

## Quantitative Histological Analysis of SM22 $\alpha$ (Transgelin) in an Adriamycin-Induced Focal Segmental Glomerulosclerosis Model

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### Key Words

SM22 $\alpha$  · Transgelin · Focal segmental glomerulosclerosis · Adriamycin nephropathy · Podocyte · Interstitial cell · Tubulointerstitium

### Abstract

**Background/Aims:** SM22 $\alpha$ , transgelin, has been revealed to be specifically expressed in glomerular epithelial cells and interstitial cells, according to the nature of the renal injury. In this study, quantitative analyses of SM22 $\alpha$  positivity were performed to investigate the pathological significance of its expression. **Methods:** Kidney samples of adriamycin nephropathy underwent immunohistochemistry with a newly established anti-SM22 $\alpha$  monoclonal antibody. The SM22 $\alpha$  positivity was quantified by an image analyzer. The correlation of the histological values with biochemical data was investigated statistically. Microstructural localization of SM22 $\alpha$  was studied by immunoelectron microscopy. **Results:** SM22 $\alpha$  was expressed along the dense basal microfilaments of degenerating podocytes, and diffusely in interstitial cells. Both the extent and intensity of SM22 $\alpha$  expression in glomerular and tubulointerstitial area were correlated with the deterioration of renal function and the severity of proteinuria. Stepwise multiple linear regression analysis revealed that the extent of its positivity in glomerular or tubu-

lulointerstitial area was the determinant of the amount of proteinuria or the deterioration of creatinine clearance (Ccr), respectively. Inversely, the deterioration of Ccr was the most important predictor of SM22 $\alpha$  expression. **Conclusion:** SM22 $\alpha$  expression in podocytes and interstitial cells represented the severity of proteinuria and the deterioration of renal function. SM22 $\alpha$  expression in renal tissues might be a hallmark of kidney diseases. Copyright © 2011 S. Karger AG, Basel

### Introduction

SM22 $\alpha$  (transgelin) was first identified as a 22-kDa protein in smooth muscles [1, 2]. It has variably been designated as p27 [3] or WS3–10 [3]. It is abundantly expressed in smooth muscle cells (SMCs) and is recognized as its differentiation marker [4, 5]. It is localized in the cytoskeletal apparatus [6] and is a member of calponin family [7, 8]. The function of SM22 $\alpha$  has not yet been completely elucidated. SM22 $\alpha$ -deficient mice normally develop and appear similar to control mice [9]. However, SM22 $\alpha$  is downregulated in SMCs in atherosclerotic lesions, and its gene ablation in apoE-deficient mice results in the extension of atherosclerotic lesions and increases the number of proliferating SMCs in plaque, indicating

that SM22 $\alpha$  may be involved in controlling the phenotypic modulation of SMCs, from contractile to proliferative, in the sclerotic artery [10]. Its expression has also been detected in several epithelial cells [7, 11, 12]. Upon tissue injuries, SM22 $\alpha$  is increasingly expressed in alveolar epithelial cells, and it may directly contribute to the lung fibrosis [13]. Moreover, SM22 $\alpha$  acts to suppress expression of the matrix metalloproteinase-9 [14], which is involved in the tissue remodeling. Loss of its expression could be involved in the development of cancer, or SM22 $\alpha$  could act as a tumor suppressor [7, 11, 12, 15]. Thus, both the functional and pathological significance of SM22 $\alpha$  expression have recently attracted attention.

Focal segmental glomerulosclerosis (FSGS) is a severe renal disease showing massive proteinuria, focal segmental glomerular consolidation and, in many cases, progressively declining renal function accompanied with increasing tubulointerstitial fibrosis. Because the pathological feature of FSGS is a foot process effacement of the glomerular epithelial cells (podocytes) followed by glomerulosclerosis, FSGS is primarily considered to be a podocyte disease [16]. The adriamycin (ADR) nephropathy has been used widely as a rat experimental model of FSGS [17].

We have reported in previous studies [18, 19] that SM22 $\alpha$  was inducibly expressed in injured podocytes in the early phase of a rat anti-glomerular basement membrane nephritis model. In addition, SM22 $\alpha$  was expressed in diverse models of glomerular and tubulointerstitial injury [20], including the 5/6 nephrectomy model, the renal ischemia-reperfusion model and puromycin aminonucleoside nephrosis. SM22 $\alpha$  might be a phenotypic marker of the injured kidney cells and be expressed in specific sites according to the nature of the injury.

In those studies, we did not perform the quantitative histological analysis of SM22 $\alpha$  expression, nor did we show how it correlated with the disease activity. In this present study we have investigated these undetermined issues to elucidate its pathological significance, using a rat FSGS model, ADR nephropathy, which showed both podocyte and tubulointerstitial injury and progressive renal dysfunction, using a newly established, highly specific anti-SM22 $\alpha$  monoclonal antibody (mAb).

## Materials and Methods

### *ADR Nephropathy*

Adult male 7-week-old Wistar rats weighing between 150 and 250 g were purchased from Charles River Japan (Tokyo, Japan), and were randomly assigned into five groups: control group (n = 5), day 7 group (n = 5), day 14 group (n = 5), day 28 group (n = 4),

and day 42 group (n = 5). ADR nephropathy was induced in ADR groups by a single caudal vein injection of 6 mg/kg ADR (adriamycin hydrochloride), which was purchased from Wako Pure Chemicals (Tokyo, Japan) dissolved in normal saline solution. The control group was injected the equivalent volume of normal saline solution. The groups of rats were sacrificed on days 7, 14, 28, 42 after the ADR injection, respectively. This study was approved by the Ethical Committee for Animal Research of Niigata University.

