Expression of SM22α (Transgelin) in Glomerular and Interstitial Renal Injury

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SM22α • Transgelin • 5/6 nephrectomy • Ischemia-reperfusion • Puromycin aminonucleoside nephrosis • Glomerular epithelial cell • Interstitial injury • α-Smooth muscle actin • Acute kidney injury

Abstract
Background/Aims: SM22α, transgelin, is abundantly expressed in smooth muscle tissues and our previous work demonstrated that it is a novel marker of injured glomerular epithelial cells in rat anti-glomerular basement membrane nephritis. In this study, we investigated SM22α expression in models of glomerular and interstitial renal injury. Methods: The 5/6 nephrectomy (Nx) model, ischemia-reperfusion (I/R) model and puromycin aminonucleoside (PAN) nephrosis of rats were studied. Immunohistochemical analyses and immunoelectron microscopic studies of SM22α expression were performed. Results: In the 5/6 Nx model, SM22α was first expressed in peritubular interstitial cells and was also expressed in injured glomerular epithelial cells at 8 weeks. In the I/R model, SM22α expression was induced in peritubular interstitial cells as early as 12 h after I/R with expression sustained at 7 days. However, SM22α was not detected in any glomerular cells or tubular epithelial cells. In PAN nephrosis, SM22α was only expressed in glomerular epithelial cells after 1 week, but expression was transient. Conclusion: SM22α was expressed in glomerular epithelial cells and interstitial cells in renal injury. SM22α is differentially upregulated in various models of renal injury and merits further study.

Introduction
SM22α is a 22-kDa cytoskeletal protein and a recognized differentiation marker of contractile smooth muscle cells (SMCs) [1–3]. It is abundantly expressed in SMCs, though its role has not been well characterized. However, SM22α expression is downregulated in atherosclerotic SMCs and may be involved in controlling the phenotypic modulation of mature SMCs [4]. SM22α is strongly expressed in tissues with abundant SMCs such as the uterus, bladder, stomach and prostate [1, 5, 6]. We reported induced expression of SM22α in injured glomerular epithelial cells in experimental rat anti-glomerular basement membrane nephritis [7]. Expression of SM22α was associated with downregulation of the specific podocyte proteins nephrin and podocalyxin [7]. Glomerular epithelial cells originate from mesenchyme [8] and contain...
major contractile proteins such as actin and myosin; therefore, they possess the contractile phenotype of SMCs [9]. Thus, it may be the case that the induction of SM22α expression results from the dedifferentiation or transdifferentiation of injured glomerular epithelial cells [7].

The 5/6 nephrectomy (Nx) model is a model of chronic progressive renal disease and is characterized by tubulointerstitial (TI) cell injury in the early stage. Glomerular injury follows and lesions resembling focal segmental glomerulosclerosis appear pathologically [10–14]. It is a model of irreversible TI and glomerular injury. The renal ischemia-reperfusion (I/R) model is characterized by acute renal failure associated with TI injury such as tubular degeneration, leukocyte infiltration and fibrosis. It is considered to be a model of acute TI injury. Depending upon the duration of renal I/R injury, renal recovery follows later [15–17]. In these renal injury models, myofibroblasts positive for smooth muscle actin (αSMA) appear in injured parts of kidney [11, 15, 18–20]. In contrast, puromycin aminonucleoside (PAN) nephrosis is a model of minimal change nephrotic syndrome and is characterized by massive proteinuria secondary to injury to glomerular epithelial cells [21]. Histologically, renal components are almost normal by light microscopy and this model is regarded as a model of reversible podocyte injury [22].

In this study, we investigated the pattern of SM22α expression in these renal disease models. We demonstrate that SM22α is a phenotypic marker of the injured kidney and may be expressed in specific sites according to the nature of the injury.

Methods

Animals

Male Sprague-Dawley rats and male Wistar-Kyoto rats (7- to 8-weeks-old) weighing 200–250 g were purchased from Charles River Japan (Yokohama, Japan). Animals were maintained in our animal facility. Animal care was performed in accordance with the guidelines of Niigata University (Niigata, Japan). Blood samples were collected for measurement of blood urea nitrogen (BUN) and serum creatinine (Cr) at the time of sacrifice. Twenty-four urine samples were collected using metabolic cages for measurement of urinary protein (UP; SRL, Tokyo, Japan) as described previously [23, 24].

