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ORIGINAL ARTICLE

Decreased urinary calbindin 1 levels in proteinuric rats and humans with distal nephron segment injuries

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Abstract

Background Several proteins have been proposed as new urinary biomarkers of kidney injuries, but they are not always capable of identifying the kidney nephron segment that has been injured. Since calbindin 1 protein is exclusively localized in the kidney distal nephron segment, it is presumed that its expression is altered during distal nephron segment injuries, resulting in changes in its urinary excretion.

Methods Calbindin 1 expression in normal rat kidneys was compared with that in the kidneys of rats that had suffered distal nephron segment injuries (unilateral ureteral obstruction [UUO] or anti-glomerular basement membrane glomerulonephritis [anti-GBM GN]) using immunohistochemical examinations and real-time polymerase chain reaction. The urinary calbindin 1 protein concentration of normal rats was also compared with that of anti-GBM GN rats and of cisplatin nephropathy rats using Western blotting. We also compared the kidney and urinary calbindin 1 protein concentrations of normal human subjects with those of proteinuric patients [immunoglobulin (Ig)A nephropathy; IgAN] with distal nephron segment injuries.

Results Calbindin 1 mRNA expression in the renal cortices and calbindin 1 protein expression in the kidney distal nephron segments were decreased in the UUO and anti-GBM GN rat kidneys. The urinary calbindin 1 protein levels of the anti-GBM GN rats were also markedly decreased, whereas those of the cisplatin nephropathy rats were slightly decreased. The human IgAN patients displayed decreased renal calbindin 1 protein expression in their dilated distal tubules, and some patients displayed decreased urinary calbindin 1 levels.

Conclusion Since it has been demonstrated that decreased urinary calbindin 1 levels are indicative of decreased calbindin 1 kidney expression due to distal nephron segment injuries, calbindin 1 might be a useful urinary biomarker for identifying distal nephron segment injuries.

Keywords Immunohistochemistry · Real-time PCR · Western blotting · Anti-GBM glomerulonephritis · IgA nephropathy

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Introduction

Several proteins have been proposed as new urinary biomarkers of kidney injury, such as NGAL, L-FABP, KIM-1, cystatin C, and interleukin-18 [1]. Although they are more useful for the early detection of acute kidney injuries than elevated serum creatinine levels, they are not always capable of identifying the injured kidney nephron segment, especially in cases where the biomarker may also be derived from serum [2]. A number of criteria for ideal biomarkers have been proposed, e.g., the biomarker must originate from the injured cells and should play a role in the pathophysiology of the affected organ [3]. Since urine

samples from proteinuric patients contain large amounts of serum protein, biomarker proteins from specific nephron segments can be easily missed.

Calbindin 1 (also known as calbindin 1, 28 kDa, or calbindin-D28K) is an intracellular calcium binding protein. In the kidneys, it has been shown to be exclusively localized in the distal tubule cells and the proximal parts of the collecting duct cells [4]. During kidney distal nephron segment injuries, calbindin 1 expression in the kidneys might be altered, resulting in changes in its urinary concentration. The aim of the present study was to investigate whether altered urinary calbindin 1 levels are indicative of changes in calbindin 1 expression in the kidneys due to distal nephron segment injuries. The first stage of this study was performed in rats, and the second stage involved humans. In both stages of the study, the renal and urinary calbindin 1 concentrations of normal subjects were compared with those of individuals with kidney diseases involving distal nephron segment injuries.

Materials and methods

Rats

Inbred male WKY rats (aged 12–16 weeks) were purchased from Charles River Inc. (Atsugi, Kanagawa, Japan) and maintained at our animal facility. All of the animal care procedures were performed in accordance with the guidelines of Niigata University (Niigata, Japan). Two rat models of kidney disease involving tubular cell injury were investigated—unilateral ureteral obstruction (UUO) [5] and anti-glomerular basement membrane glomerulonephritis (anti-GBM GN) [6]. Cisplatin nephropathy [7] was also investigated as a tubular specific injury model.

UUO

Five rats were subjected to UUO. Briefly, the middle portion of the left ureter was ligated and then cut between the two ligated points. The rats were sacrificed at 14 days after the procedure. The obstructed left (ipsilateral) and right (contralateral) kidneys were harvested for renal histological examination, immunohistochemistry, and RNA isolation.