### *Blood and Urine Biochemical Variables*

Blood samples were collected for measurement of blood urea nitrogen (BUN) and serum creatinine (Scr) at the time of sacrifice. 24-Hour urine samples were collected using metabolic cages for measurement of urinary protein (UP) (SRL, Tokyo, Japan) as described previously [21, 22].

### *Recombinant Protein of the Rattus norvegicus SM22 $\alpha$*

The rat SM22 $\alpha$  recombinant protein (rSM22 $\alpha$ ) was prepared as previously described [18]. Briefly, the pQE-30UA vector into which the in-frame 260 bp of rat SM22 $\alpha$  cDNA corresponding to amino acids 85–170 prepared by PCR was ligated and transformed into *Escherichia coli* strain JM109 (Toyobo, Osaka, Japan). The production of 6 $\times$  histidine-tagged SM22 $\alpha$  was induced by isopropyl-D-1-thiogalactopyranoside in Lennox Broth medium and the cells were then lysed and centrifuged. The supernatant was subjected to affinity purification with QIAexpress Type IV kit (Qiagen, Tokyo, Japan).

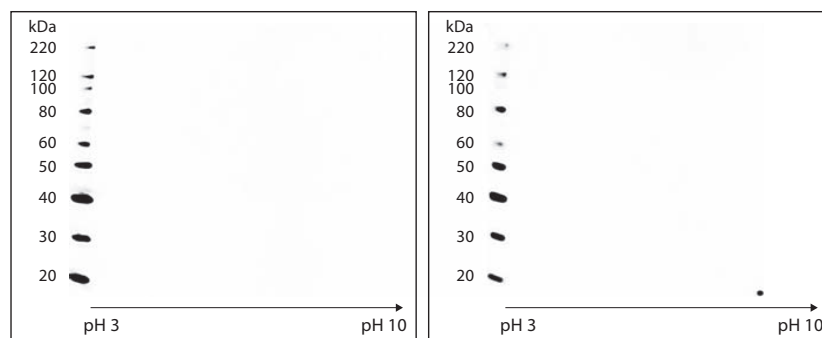
### *Production of mAb against rSM22 $\alpha$*

The mAb against rSM22 $\alpha$  was raised by Medical & Biological Laboratories Co. Ltd (Nagano, Japan) [23, 24]. Briefly, BALB/c mice were immunized with rSM22 $\alpha$  and complete Freund's adjuvant (1:1). The lymph node cells were then removed from immunized mice and fused with P3U1 myeloma cells at a ratio of 5:1 by the polyethyleneglycol-400 procedure. Hybridoma supernatants were screened by ELISA using the immunogen. After cloning the hybridomas, the mAbs were purified by protein G Sepharose column chromatography. Among the hybridomas, clone 75-7 was selected based on its ability to perform immunohistochemistry. Its isotype was determined as IgG1.

### *Two-Dimensional Western Blot Analysis*

100  $\mu$ g of proteins of lysate from normal Wistar rat aorta was loaded. The first dimension was carried out on a Zoom<sup>®</sup> IPGRunner system (Invitrogen Corp.) using pH 3–10 gel strips. Strips were rehydrated at room temperature (RT) for 12 h in 12  $\mu$ l of sample. Isoelectric focusing was performed at RT under the following conditions: 15 min at 175 V, 45 min at 175–2,000 V, and 30 min at 2,000 V. In order to eliminate disulfide bonds in the focused proteins prior to SDS-PAGE, IPG strips were incubated for 15 min in equilibration buffer which consisted of 9 ml 1 $\times$  NuPage<sup>®</sup> LDS sample buffer and 1 ml of 1 M DTT. The IPG strips were then soaked in alkylation buffer for an additional 15 min to alkylate the sulfhydryl groups. Alkylation buffer was produced by 125 mM iodoacetamide soluted in 10 ml of 1 $\times$  NuPage<sup>®</sup> LDS sample buffer. The strips were then embedded in 0.5% w/v agarose on top of Novex<sup>®</sup> 4–12% Bis-Tris Zoom<sup>®</sup> gels and the second dimension separation was carried out at RT at constant voltage of 200 V for 50 min. The protein was transferred to a PVDF membrane,

**Fig. 1.** Specificity of anti-rSM22 $\alpha$  mAb (75-7) by two-dimensional Western blot. The lysate of the normal rat aorta was electrophoresed and immunoblotted by non-specific mouse IgG1 (left membrane) or by the anti-rSM22 $\alpha$  mAb, 75-7 (right membrane).



blocked with 10% powdered milk in TBST (20 mM Tris-HCl, pH 8.0, 0.5 M NaCl, 0.5% Tween 20), then incubated with anti-rSM22 $\alpha$  mAb, 75-7, overnight at 4°C.

Peroxidase-conjugated goat anti-mouse IgG Ab (Dako) was used as the secondary Ab, and the immune complex was visualized using ECL (Western blotting detection reagents; Amersham). Normal mouse IgG1 was used as a control of the primary antibody.

#### Immunohistochemistry

The 5- $\mu$ m sections were deparaffinized in xylene and ethanol, and rehydrated in water. The sections were then washed in PBST (0.05% Tween 20) for 5 min. Slides were incubated in endogenous peroxidase buffer (a mixture of 5 ml 3% H<sub>2</sub>O<sub>2</sub> and 45 ml methanol) for 20 min at RT. The sections were then washed in PBST for 5 min. Before incubating the slides with anti-rSM22 $\alpha$  mAb, 75-7, as the primary antibody for 2 h at RT, block slides with 3% BSA were done for 60 min. After washing the sections for 15 min with PBST, the slides were incubated with peroxidase-conjugated goat anti-mouse IgG (Dako, Carpinteria, Calif., USA) as the secondary antibody for 30 min at RT. They were then gently rinsed with distilled water for about 15 min. The immune complex was detected with 3,3-diaminobenzidine tetrahydrochloride (Dako), and counterstaining was performed with hematoxylin. Control sections were treated with mouse IgG1 as the primary antibody.