5/6 Nephrectomy Model

Male Sprague-Dawley rats were randomly divided into two groups: the 5/6 Nx group (n = 11) and the sham surgery control group (n = 7). In the 5/6 Nx group, rats were anesthetized with isoflurane and underwent a right Nx and removal of the two poles of left kidney at the same time (the one-step 5/6 Nx method). In the sham surgery control group, rats underwent a laparotomy only. The remnant kidneys were harvested after 1, 2, 4 and 8 weeks. Renal tissue was fixed in methyl Carnoy’s fixative for immunohistochemical analysis and periodic acid-Schiff (PAS) stain for histological analysis.

Ischemia-Reperfusion Model

Male Sprague-Dawley rats were randomly divided into two groups; the I/R group (n = 13) and the sham surgery control group (n = 7). In the I/R group, rats were anesthetized with isoflurane and renal ischemia was induced by occlusion of the left renal pedicle with a clamp for 45 min. In the sham surgery control group, rats underwent a laparotomy only. The kidneys were harvested after reperfusion at 0, 6 and 12 h and 1, 2 and 7 days. Renal tissue were fixed in methyl Carnoy’s fluid for immunohistochemical analysis and PAS stain for histological analyses.

Puromycin Aminonucleoside Nephrosis

Male Wistar-Kyoto rats were randomly divided into two groups: the PAN nephrosis group (n = 11) and the control group (n = 7). In the PAN nephrosis group, disease was induced in the rats by a single intravenous injection of PAN (Sigma, St. Louis, Mo., USA) at a dose of 50 mg/kg. In the control group, rats were injected with saline. The kidneys were harvested after 1, 2, 4, 6 and 8 weeks. Renal tissues were fixed in methyl Carnoy’s fluid for immunohistochemical analyses and in PAS stain for histological analyses.

Recombinant Protein and Antibody

Rat SM22α recombinant protein (rSM22α) was prepared as previously described [7]. Briefly, the pQE-30UA vector into which the in-frame 260 bp of rat SM22α 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 6x histidine-tagged SM22α was induced by isopropyl-β-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 (Qia-gen, Tokyo, Japan). To obtain the rabbit anti-rSM22α antibody (Ab), 1 mg of histidine-tagged rat rSM22α was blended with an equal volume of complete Freund’s adjuvant and injected subcutaneously into a rabbit. After 3 weeks, 1 mg of rSM22α in an equal volume of incomplete adjuvant was injected every 3 weeks for a total of 4 times. Rabbit anti-rSM22α serum was purified by immunoadfinity chromatography using HiTap Protein G HP (Amersham Biosciences, Wikstoms, Sweden).

Immunohistochemistry

Immunohistochemistry was undertaken following the methods of our previous work [25, 26]. Paraffin-embedded kidney tissue was cut into 10-μm thick sections. Tissue sections were deparaffinized in xylene and rehydrated in 100% ethanol. Slides were blocked with 3% H2O2 in PBS for 20 min, and 3% BSA in PBS for 30 min. For the detection of SM22α, the slides were incubated with rabbit anti-rSM22α IgG (2 μg/ml) overnight at 4°C, and treated with horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (1:200 dilution; DAKO, Carpinteria, Calif., USA) as the secondary Ab. The immune complex was detected with 3,3’-diaminobenzidine tetrahydrochloride (DAKO) and counterstaining was performed with hematoxylin. Control sections were incubated with normal rabbit IgG (Sigma-Aldrich, Tokyo, Japan).
Interstitial cells after 1–8 weeks (fig. 1). Tubular dilatation and degeneration was also apparent. At 8 weeks, focal glomerulosclerosis with segmental PAS-positive sclerotic lesions and adhesions to Bowman’s capsule (fig. 2) were observed in 8% of glomeruli (7/89). SM22α was expressed in a glomerular epithelial pattern in such injured glomeruli (fig. 2), but was not expressed in tubular epithelial cells. SM22α was detected in the vessel walls in both control and diseased tissues.