Anti-GBM GN

Twenty-five rats were intravenously injected with 25 μl of anti-GBM antibody [containing 325 μg of rabbit immunoglobulin (Ig)G] per 100 g body weight. Groups of five rats were sacrificed on days 2, 4, 7, 14 and 21, and their kidneys were obtained. Five normal rats were also sacrificed as controls. Urine specimens were collected before sacrifice on day 0 (from −24 to 0 h), day 2 (from 24−48 h), day 4 (from 72−96 h), day 7 (from 144−168 h), day 14 (from 312−336 h), and day 21 (from 240−264 h) by housing the animals in metabolic cages. The specimens were used for urinary protein measurement, creatinine measurement, calcium measurement, and Western blotting. The amount of urinary protein was determined using a protein assay kit (Bio-Rad Laboratories, Tokyo, Japan). The amount of creatinine present in the urine and sera from the rats was determined by an enzymatic assay method (Kainos CRE-EN; Kainos Laboratories, Tokyo, Japan). The amount of urinary calcium was determined using a calcium assay kit (Cayman Chemical Co., Ann Arbor, MI, USA).

Cisplatin nephropathy

Ten rats were given a single intraperitoneal injection of cisplatin (6 mg/kg). Groups of five rats were sacrificed on days 2 and 8, and their kidneys obtained. Urine specimens were collected before sacrifice on day 2 (from 24−48 h), and day 8 (from 168−192 h). The amount of serum creatinine was determined as described above.

Humans

All human samples (kidney tissue, and serum and urine samples) were obtained before treatment, and informed consent was obtained from the patients and/or their parents prior to the tissue being harvested. The usage of the kidney tissues, serum, and urine in the current study was approved by the Committee of Ethics for Life and Genes of the Graduate School of Medical and Dental Sciences, Niigata University, and Niigata National Hospital. Normal sections of kidney tissue were obtained by nephrectomy from patients with renal neoplasia and used to assess the normal (control) levels of cortical/glomerular proteins via Western blotting. Ten renal biopsy tissue samples were obtained from pediatric patients (aged 6–18-years) with chance hematuria and/or proteinuria—5 kidneys were histologically normal and 5 kidneys had been diagnosed with immune-mediated glomerulonephritis (IgA nephropathy; IgAN) involving significant tubulointerstitial injury. Tubular injury was scored semi-quantitatively as described previously [8]. Tubular injury was defined as tubular dilation, tubular atrophy, tubular cast formation, sloughing of tubular epithelial cells, or thickening of tubular basement membrane and scored on a 1–5 scale. Only cortical tubules were included in the following scoring system—0, no tubular injury; 1, <10 % of tubules injured; 2, 10–25 % of tubules injured; 3, 26–50 % of tubules injured; 4, 51–75 % of tubules injured; and 5, >76 % of tubules

injured. All ten subjects were asymptomatic and displayed normal renal function. The kidney tissues were fixed in 4 % paraformaldehyde solution and subjected to histological diagnosis by an expert pathologist (TY) as well as immunohistochemical staining. There were no significant differences between the normal and IgAN patients with regard to their serum creatinine levels, or their serum calcium concentrations (which were corrected for albumin level—[(normal albumin – patient's albumin) × 0.8] + patient's measured total calcium) (data not shown).

Real-time polymerase chain reaction (rats)

Calbindin 1 expression in the rat kidneys was quantified by real-time polymerase chain reaction (PCR). Total kidney cortex RNA was extracted from the ipsilateral and contralateral kidneys of the UUO rats and the kidneys of the anti-GBM GN rats using TRIzol® reagent according to the manufacturer's instructions (Invitrogen, Carlsbad, CA, USA), before being reverse transcribed to cDNA with random hexamers as primers and the Superscript II First-Strand Synthesis System (Invitrogen). PCR reactions were performed with the SYBR Green method using the SYBR Premix Ex Taq II Kit (Takara Bio, Otsu, Japan) on a Thermal Cycler Dice Real-Time System (Takara Bio). Real-time PCR conditions consisted of initial denaturation at 95 °C for 30 s; 40 cycles of denaturation at 95 °C for 5 s, followed by annealing and detection at 60 °C for 30 s; and a confirmation step, in which a dissociation curve was used to ensure the quality of the PCR products. The concentration of each product was calculated using the relative standard curve method. In each sample, the mRNA concentration of calbindin 1 was normalized to that of glyceraldehyde 3-phosphate dehydrogenase. The sequences of the primers used in this study were as follows—calbindin 1, forward 5′-acagtggcttcatagaaactgag-3′ (corresponding to bp 341–363 of human CALB1; hsa: 793, from the KEGG genes database on GenomeNet), reverse 5′-ccacacattttgattccctgga-3′ (corresponding to bp 542–563 of human CALB1); rat GAPDH, forward 5′-taaagggcatcctgggctacact-3′ (corresponding to bp 803–825 of rat GAPDH; rno: 24383, KEGG genes), reverse 5′-ttactccttggaggccatgtagg-3′ (corresponding to bp 980–1002 of rat GAPDH); rat AQP1, forward 5′-ccgagacttaggtggctcag-3′ (corresponding to bp 483–502 of rat AQP1; rno: 25240, KEGG genes), reverse 5′-tcatgcggtctgtaaagtcg-3′ (corresponding to bp 708–727 of rat AQP1); rat AQP2, forward 5′-ccacgctccttttttgtcttc-3′ (corresponding to bp 56–75 of rat AQP2; rno: 25386, KEGG genes), reverse 5′-catagaaggcagctcgaagg-3′ (corresponding to bp 249–268 of rat AQP2); rat AQP3, forward 5′-accatcaacttggcttttgg-3′ (corresponding to bp 172–191 of rat AQP3; rno: 65133, KEGG genes), reverse 5′-aggcccagattgcatcatag-3′ (corresponding to bp 369–388 of rat AQP3).