#### Evaluation of SM22 $\alpha$ -Positive Staining

All sections were stained at the same laboratory by a researcher following exactly the same protocol of IHC using the same antibodies. Photos were also taken under precisely the same exposure time and shutter speed, to avoid any errors, using a Nikon Eclipse E-600 microscope (Tokyo, Japan) coupled to a Pro-Series High-Performance CCD camera. The SM22 $\alpha$  staining was digitized using Image-pro Plus (Media Cybernetics, Silver Spring, Md., USA). Before detections of SM22 $\alpha$ -positive staining, the integrated optical density (IOD) was calibrated and the glomerular area (GA) and tubulointerstitial area (TA) were selected arbitrarily. Then, in those selected areas, the SM22 $\alpha$ -positive area (PA) was counted. The statistical data of PA in GA (PA/GA), IOD in GA (IOD/GA), PA in TA (PA/TA) and IOD in TA (IOD/TA) were obtained. All the values were determined by measuring three times

each glomerulus and TA, which were randomly selected in five serial sections of a single kidney of rats (see fig. 5).

#### Statistical Analyses

All data are shown as mean  $\pm$  SD. One-way factorial ANOVA with a Bonferroni-Dunn post-hoc test was applied for comparing values of the experimental groups. Pearson correlation coefficients were used to evaluate correlations between histological values and biochemical data. The stepwise multiple linear regression analysis was used to determine the histological values contributing to changes of biochemical data, and vice versa. Statistical analyses were performed using SPSS 12.0 Software (SPSS Inc., Chicago, Ill., USA). The difference was considered statistically significant at  $p < 0.05$ .

## Results

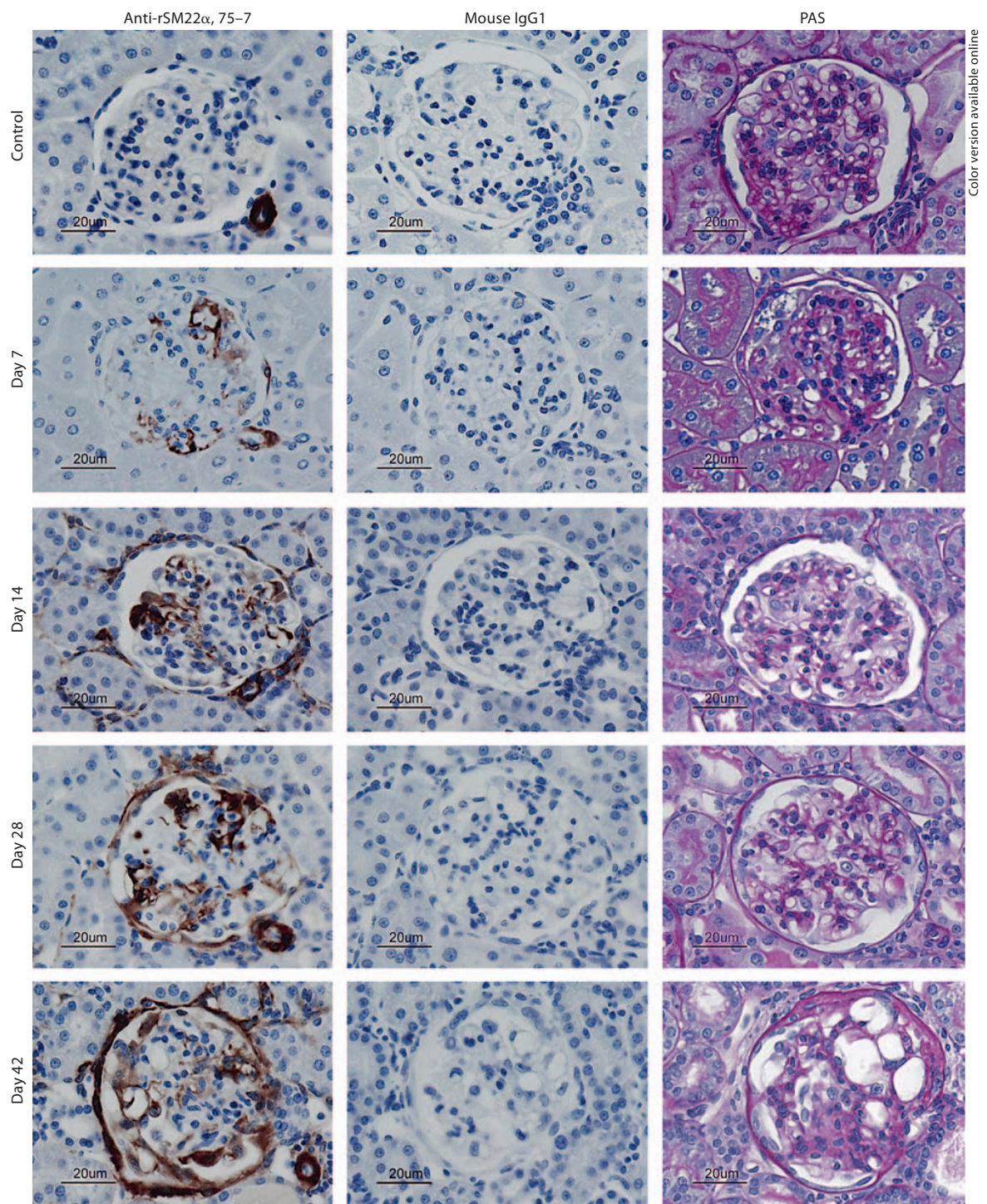
#### Specificity of mAb against rSM22 $\alpha$ , 75-7

To study the pathophysiological features of SM22 $\alpha$  in renal diseases reproducibly, we established some stable hybridoma clones using the recombinant rat SM22 $\alpha$  as an immunogen, which had also been employed for making the polyclonal Ab in our previous study. Among the clones, we picked up clone 75-7 because of its strong reactivity for immunohistochemistry. In order to check the specificity, we performed two-dimensional Western blot analysis using the rat aorta. As shown in figure 1, clone 75-7 recognized a single spot of 22 kDa protein at around pI 9.0, which is consistent with previously published data [25].

