concomitant damage to mesangial cells and podocytes caused renal lesions with the characteristic proteinuria kinetics of chronic glomerulonephritis.

Whereas injury to either mesangial cells or podocytes caused reversible lesions [3, 9], simultaneous injection of 2 mAbs evoked irreversible changes, which suggested a cooperative protective role between mesangial cells and podocytes. In previous studies, rats injected with mAb 1-22-3 5 days after the injection of puromycin aminonucleoside (PAN) exhibited irreversible mesangial alteration with persistent proteinuria. Mesangial matrix expansion and tubulointerstitial injury, including inflammatory cell infiltration, were evident in light micrographs, and deposition of collagen type I in glomeruli and the tubulointerstitium were increased in immunofluorescence microscopy. As effacement of foot processes was also detected by electron microscopy, podocyte insult was considered an important factor that exacerbated mesangial cell proliferation and matrix expansion [5]. Mesangial cell

expansion and matrix accumulation are believed to contribute to the development of glomerulosclerosis and various human glomerular diseases, including IgA nephropathy. The characteristic proteinuria kinetics produced in this study differed markedly from those of previous ones. Many cases of chronic glomerulonephritis exhibit an acute attack of the disease that develops into the chronic form years later [12]. Our method appears to reproduce the clinical progression of chronic glomerulonephritis, which exists largely undetected while gradually worsening.

The present model of a simultaneous injection of 2 mAbs produced the immediate onset of massive synergistically increased proteinuria that peaked on day 1, steadily decreased by day 35, and then began gradually increasing again from 3 months. There have been no other studies inducing such proteinuria kinetics with a prolonged subclinical period.

In another report, a single injection of mAb 5-1-6 in rats triggered an immediate proteinuria spike that peaked on day 8 and then fell to normal levels by day 18. This mAb binds to the surface of glomerular epithelial foot processes, mainly slit diaphragms, but no histologic abnormalities or ultrastructural changes were evident apart from partial retraction of foot processes in electron microscopy [3]. In this study, deposition of collagen type I was increased in glomeruli according to immunofluorescence, and both effacement of foot processes and accumulation of collagen fibrils were disclosed by electron microscopy.

Double consecutive injections of mAb 1-22-3 5 days after the injection of PAN caused irreversible persistent proteinuria without a subclinical period [11]. In previous studies, the slit diaphragm of podocytes played an important role as the main barrier in the glomerular capillary wall for the maintenance of normal permselectivity [5, 11]. These results indicated the possibility of an interactive protective role by podocytes and mesangial cells.

A single injection of mAb 1-22-3 in rats bound to the limited areas of the mesangial cell surface facing endothelial cells [1]. While this injection caused more severe mesangial cell injury, it was reversible unless administered to nephrectomized rats. However, 2 consecutive injections created an irreversible model of renal damage with persistently increased proteinuria. The present study showed that a single simultaneous

injection of 2 different mAbs into rats caused mesangial cell proliferation and matrix accumulation that was considered to contribute to the development of glomerulosclerosis without either nephrectomy or multiple injections. To the best of our knowledge, our report is the first describing a single injection inducing irreversible renal damage.

There were several limitations to this study. First, the group differences for proteinuria and serum biochemistry were large, but not statistically significant. Second, the observation period should be extended to better detect significant factors in the progression of renal lesions. Third, electron microscopy will be needed to identify early relevant findings in the subclinical period.

In conclusion, a single simultaneous injection of 2 mAbs caused novel progressive renal lesions with the characteristic proteinuria kinetics of chronic glomerulonephritis. This new model may enable precise identification of the mechanisms and treatments of gradual renal lesion progression.

### Acknowledgments

The author is indebted to Prof. Fujio Shimizu and Prof. Hiroshi Kawachi for providing the test mAbs and diverse research opportunities. I am also grateful to the members of the Department of Cell Biology at Niigata University for their helpful suggestions. I thank Prof. Ryoji Hirota, Prof. Ayako Nakayama, Mr. Yuuki Tsunoda, and Koito Fujioka, Akira Fujioka, Tsukasa Fujioka, Yayoi Fujioka, and Takejiro Fujioka for their special encouragement and support.