Immunohistochemistry (rats and humans)

Sections (3-μm-thick) of kidney tissue that had been fixed in 4 % paraformaldehyde and then embedded in paraffin were dewaxed and used for periodic acid-Schiff (PAS) staining and the immunohistochemical detection of calbindin 1. After being heated for 20 min in a microwave in 0.01 M sodium citrate buffer (pH 6.0), the sections were incubated with normal goat serum (1:20 dilution) for 20 min to block non-specific reactions, before being incubated overnight at 4 °C with monoclonal antibody against calbindin-D-28k (Sigma Aldrich, Tokyo, Japan, 1:1000 dilution). Antibodies for AQP1 [mouse anti-human AQP1 mAb (B11), Santa Cruz, CA, 1:200 dilution] and AQP2 [rabbit anti-human AQP2 pAb (N-terminus), LifeSpan BioSciences, WA, 1:25 dilution] were also used to compare protein localization in the nephron segments. After being washed in phosphate-buffered saline (PBS), the sections were treated with horseradish peroxidase-conjugated goat anti-mouse (for Calbindin 1 and AQP1) or anti-rabbit (for AQP2) immunoglobulins (EnVision) for 30 min. The peroxidase-conjugated reaction products were visualized by using 0.5 mg/ml of 3′-diaminobenzidine tetrahydrochloride−0.01 % hydrogen peroxide as a substrate. In the immunohistochemical examinations, staining intensity of calbindin 1 was evaluated semi-quantitatively by an investigator who was blinded to the underlying diagnosis. The combined intensity and distribution of the immunostaining in the distal tubule cells was determined on a scale of 0–3 (0, absent; 1, weak staining; 2, moderate staining; and 3, strong staining). The mean value of at least 5 fields of view observed at a magnification of 100× was calculated for each individual.

Western blotting

Spot urine samples from human subjects, and 24-h urine samples from rats were used for Western blotting. The urine samples were centrifuged to remove any sediment, and urinary proteins were extracted using the Proteospin Urine Protein Concentration Micro Kit, according to the manufacturer's instructions (Norgen Biotek Corporation, Thorold, ON, Canada). One to 10 ml of human urine samples were used depending on the protein concentration. Next, 20 μl (containing approximately 50–200 μg protein) of concentrated urine samples were loaded onto 15 % sodium dodecyl sulfate–polyacrylamide gel, and the resultant bands were transferred to polyvinylidene difluoride membranes. The membranes were preincubated with 5 % non-fat milk in PBS containing 0.05 % Tween 20 and then incubated overnight with mouse monoclonal antibody against calbindin-D-28k (Sigma Aldrich, 1:10,000 dilution), before being washed in PBS containing 0.05 % Tween. Finally, they were incubated with a 1:1,000 diluted