#### Immunohistochemistry of SM22 $\alpha$ in Glomeruli of Rat ADR Nephropathy

As shown in figures 2 and 3, in control rats, SM22 $\alpha$  was only detected in vessel walls. Seven days after ADR injection, SM22 $\alpha$  was newly detected in glomeruli seg-

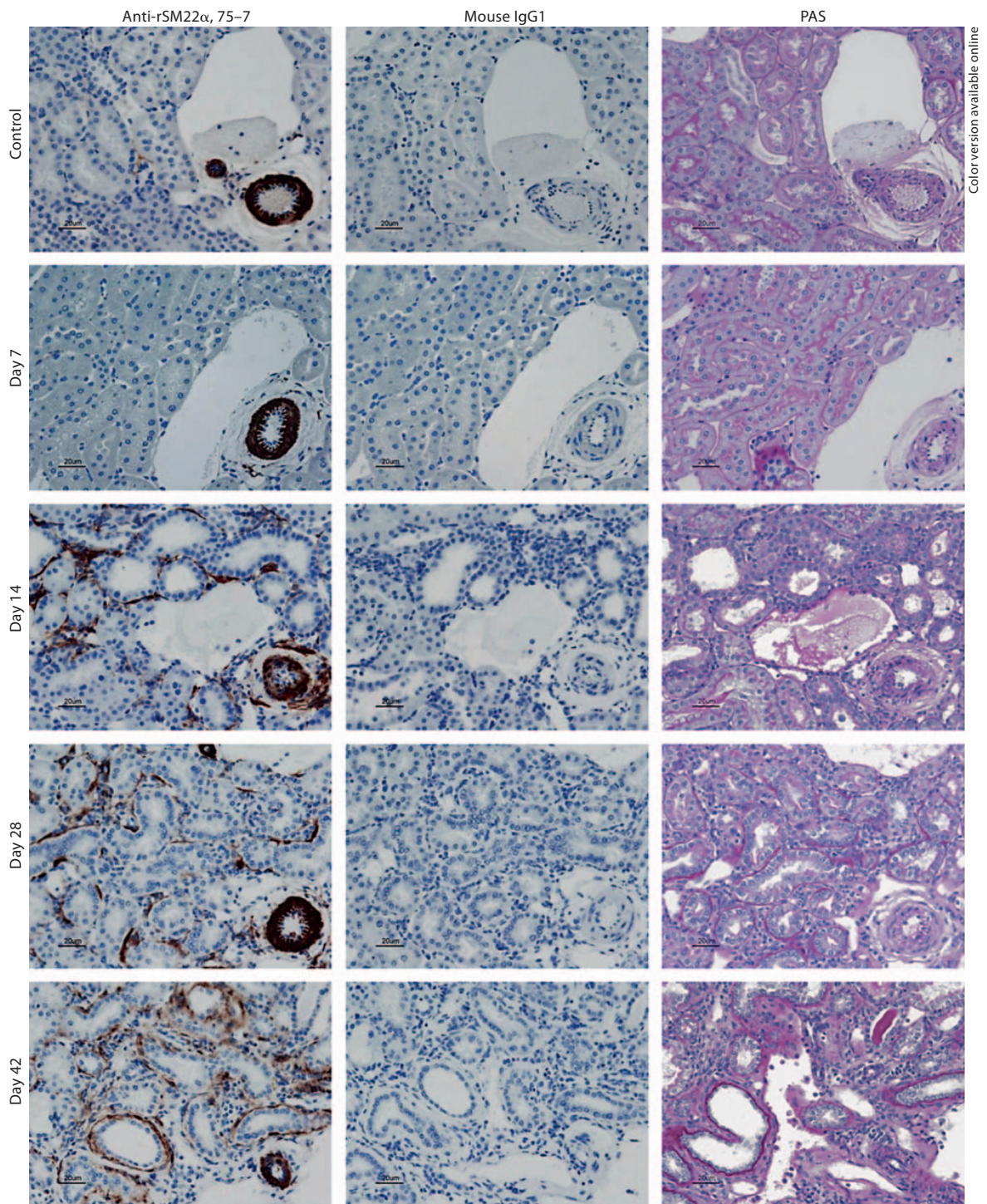




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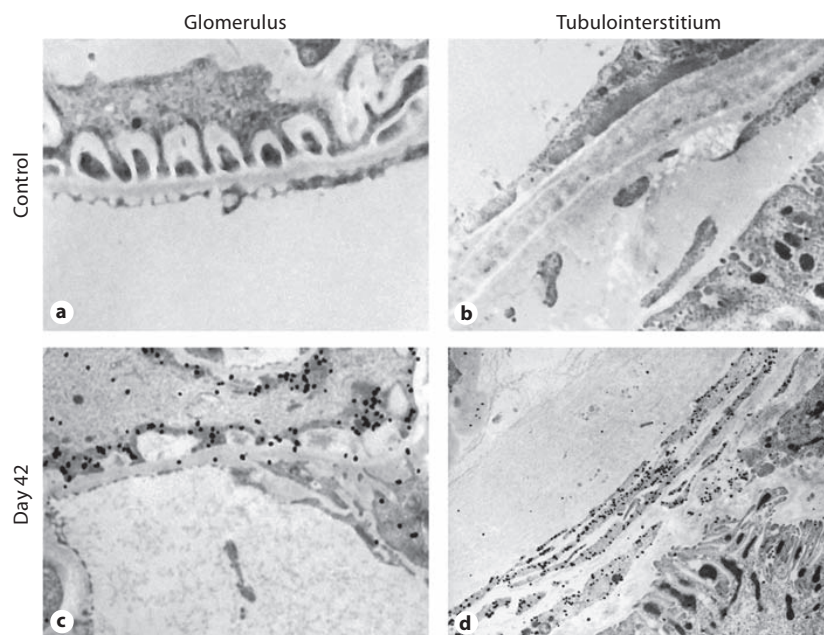
**Fig. 2.** Immunohistochemistry of SM22 $\alpha$  in glomeruli of ADR nephropathy. The left column was stained with anti-rSM22 $\alpha$  mAb (75-7), the middle column shows the negative controls which are incubated with nonspecific isotype-matched mouse IgG1, and the right column shows PAS staining. Serial sections were used for each staining. Orig. magnif.  $\times 800$ .





Color version available online

**Fig. 3.** Immunohistochemistry of SM22 $\alpha$  in the TA of ADR nephropathy. The left column was stained with anti-rSM22 $\alpha$  mAb (75-7), the middle column shows the negative control tissues which are incubated with nonspecific isotype-matched mouse IgG1, and the right column shows PAS staining. Serial sections were used for each staining. Orig. magnif.  $\times 800$ .



**Fig. 4.** SM22 $\alpha$  expression in immunoelectron microscopy. Kidney samples of normal control tissue (**a, b**) and ADR nephropathy (42 days after ADR injection) (**c, d**) were examined for SM22 $\alpha$  by immunoelectron microscopy using the immunogold technique. Cells in the glomeruli (**a**) or