SM22α Expression in the I/R Model
Because SM22α was expressed in glomerular and interstitial cells following injury, we next examined the I/R model: an injury model characterized by tubular cell injury (fig. 3). In this model, glomeruli are minimally changed [15] and SM22α was not detected at 0 or 6 h after reperfusion except in blood vessels. At 12 h, SM22α was expressed in peritubular and periglomerular interstitial cells and its expression was sustained until 7 days after I/R injury. However, SM22α was not detected in any glomerular cells or tubular epithelial cells after I/R injury.

SM22α Expression in the PAN Nephrosis Model
We then investigated the expression of SM22α in the PAN nephrosis model characterized by specific injury to glomerular epithelial cells (fig. 4). PAN nephrosis is considered as a model of minimal change nephrotic syndrome with only minor glomerular abnormalities found by light microscopy.

SM22α was detected by immunohistochemistry in a glomerular epithelial pattern and partially in Bowman’s capsule 1 week after PAN injection. Indeed, SM22α expression peaked at 1 week with expression declining thereafter. SM22α was rarely detected in interstitial cells, whereas it was prominent in both the 5/6 Nx model and the I/R model. Urinary protein excretion significantly increased and peaked at 1 week compared with the control group and fell thereafter (fig. 4B). There was no significant difference in BUN or creatinine between the two groups (data not shown). SM22α expression thus appeared to correlate with the extent of urinary proteinuria.

SM22α Is Expressed by Glomerular Epithelial Cells and Interstitial Cells by Immunoelectron Microscopy
In order to identify SM22α expressing cells microanatomically in these distinct injury models, we performed immunoelectron microscopy with anti-SM22α Ab and an immunogold technique. SM22α was not detected in normal tissue by immunogold microscopy (fig. 5a, b). In the PAN nephrosis model with glomerular epithelial cell

![Fig. 1. SM22α expression in the 5/6 Nx model. Serial sections of kidney samples from control (a, f) and rats at 1 week (b, g), 2 weeks (c, h), 4 weeks (d, i) and 8 weeks (e, j) after surgery were immunostained with anti-SM22α Ab (f-j) or control normal rabbit IgG (a-e) SM22α was mainly expressed in peritubular and periglomerular cells throughout the time course (original magnification ×200).](image-url)

SM22α in Renal Injury

Kidney tissues were fixed with PLP, dehydrated through a graded series of ethyl alcohols and embedded in hydrophilic methacrylate resin. Ultrathin sections (80 nm) were mounted on nickel grids. After drying, the grids were blocked with 1% BSA buffer for 1% uranyl acetate and 1% lead citrate. After rinsing with distilled water, the gold on the grids was enhanced by enhancement reagents (IntenSE M, GE Healthcare) for 10 min. After rinsing with PBS, the grids were incubated in secondary Ab attached to 10-nm gold particles (Gold Conjugates, BB International) for 2 h at room temperature. After rinsing with distilled water, the grids were stained for contrast with 1% uranyl acetate and 1% lead citrate. After rinsing with distilled water and drying, the grids were observed with a transmission electron microscope (H-600A; Hitachi High-Technologies Corp., Tokyo, Japan).

Statistical Analysis
All data are presented as means ± SD. Repeated measures ANOVA with a Bonferroni/Dunn post hoc test was used to analyze data. The unpaired Student t test was also used to compare control versus experimental groups. p < 0.05 were considered significant for both analyses.

Results
SM22α Expression in the 5/6 Nx Model
Rats developed significant proteinuria and renal impairment at 8 weeks compared to control rats (urinary protein: 115.5 ± 13.8 vs. 17.7 ± 6.0 mg/day, p < 0.01; BUN: 58.8 ± 16.4 vs. 23.4 ± 1.9 mg/dl, p < 0.05; creatinine: 0.77 ± 0.29 vs. 0.23 ± 0.04 mg/dl, p < 0.05; 5/6 Nx rats vs. control rats; unpaired Student’s t test, n = 4/group).