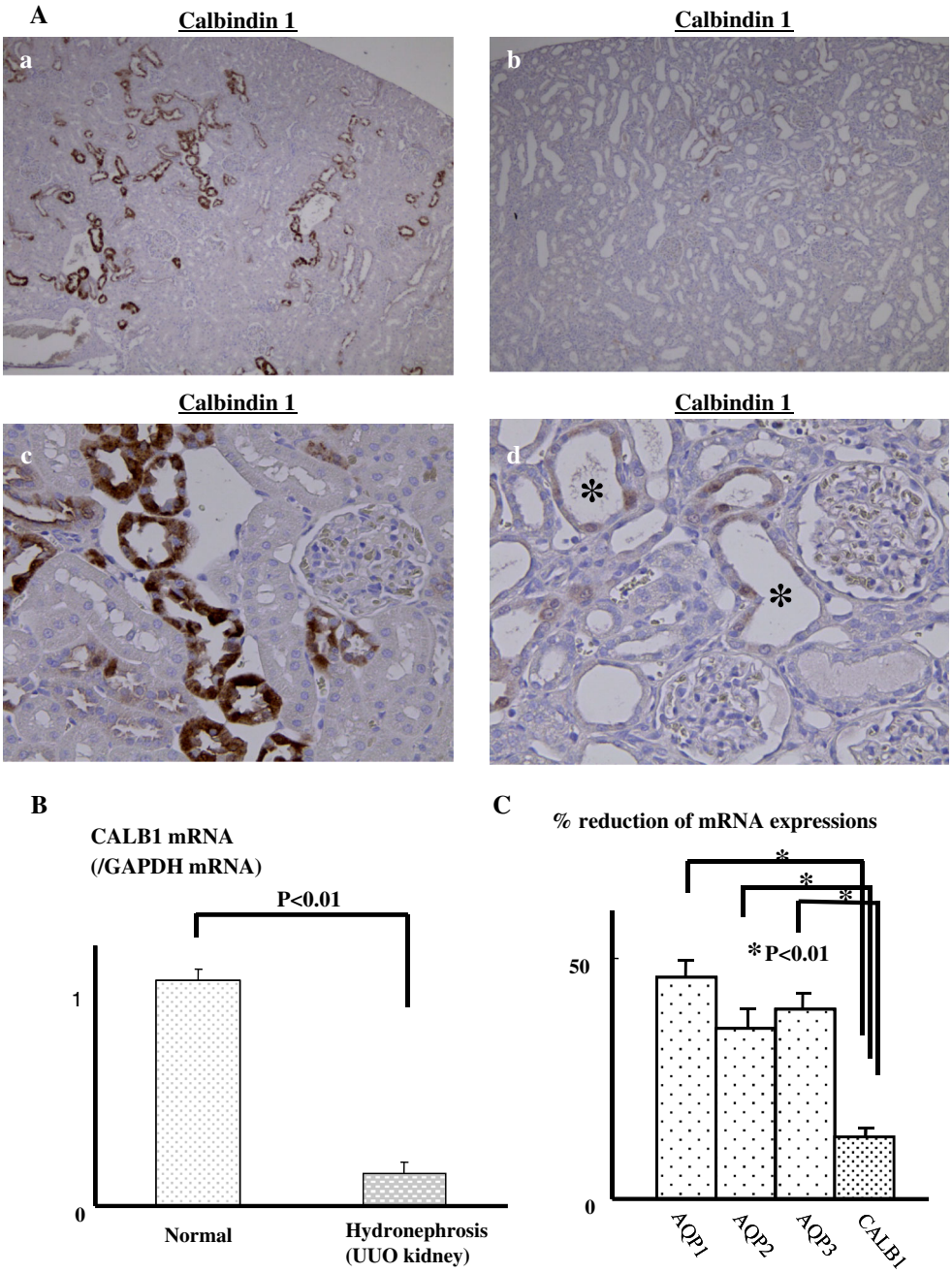
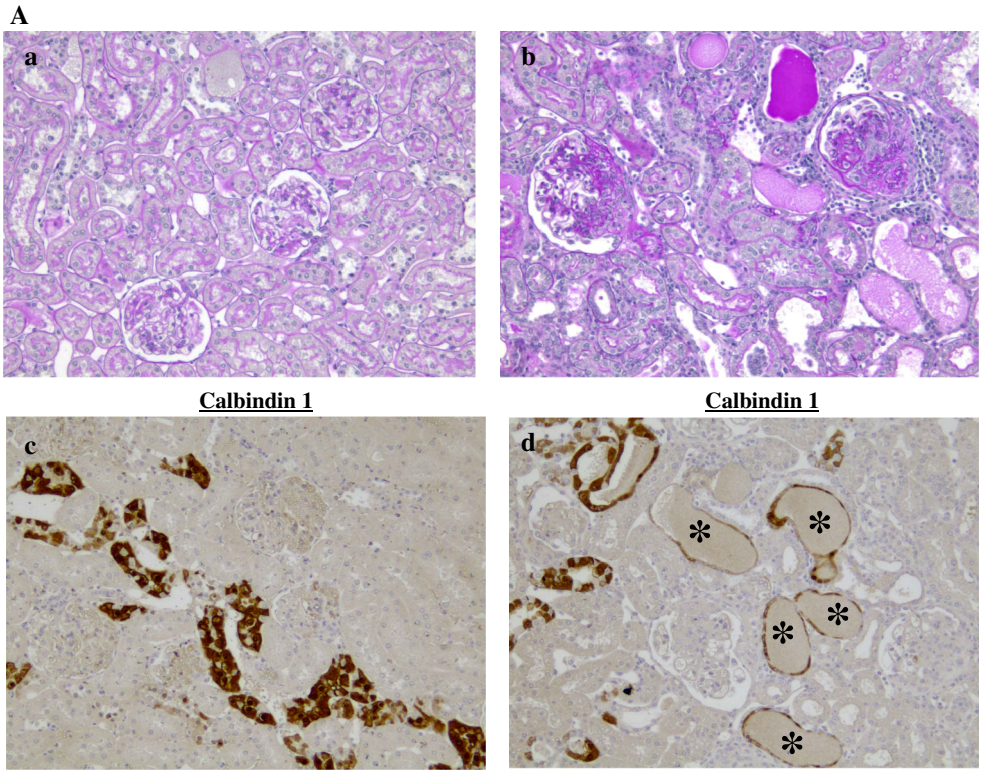