periglomerular interstitial area (**b**) of normal control tissue were negative for immunogold label, whereas podocytes (**c**) and interstitial cells (probably fibroblasts) (**d**) in ADR nephropathy were positive. There was no obvious labeling of other types of cells, including endothelial cells or tubular cells. Orig. magnif.  $\times 4,000$ .

mentally in an epithelial pattern. After day 28 it became positive in interstitial cells, as well as glomerular cells. On day 42, when lesions of FSGS and interstitial fibrosis were apparent, SM22 $\alpha$  was detected in the sclerotic lesions of glomeruli. The staining of TAs is shown in figure 3. After day 14, interstitial cells were positive for SM22 $\alpha$ .

#### *Immunoelectron Microscopy*

To investigate SM22 $\alpha$  expression in the cells in detail, we performed immunoelectron microscopy by immunogold technique. In control normal rats, SM22 $\alpha$  was negative in glomerular epithelial cells (fig. 4a) or periglomerular interstitial cells (fig. 4b). In rats on day 42 after ADR injection, podocytes became swollen and showed the foot process effacement in part. SM22 $\alpha$  was preferentially expressed along the dense basal microfilaments of podocytes (fig. 4c). The interstitial cells (probably fibroblasts) around the glomeruli were also positive for SM22 $\alpha$  (fig. 4d).

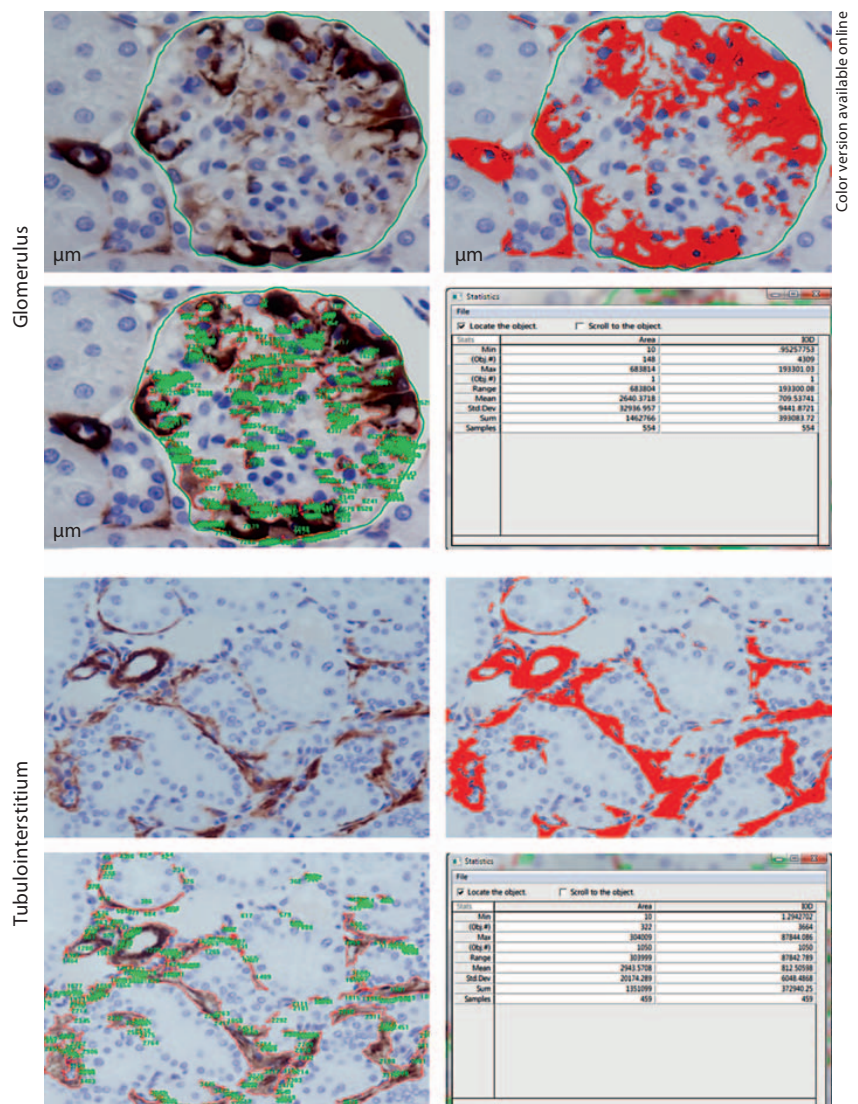
#### *Quantitative Analysis of Sections Stained with Anti-rSM22 $\alpha$ mAb (75-7) and Biochemical Parameters*