We performed immunohistochemical analysis of the 5/6 Nx model using the anti-SM22α Ab (fig. 1). SM22α was mainly expressed in peritubular and periglomerular

instead of anti-rSM22α IgG. The Ab absorption test was performed as follows: 2 × 10^{-5} μM of anti-rSM22α IgG in 1% BSA-PBS was incubated with immunogen (rSM22α; 1 × 10^{-3} μM) or the buffer used for extraction (QIAexpress type IV kit) overnight at 4°C. Then, the mixtures were briefly centrifuged at 14,000 g at 4°C and the supernatants were used for immunostaining.
Injury, podocytes were positive for SM22α/H9251 (fig. 5c) where foot process effacement was observed. Endothelial cells or mesangial cells were negative for SM22α. In contrast, in the I/R model with TI injury, interstitial cells (probably fibroblasts) were positive for SM22α/H9251 (fig. 5d) while tubular epithelial cells were SM22α-negative.

Specificity of Anti-SM22α Ab

In order to ensure that the anti-SM22α Ab used in this study was specific, we carried out the Ab absorption test using the antigen used to generate the Ab. As shown in figure 6, the anti-SM22α Ab completely lost its tissue reactivity in immunohistochemical staining of the PAN nephrosis kidney tissue following absorption with the immunogen rSM22α Ag. Taken together with the previous Western blot data in our previous study [7] indicating that this Ab recognized a single 22-kD band in rat aorta lysate, the anti-SM22α Ab could be considered specific for rat SM22α.

Discussion

Our previous studies demonstrated that SM22α is a novel marker of injured glomerular epithelial cells in the rat anti-glomerular basement membrane nephritis model [7]. In the current study, we have demonstrated that SM22α was also expressed in the injured in three diverse models of renal injury; 5/6 Nx model, I/R injury and PAN nephrosis.

The 5/6 Nx model is characterized by chronic renal injury and leads to chronic renal failure [10–14] with TI injury evident at an early stage and focal glomerulosclerosis lesions appearing later. SM22α expression was detected in peritubular and periglomerular interstitial cells in the early stages and in glomerular epithelial cells in the later stages of disease.

Renal I/R injury is a model of acute renal failure with acute tubular necrosis. Renal dysfunction is evident early and recovers after about 1 week [15, 17]. Tubular epithelial cells are mainly injured in this model with minimal injury to glomerular cells [15, 16]. SM22α expression was mainly detected in peritubular interstitial cells. Interestingly, SM22α was expressed as early as 12 h after I/R injury, whereas αSMA-positive cells were reported to appear only after 2 days [15]. Expression of SM22α was sustained until day 7. There was no expression of αSMA in any glomerular cell in this model. Markers of fibrosis such as TGFβ, collagens and αSMA-positive myofibroblasts increase in the interstitial area of both the 5/6 Nx model and the I/R injury model, and are evident later in glomeruli of the 5/6 Nx model [11, 12, 18, 20]. Thus, SM22α might be another marker of interstitial fibrosis and glomerulosclerosis, as it is potentially correlated with...
Fig. 3. Interstitial SM22α expression in the I/R model. Kidney samples of control tissue (h) and tissue removed at 0 h (i), 6 h (j), 12 h (k), 1 day (l), 2 days (m) and 7 days (n) after I/R injury were immunostained with normal rabbit IgG (a–g) or anti-SM22α Ab (h–n) (original magnification ×200). SM22α was not detected at 0 or 6 h after I/R, except in blood vessels. SM22α was partially expressed at 12 h after I/R in peritubular and periglomerular interstitial cells with expression increasing over time and sustained until day 7. SM22α was not detected in any glomerular cells.
**Fig. 4.** SM22α expression in PAN nephrosis. **A** Kidney samples of control tissue (g) and tissue removed after 1 week (h), 2 weeks (i), 4 weeks (j), 6 weeks (k) and 8 weeks (l) were immunostained with normal rabbit IgG (a-f) or anti-SM22α Ab (g-l) (original magnification ×400). SM22α was preferentially expressed in a glomerular epithelial pattern with partial expression evident in the Bowman’s capsule 1 week after PAN injection. SM22α expression peaked at 1 week and was rarely detected in peritubular and periglomerular interstitial cells. **B** Urinary proteinuria significantly increased in rats treated with PAN and peaked at 1 week and fell thereafter (n = 9 for PAN nephrosis, n = 5 for control). Values are means ± SD. Analysis was performed by repeated measures ANOVA (p < 0.01 for difference of response, *p < 0.05 by Bonferroni/Dunn post hoc comparison).
**Fig. 5.** SM22α expression detected by immunoelectron microscopy. Kidney samples of normal control tissue (a, b), PAN nephrosis tissue at day 7 (c) and I/R injury tissue 48 h after injury (d) were examined for SM22α by immunoelectron microscopy using the immunogold technique. Cells in the glomeruli (a) or interstitial area (b) of normal control tissue were negative for immunogold label, whereas podocytes in PAN nephrosis (c) and interstitial cells (probably fibroblasts) in the I/R injury model (d) were positive. There was no obvious labeling of other types of cells, including endothelial cells or tubular cells. Arrows indicate SM22α-positive cells. PTC = Peritubular capillary (original magnification ×4,000).