Fig. 1 **A** Calbindin 1 protein expression was decreased in the dilated distal tubules of the kidney cortices of UUO rats. In an immunohistochemical examination, calbindin 1 protein was clearly detected in the distal tubular cells of the contralateral (normal) kidney cortices (**a**, **c**), whereas its expression was markedly decreased in the ipsilateral hydronephrotic UUO kidney cortices, especially in distal tubules with dilated lumens (*asterisks*) (**b**, **d**). Original magnification: **a**, **b**: ×30; **c**, **d**: ×150. **B** Calbindin 1 mRNA expression was decreased in the kidney cortices of UUO rats. In real-time PCR, the cortices of the hydronephrotic UUO kidneys displayed significantly decreased calbindin 1 mRNA expression compared with the normal contralateral kidney cortices ($p < 0.01$). **C** Calbindin 1 mRNA was more prominently decreased than those of AQP 1, 2, and 3 in the kidney cortices of UUO rats. The mRNA expressions of AQP 1, 2, and 3 were also shown to be decreased in the ipsilateral kidney cortices of UUO rats compared with their contralateral (normal) kidney cortices, but the % reduction of the calbindin 1 mRNA was more pronounced than those of AQP 1, 2, and 3

goat anti-mouse immunoglobulin conjugated with a peroxidase-labeled dextran polymer (mouse EnVision; DAKO, Carpinteria, CA, USA), and the resultant immunoreactivity was visualized using the ECL plus Western blotting detection system (GE Healthcare, Buckinghamshire, UK). Densitometric analysis was performed using the NIH image software package (version 1.62, NIH), and the results were corrected for urinary creatinine concentration. Diluted (20 µl) normal human serum protein samples (1:14, containing approximately 100 µg protein), 2 µl normal human kidney glomerular protein samples (containing approximately 10 µg protein), and 2 µl normal human kidney cortical protein samples (containing approximately 10 µg protein) were also loaded for comparison. For rat urine samples, 1 ml from each animal was used; 20 µl (containing approximately 200–2,000 µg protein) of concentrated urine samples were loaded onto gel for Western blotting.

Statistical analysis

Results are expressed as the mean ± SE. Mann–Whitney's *U* test was used for comparisons between two groups. Statistical significance was defined as $p < 0.05$.