In order to investigate the relationship between SM22 $\alpha$  expression and parameters of urine and blood data, we first quantified the extent and intensity of SM22 $\alpha$  expres-

sion by using Image-pro Plus software. SM22 $\alpha$ -PA and IOD in glomerular or tubulointerstitial areas were measured. The data were divided by the values of selected GA or TA for standardization (fig. 5). The data of groups are shown in figure 6. Urine and blood biochemical data such as creatinine clearance (Ccr), Scr, BUN, the amount of proteinuria (UP) and the ratio of UP to the concentration of urinary creatinine (UP/Ucr) of groups are shown in figure 7.

Correlation analysis (fig. 8) showed that the histological parameters of SM22 $\alpha$  expression, such as PA/GA, IOD/GA, PA/TA and IOD/TA, were significantly associated with biochemical data presenting renal function including Ccr, BUN and Scr, and the severity of proteinuria including UP and UP/Ucr. Especially, as for renal function, Ccr was most strongly associated with PA/TA ( $R^2 = 0.8460$ ,  $p < 0.0001$ ). As for severity of proteinuria, UP/Ucr was moderately associated with PA/GA ( $R^2 = 0.4348$ ,  $p = 0.0029$ ). A stepwise multiple linear regression incorporating all the histological parameters of SM22 $\alpha$  expression showed that PA/TA or PA/GA were predictors of Ccr or UP/Ucr, respectively (table 1a). Inversely, the deterioration of Ccr was the most important predictor of all parameters of SM22 $\alpha$  expression (table 1b).



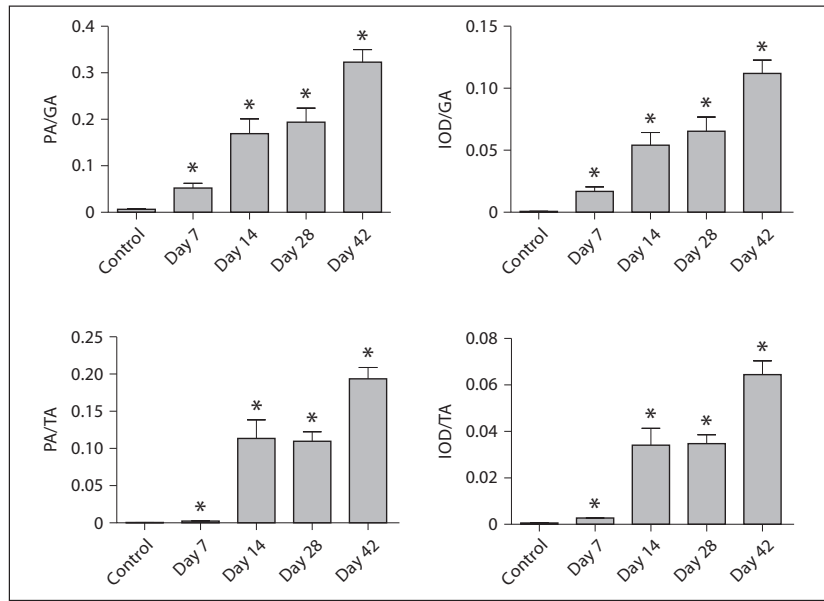


**Fig. 5.** Quantification of SM22 $\alpha$ -positive staining. The immunohistochemical sections were studied by using Image-Pro Plus software on SM22 $\alpha$  immunostained sections to quantitate the area of positive staining (PA), the whole area of the selected glomerulus (GA), the IOD of PA and the whole area of the selected TA. The positive staining was identified in the statistical red color, and calculated in green numbers. The data are shown in the forms. The values of PA/GA, IOD/GA, PA/TA and IOD/TA were used for statistical analyses.

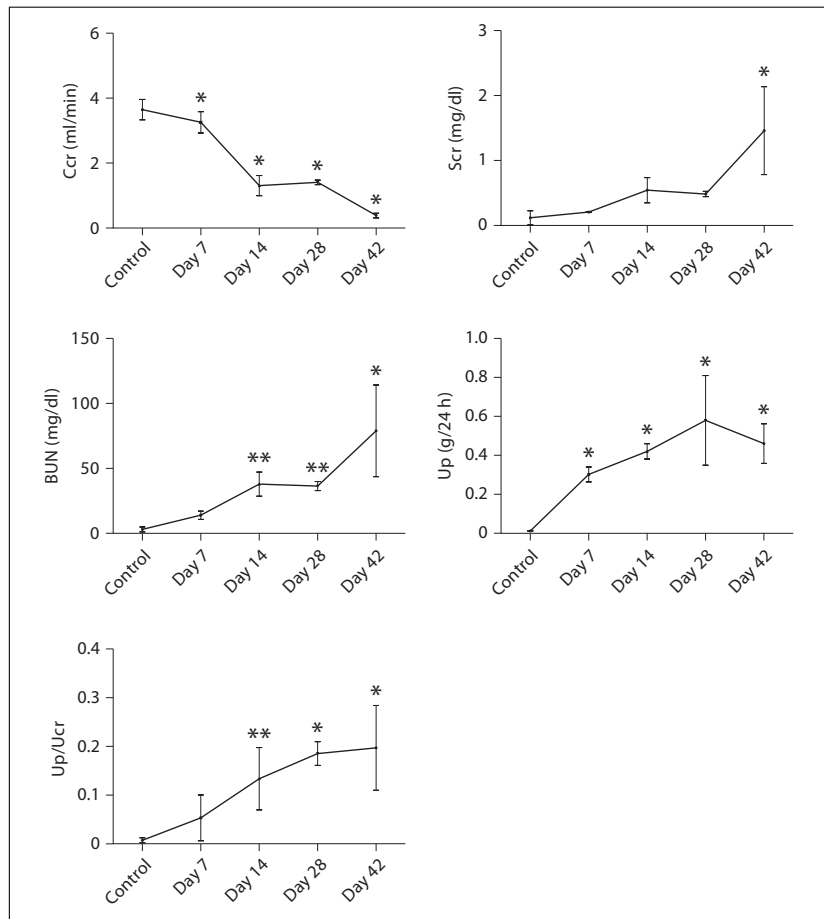
**Table 1.** Stepwise multiple linear regression models