**Fig. 6.** The anti-SM22α Ab is specific. The anti-SM22α Ab used in this study underwent the Ab-absorption test using the antigen (Ag) that was employed as the original immunogen for Ab generation. The anti-SM22α Ab was incubated in an excess amount of immunogen (molecular ratio IgG:Ag = 1:20) or vehicle as described in Methods. Then, serial sections of renal tissue from PAN nephrosis (day 7) were immunostained with either anti-SM22α Ab treated with vehicle (a) or anti-SM22α Ab treated with Ag (b), followed by treatment with HRP-conjugated goat anti-rabbit IgG (original magnification ×200). Ab immunoreactivity against both injured glomeruli and blood vessels was completely abolished by pre-absorption with the immunogen.
other markers of fibrosis. In contrast, SM22α expression was only detected in glomerular epithelial cells in the PAN nephrosis model that exhibits specific podocyte injury.

Furthermore, SM22α expression peaked at the 1-week time point and fell thereafter, which directly paralleled the pattern of urinary protein excretion. Our previous histological analyses of SM22α expression [7] and the present studies of renal injury indicate that SM22α expression may be induced in glomerular epithelial cells, including podocytes and epithelial cells of Bowman’s capsule and interstitial cells that are likely to be fibroblasts. SM22α expression is sustained in persistent injury but short-lived in transient injury. SM22α expression was not detected in tubular epithelial cells or glomerular endothelial cells in any renal injury model studied, whereas it was reported that αSMA may be expressed by tubular epithelial cells in the 5/6 Nx model [20].

It has recently been reported that SM22α may be expressed in alveolar epithelial type II cells under the action of TGF/Smad3 signaling and is involved in lung fibrosis [27]. TGFβ is a key mediator for transdifferentiation of renal cells, glomerulosclerosis and TI fibrosis in chronic kidney diseases and induces Smad3 signaling [28]. Also, because TGFβ and Smad3 have been reported to increase SM22α gene transcription [29, 30], SM22α expression might be related to cellular transdifferentiation following injury possibly induced by TGFβ.

It was reported that some intermediate filaments such as desmin, nestin and vimentin were upregulated in podocytes in response to injury [22, 31], whereas the podocyte-specific marker nephrin was downregulated [21]. Among those other markers of podocyte injury, the level of desmin expression was in parallel with urinary proteinuria excretion like SM22α. Because SM22α was known to be associated with actin-stress fibers and stabilize actin gels [1], it might be involved in promoting the reorganization of the actin cytoskeleton of injured cells and increasing the mechanical stability of cells in coordination with intermediate filaments. However, the exact cellular function of SM22α in renal injury remains unclear and should be further investigated.

In conclusion, SM22α was expressed in diverse models of glomerular and TI injury in addition to anti-glomerular basement membrane nephritis. SM22α may represent a useful phenotypic marker of kidney cells following renal injury in both glomerular and TI disease.

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