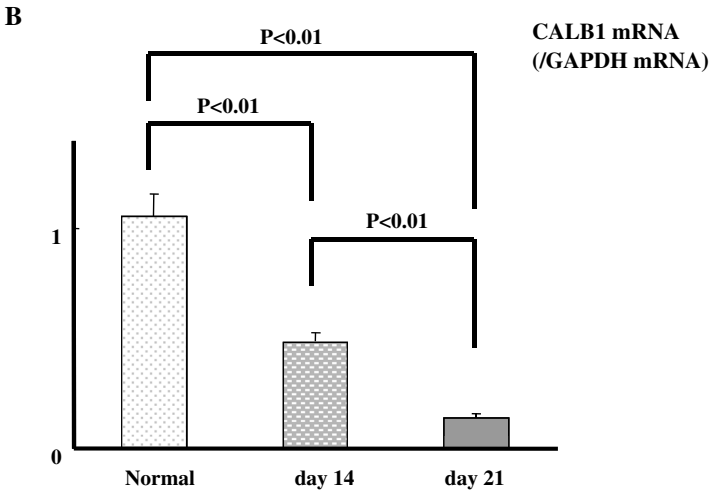
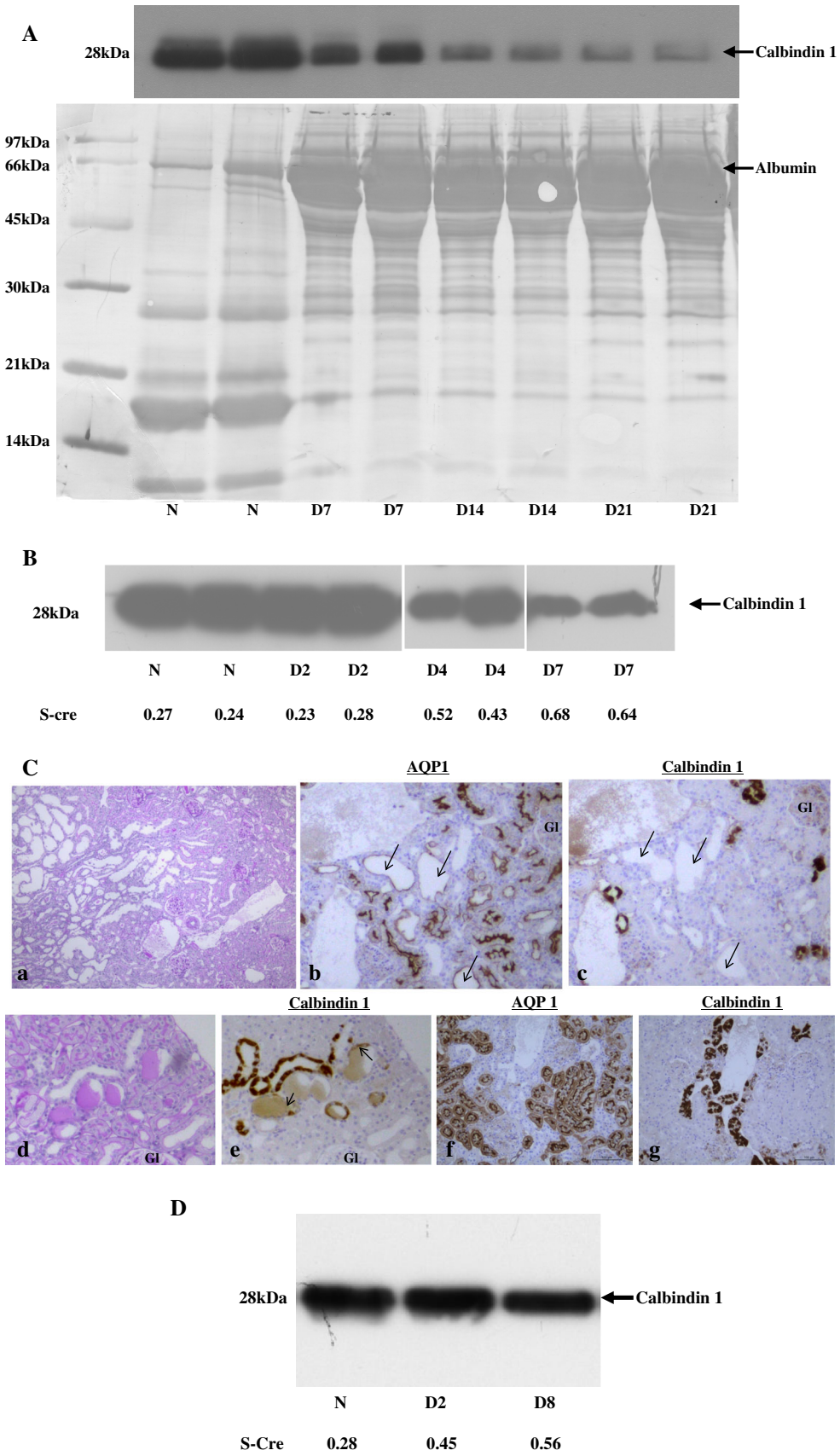


Fig. 2 **A** Calbindin 1 protein expression was decreased in the dilated distal tubules of the kidney cortices of anti-GBM GN rats. In an immunohistochemical examination, calbindin 1 protein was clearly detected in the distal tubular cells of the normal kidney cortices (**a**, **c**), whereas its expression was decreased in the anti-GBM GN kidney cortices, especially in distal tubules with dilated lumens (*asterisks*) (**b**, **d**). Original magnification: **a–d**: ×100. **B** Calbindin 1 mRNA expression was significantly decreased in the kidney cortices of anti-GBM GN rats. In real-time PCR, the kidney cortices of the anti-GBM GN rats displayed significantly decreased calbindin 1 mRNA expression compared with the cortices of the normal rats

Results

Calbindin 1 protein and mRNA expression in the kidney cortices of the UUO rats

Our immunohistochemical examination revealed that calbindin 1 protein expression was significantly decreased in the dilated distal tubules of the ipsilateral kidney cortices of the UUO rats (Fig. 1Ab, d), but was easily detected in their contralateral (normal) kidney cortices (Fig. 1Aa, c) (mean immunostaining values 0.60 ± 0.11 vs 2.44 ± 0.04; $p < 0.01$). In real-time PCR, calbindin 1 mRNA expression was found to be significantly decreased in the ipsilateral kidney cortices of the UUO rats compared with their contralateral (normal) kidney cortices (0.15 ± 0.08 vs 1.04 ± 0.05, $p < 0.01$) (Fig. 1B). The mRNA expressions of AQP 1, 2, and 3 were also shown to be decreased in the ipsilateral kidney cortices of the UUO rats compared with their contralateral (normal) kidney cortices, but the % reduction of the calbindin 1 mRNA was more pronounced than those of AQP 1, 2, and 3 (Fig. 1C).