Dependent variable and model	Independent variable	Unstandardized		Standardized		F	p	
		$\beta$ -coefficient	constant	$\beta$ -coefficient	R <sup>2</sup>			
<b>a</b> Regression model including PA/GA, IOD/GA, PA/TA, IOD/TA								
Ccr	One step	PA/TA	-13.984	3.007	-0.92	0.836	87.897	0.0001
UP/Ucr	One step	PA/GA	0.486	0.049	0.659	0.399	12.31	0.003
<b>b</b> Regression model including Ccr, Scr, BUN, UP, UP/Ucr								
PA/GA	One step	Ccr	-0.087	0.323	-0.883	0.765	56.356	0.0001
IOD/GA	One step	Ccr	-0.03	0.11	-0.859	0.722	45.134	0.0001
PA/TA	One step	Ccr	-0.06	0.202	-0.92	0.836	87.897	0.0001
IOD/TA	One step	Ccr	-0.019	0.065	-0.901	0.8	68.847	0.0001

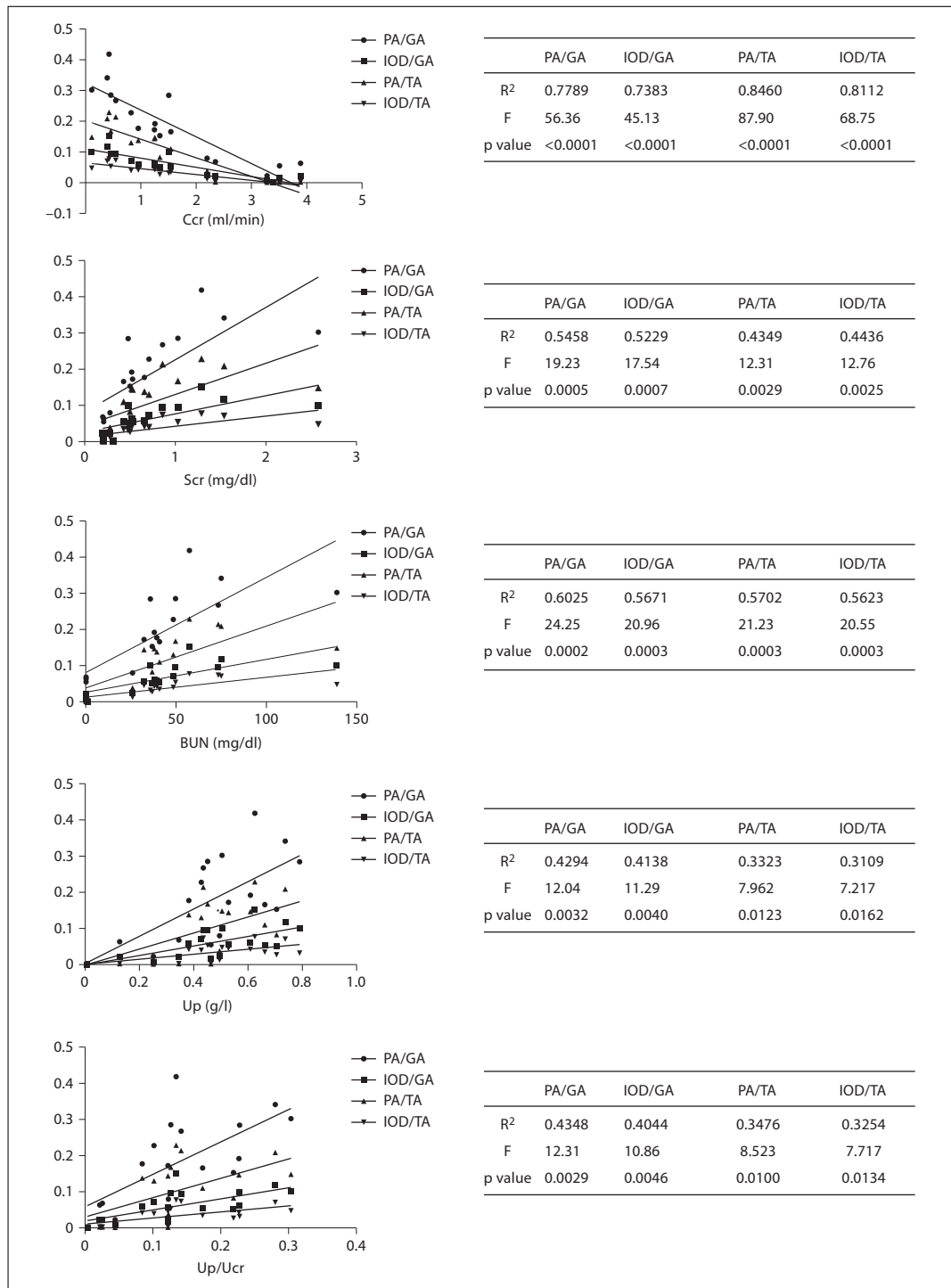
**Fig. 6.** The extent and the intensity of SM22 $\alpha$ -positive staining. The values of PA/GA, IOD/GA, PA/TA and IOD/TA of control and ADR-treated groups, calculated in figure 5, are shown. One-way factorial ANOVA with a Bonferroni-Dunn post-hoc test was applied for comparing values of the experimental groups (\*  $p < 0.01$ ).