### Sources

- [1] Kawachi H et al. Mesangial sclerotic change with proteinuria in rats after two consecutive injections of monoclonal antibody 1-22-3. *Clin Exp Immunol.* 1992, 90, p.129-134.
- [2] Shimizu F et al. Role of mesangial cell damage in progressive renal disease. *Kidney Blood. Press.* 1999, 22, p.5-12.
- [3] Orikasa M et al. Massive proteinuria induced in rats by a single intravenous injection of a monoclonal antibody. *J Immunol.* 1988, 141, p.807-814.
- [4] Shimizu F et al. Pirfenidone prevents the progression of irreversible glomerular sclerotic lesions in rats. *Nephrology.* 1997, 3, p.315-332.
- [5] Morioka Y et al. Podocyte injuries exacerbate mesangial proliferative glomerulonephritis. *Kidney International.* 2001, 60, p.2192-2204.
- [6] Wan Y et al. Multi-glycoside of Tripterygium wilfordii Hook f. ameliorates proteinuria and acute mesangial injury induced by anti-Thy1.1 monoclonal antibody. *Nephron Exp Nephrol.* 2005, 99, p.121-129.
- [7] Fujioka Y. et al. Early intervention in reducing dietary protein intake ameliorates irreversible mesangioproliferative glomerulonephritis in Rats. *Acta Medica et Biologica.* 2005; 53(4):95-107.
- [8] Suzuki K, et al. Progressive Glomerulonephritis with increasing proteinuria induced by a second attack to the Mesangial Cell. *Acta Medica et Biologica.* 2005, 53, p.117-124.
- [9] Kawachi H et al. Epitope-specific induction of mesangial lesion with proteinuria by a MoAb against mesangial cell surface antigen. *Clin Exp Immunol.* 1992, 88, p.399-404.
- [10] Suzuki K et al. Angiotensin II type 1 and type 2 receptors play opposite roles in regulating the barrier function of kidney glomerular capillary wall. *Am J Pathol.* 2007, 170, p.1841-53.
- [11] Kikuchi H et al. Severe proteinuria, sustained for 6 months, induces tubular epithelial cell injury and cell infiltration in rats but not progressive interstitial fibrosis. *Nephrol Dial Transplant.* 2000, 15, p.799-810.
- [12] National kidney Foundation  
<https://www.kidney.org/atoz/content/glome>

(Received February 19, 2018; accepted December 28, 2018)



## 2つのモノクローナル抗体の1回同時注入により惹起させた、 特徴的な蛋白尿動態を示す進行性腎病変

藤岡 由美子<sup>1</sup>

<sup>1</sup>松本大学人間健康学部健康栄養学科

キーワード：進行性腎病変，蛋白尿，モノクローナル抗体

### Abstract (Japanese)

臨床では一度は治癒したかにみえた腎病変が長い潜伏期間を経た後再発し、腎不全に進展する症例が頻繁に経験される。しかし、その様な病態を再現するモデルはこれまで限られていた。先行研究において、糸球体上皮細胞傷害が軽症でも後に傷害したメサンギウム細胞病変の増悪により進行性腎炎が惹起されたとの報告を基に、双方の細胞を同時期に傷害した場合どのような病変が起こり得るかを検討した。

メサンギウム細胞を標的とする抗モノクローナル Thy1.1 抗体 (1-22-3) と糸球体上皮細胞を標的とする抗 nephrin 抗体 (5-1-6) を同時に 1 回で注入したところ、相乗的な大量の蛋白尿が発現した後、一度は鎮静化したものの、3 ヶ月後に再び出現した後は 9 ヶ月まで徐々に増加するという特徴的な蛋白尿の経過を辿る腎病変が惹起された。9 ヶ月後の光学顕微鏡像では、糸球体硬化、尿細管の萎縮及び細胞浸潤を伴う間質の線維化を確認し、蛍光顕微鏡では糸球体内に I 型 collagen の沈着を、電子顕微鏡像では足突起の消失と尿細管間質での collagen 線維の沈着を確認した。蛋白尿が再発する 3 ヶ月後では、光学顕微鏡像で、既にメサンギウム細胞の増殖が僅かに観察され、蛍光抗体法では糸球体内 I 型 collagen が沈着していた。一方、各々の抗体を単独で投与した群では、何れも一時的な蛋白尿を伴う可逆性の腎病変に過ぎず、光学、蛍光、電子顕微鏡像の何れも特徴的な病変は観察されなかった。これらの結果から、メサンギウム細胞と糸球体上皮細胞には相互的な保護作用がある可能性が示唆された。これまでに抗体を 1 回注入しただけで進行性腎病変を惹起させたモデルはなく、今後はこの新規な病態モデルの精度を高め、徐々に進行する腎病変のメカニズムや治療法の解析へ使用されることに期待している。

### Yumiko Fujioka

Department of Health and Nutrition Science, Faculty of Human Health Science, Matsumoto University

My field of research and education is clinical nutrition. My major interest is renal nutrition.

### Representative publications

- [1] Fujioka Y et al. Early intervention in reducing dietary protein intake ameliorates irreversible mesangioproliferative glomerulonephritis in Rats. *Acta Medica et Biologica*. 2005, 53, p.95-107.
- [2] Fujioka Y et al. Effects of Dietary Protein Amount on the progression of mesangioproliferative glomerulonephritis model. 12<sup>th</sup> International Congress of Nutrition and Metabolism in Renal Disease. Program and abstracts, 2004, p.119.
- [3] Fujioka Y et al. Differences in National Character between Japan and Korea more strongly Highlight the Correlation between Eating with Others and Daily Mental States of University Students as it Relates to Cooking one's Meals, or not. *Nutrition & Dietetics*, 2012, 69, p.97 (Best Non English Speaking background Poster).
- [4] Fujioka Y et al. The Relationships between Mental State and Eating Habits, and Meal Time Environment: A Comparative Study between Japan, Korea, and Gender. *Annals of Nutrition & Metabolism*, 2009, 55, p.482.
- [5] Fujioka Y et al. The Feeling Enjoying Meals and Conversation during Meals are Important Factors in Maintaining Mental Health. 15<sup>th</sup> International Congress of Dietetics Abstract Book, 2009, p.193