A
28kDa
Calbindin 1
97kDa
66kDa
45kDa
30kDa
21kDa
14kDa
Albumin
N N D7 D7 D14 D14 D21 D21
B
28kDa
Calbindin 1
N N D2 D2 D4 D4 D7 D7
S-cre 0.27 0.24 0.23 0.28 0.52 0.43 0.68 0.64
C
AQP1
Calbindin 1
GI
a
b
c
Calbindin 1
AQP 1
Calbindin 1
d
e
f
g
D
28kDa
Calbindin 1
N D2 D8
S-Cre 0.28 0.45 0.56

◀**Fig. 3** **A** Urinary calbindin 1 protein concentration was decreased in the proteinuric anti-GBM GN rats. Western blotting demonstrated that the urinary calbindin 1 concentration (*arrow*, 28 kDa) was decreased in proteinuric anti-GBM GN rats in comparison with the normal rats, even though the former rats exhibited increased levels of urinary albumin (*arrow*, 67 kDa). *Upper photograph*: Western blotting; *lower photograph*: Coomassie blue staining. *Lanes 1, 2*: normal (*N*), *lanes 3, 4*: anti-GBM GN day 7 (*D7*). *Lanes 5, 6*: anti-GBM GN day 14 (*D14*). *Lanes 7, 8*: anti-GBM GN day 21 (*D21*). The approximate amount of loading protein: *lane 1, 2*: 200 μg, *lane 3–8*: 2,000 μg. **B** Decreased urinary calbindin 1 protein levels were demonstrated on day 4 in the anti-GBM GN rats, and coincided with serum creatinine elevation. Decreased urinary calbindin 1 protein levels were demonstrated on day 4 in the anti-GBM GN rats, when serum creatinine levels were significantly elevated. Their earlier decrease in comparison to serum creatinine elevation could not be demonstrated on day 2 of this model. *Lanes 1, 2*: normal (*N*), *lanes 3, 4:* anti-GBM GN day 2 (*D2*). *Lanes 5, 6*: anti-GBM GN day 4 (*D4*). *Lanes 7, 8*: anti-GBM GN day 7 (*D7*). **C** Calbindin 1 protein expression was slightly decreased in the kidneys of cisplatin nephropathy rats. Pathological changes in the kidneys were conspicuously observed in the deep cortices and the outer stripe of the outer medullas (**a**). *Right*; the superficial cortex, *left*; the deep cortex–the outer stripe of the outer medulla. The sequential serial sections on day 8 of the cisplatin nephropathy for AQP1 (**b**, proximal tubule cells marker) and for calbindin 1 (**c**) demonstrated that the most of the injured tubules with dilation in the deep cortices were AQP1-positive proximal tubules (*arrows*) (*Gl*; glomerulus). Dilated tubules with protein casts were rarely observed in the superficial cortices (**d**), some of these were shown as distal tubules with decreased calbindin 1 expression (**e**) (*arrows*). **d, e** sequential serial sections (*Gl*; glomerulus). There were no overlaps of AQP1-positive cells (**f**) and calbindin 1-positive cells (**g**) in normal rat kidneys (sequential serial sections). Original magnification: **a**: ×20, **b**, **c**: ×50; **d, e**: ×100; **f, g**: ×50. **D** Urinary calbindin 1 protein was slightly decreased on day 8 in the cisplatin nephropathy rats. Urinary calbindin 1 protein levels were not decreased on day 2, but slightly decreased on day 8 in the cisplatin nephropathy rats, although serum creatinine levels were significantly elevated as early as day 2. *Lane 1*: normal (*N*), *lane 2*: cisplatin day 2 (*D2*), *lane 3*: cisplatin day 8 (*D8*)

Calbindin 1 protein and mRNA expression in the kidney cortices of anti-GBM GN rats

In comparison with normal kidneys (Fig. 2Aa), crescentic glomerulonephritis involving interstitial mononuclear cell infiltration and tubular dilation was detected on day 14 after anti-GBM antibody administration (Fig. 2Ab), when moderately elevated serum creatinine levels were also observed [0.28 ± 0.01 mg/dl on day 0, 0.62 ± 0.04 mg/dl (day 14, $p < 0.01$ vs normal), and 0.68 ± 0.04 mg/dl (day 21, $p < 0.01$ vs normal; ns vs day 14)]. Immunohistochemistry demonstrated that calbindin 1 protein expression was decreased in the dilated distal tubules of the kidney cortices of anti-GBM GN rats (Fig. 2Ad) but it was clearly detected in their normal kidney cortices (Fig. 2Ac) [mean immunostaining values 2.40 ± 0.11 (normal), 1.60 ± 0.13 (day 14, $p < 0.01$ vs normal) and 1.52 ± 0.12 (day 21, $p < 0.01$ vs normal; ns vs day 14)]. Real-time PCR revealed that calbindin 1 mRNA expression was significantly decreased in the anti-GBM GN kidney cortices compared with the normal kidney cortices (Fig. 2B) [1.06 ± 0.10 (normal), 0.48 ± 0.04 (day 14, $p < 0.01$ vs normal) and 0.14 ± 0.02 (day 21, $p < 0.01$ vs normal; $p < 0.01$ vs day 14)].