**Fig. 7.** Biochemical data of blood and urine of ADR nephropathy. Biochemical data of blood and urine of control and ADR-treated groups are shown. One-way factorial ANOVA with a Bonferroni-Dunn post-hoc test was applied for comparing values of the experimental groups (\*  $p < 0.01$ , \*\*  $p < 0.05$ ).







**Fig. 8.** Pearson correlation matrix among variables of blood/urine biochemical data and SM22 $\alpha$  expression. The correlation between blood/urine biochemical data and the histological parameters of control and ADR nephropathy rats was investigated. Scatter diagrams of bivariate correlations are shown as graphs with tables of results.

## Discussion

In the present study, using a highly specific monoclonal anti-rat SM22 $\alpha$  mAb (75–7), we quantified the extent and intensity of SM22 $\alpha$  expression in a well-known model of FSGS, ADR nephropathy, which demonstrated damages of glomerular epithelial cells first, and tubulointerstitial cells later. In kidney diseases, in general, various kinds of histological damage, such as glomerular and tubulointerstitial disorders, are mixed together. Then, taken together with all data of stages of ADR nephropathy, we investigated the correlation between histological parameters of SM22 $\alpha$  expression and biochemical data. Both the extent and intensity of SM22 $\alpha$  expression in glomerular and TA were correlated with the deterioration of renal function and the severity of proteinuria. In order of importance, the extent of SM22 $\alpha$  expression in tubulointerstitial area (PA/TA) was the predictor of the deterioration of renal function (Ccr), and that in the glomerular area (PA/GA) was the predictor of the severity of proteinuria (UP/Ucr). Thus, it seemed that SM22 $\alpha$  expression in glomerular cells, mainly podocytes, reflected the dysfunction of those cells, resulting in proteinuria, and that its expression in interstitial cells represented the tubulointerstitial damage, which is a common appearance of kidney diseases presenting progressive renal dysfunction.

The immunostaining results using our mAb, 75–7, were basically identical to those obtained by anti-SM22 $\alpha$  polyclonal Ab in our previous studies [18, 20], although disease models were different from each other. SM22 $\alpha$  was detected in glomerular epithelial cells first, and also in tubulointerstitial cells later in this model. By immunoelectron microscopy, as the results in our previous study [20], SM22 $\alpha$  was positive in the basal microfilament layer of podocytes, which showed foot process effacement, and on the other hand, it was detected diffusely in the cytoplasm of tubulointerstitial cells. It seemed to be a universal phenomenon that SM22 $\alpha$  was de novo expressed in specific sites, such as glomerular epithelial cells and tubulointerstitial cells, according to the nature of the injury.

The inducible expression of SM22 $\alpha$  might imply the acquisition of contractile or degenerative properties by the kidney cells. Because SM22 $\alpha$  is originally localized in the cytoskeleton of SMCs, the difference of its distribution between glomerular epithelial cells and tubulointerstitial cells might be derived from the anatomical relationship of the cells to surrounding extracellular matrices. The modified environment for the attach-

ment of cells to extracellular matrices and tension generation might lead to the SM22 $\alpha$  expression in accordance with the actin reorganization under the diseased conditions.

The functional consequence of SM22 $\alpha$  expression in the glomerular cells, including injured podocytes, remains unclear. In podocytes, upon the glomerular injury, actin fibers might be reorganized and form dense microfilament bundles containing SM22 $\alpha$ . The cytoskeletal changes in podocytes were precisely investigated in previous studies [26], reporting that the dense microfilament network appeared along the basal cell membrane of podocytes. The dense bundles contained  $\alpha$ -actinin, which might serve as a cross-linker for the microfilaments and represent the contractile phenotypic change of podocytes upon glomerular injury. Because SM22 $\alpha$  is known to be associated with actin stress fibers and stabilize actin gels *in vitro* [7], it might also work as a factor that promoted the reorganization of the actin cytoskeleton of podocytes and supported some functions of podocytes under the diseased condition induced by the cytotoxic reagent, ADR. In this present study it was revealed that SM22 $\alpha$  expression in renal cells represented the severity of proteinuria and the deterioration of renal function. Its expression in podocytes might demonstrate the severity of cell damage, where it might work to maintain configuration of podocytes as a barrier for protein loss. Its expression in interstitial cells might reflect the tubulointerstitial disorder, which was an outcome of disease progression. In our study using the late phase of an anti-glomerular basement membrane glomerulonephritis model, when interstitial fibrosis was evident, SM22 $\alpha$  was costained with  $\alpha$ SMA ( $\alpha$ -smooth muscle actin) in interstitial cells (data not shown), indicating that SM22 $\alpha$ -positive cells in the interstitium might be myofibroblasts. On the other hand, SM22 $\alpha$ -positive podocytes were never costained with  $\alpha$ SMA.

However, the functional significance of SM22 $\alpha$  in kidney diseases should be further investigated so that the pathophysiology, or the mechanism of the development, of the diseases can be better understood. Moreover, revealing the functional role of this molecule might lead to the therapeutic application for renal diseases by controlling its function.



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## Disclosure Statement

The authors have no conflicts of interest to disclose.

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