Urinary calbindin 1 protein expression in normal and anti-GBM GN rats

Western blotting demonstrated that the anti-GBM GN rats displayed a lower urinary calbindin 1 protein level than the normal rats (Fig. 3A) [mean expression values (densitometry/urinary creatinine) 5.23 ± 0.17 (normal), 2.58 ± 0.17 (day 7, $p < 0.01$ vs normal), 1.50 ± 0.26 (day 14, $p < 0.01$ vs normal; $p < 0.01$ vs day 7), and 1.05 ± 0.20 (day 21, $p < 0.01$ vs normal; ns vs day 14). The urinary calcium excretion (/creatinine) was also shown to be significantly enhanced in the anti-GBM GN rats compared with the normal rats [0.12 ± 0.01 (normal), 0.21 ± 0.03 (day 7, $p < 0.01$ vs normal), 0.25 ± 0.05 (day 14, $p < 0.01$ vs normal; ns vs day 7), and 0.19 ± 0.03 (day 21, $p < 0.01$ vs normal; ns vs day 14)].

Urinary calbindin 1 protein levels of the earlier stage of this model (days 2 and 4) were also studied (Fig. 3B). Urinary calbindin 1 protein levels were shown to be decreased on day 4—5.67 ± 0.38 (day 2, ns vs normal), and 3.50 ± 0.25 (day 4, $p < 0.01$ vs normal; $p < 0.01$ vs day 2). Serum creatinine levels were shown to be increased on day 4—0.27 ± 0.02 mg/dl (day 2, ns vs normal), and 0.49 ± 0.04 mg/dl (day 4, $p < 0.01$ vs normal; $p < 0.01$ vs day 2). Decreased urinary calbindin 1 protein levels were demonstrated on day 4 in the anti-GBM GN rats, which coincided with elevated serum creatinine levels. The earlier decrease of urinary calbindin 1 protein in comparison to serum creatinine elevation could not be demonstrated.

Renal and urinary calbindin 1 protein expression in the cisplatin nephropathy rats

The main pathological changes in the kidney were the tubules in the deep cortices and the outer stripe of the outer medullas, in the form of flattened tubular cells with diluted lumen (Fig. 3Ca). The sequential serial sections on day 8 for AQP1 (Fig. 3Cb, proximal tubule cells marker) and for calbindin 1 (Fig. 3Cc) demonstrated that most of the injured tubules with dilation in the deep cortices were AQP1-positive proximal tubules (arrows). In contrast, tubules in the superficial cortices showed little changes; dilated tubules with protein cast were rarely observed, and some of these were shown as distal tubules with faint calbindin 1 expression (Fig. 3Cd, e) (arrows).

Urinary calbindin 1 protein levels were not decreased on day 2, but were slightly decreased on day 8 (Fig. 3D), although serum creatinine levels were significantly elevated on day 2. Urinary calbindin 1 protein

levels were 5.23 ± 0.17 (normal), 5.49 ± 0.30 (day 2, ns vs normal), 4.43 ± 0.16 (day 8, $p < 0.05$ vs normal; $p < 0.05$ vs day 2), and serum creatinine levels were 0.28 ± 0.02 (normal), 0.45 ± 0.02 (day 2, $p < 0.01$ vs normal), 0.56 ± 0.5 (day 8, $p < 0.01$ vs normal; ns vs day 2).

Calbindin 1 protein expression in the distal tubules of human IgAN patients

All 5 IgAN kidneys were shown to have mild but significant tubulointerstitial injuries (tubular injury score 1–2). Immunohistochemistry detected calbindin 1 protein in the distal tubule cells of normal human kidney cortices (Fig. 4a), but its expression was decreased in the kidney cortices of the IgAN patients (Fig. 4b); this was similarly observed in the rat kidneys with distal nephron injuries. The mean immunostaining values of calbindin 1 were 2.36 ± 0.12 (normal) versus 1.52 ± 0.16 (IgAN, $p < 0.01$). The protein expressions of AQP2 (collecting tubule cells marker) were not obviously decreased in the kidney cortices of IgAN patients (Fig. 4d) compared with normal human kidney cortices (Fig. 4c), as shown by immunohistochemical examination. The sequential serial sections of the kidneys of IgAN patients for AQP1 (Fig. 4e, proximal tubule cells marker) and for calbindin 1 (Fig. 4f) demonstrated that some of the dilated tubules with protein casts (asterisk) were not AQP1-positive proximal tubules. This should be called a distal tubule, since only a faint staining signal of calbindin 1 was observed (arrow).

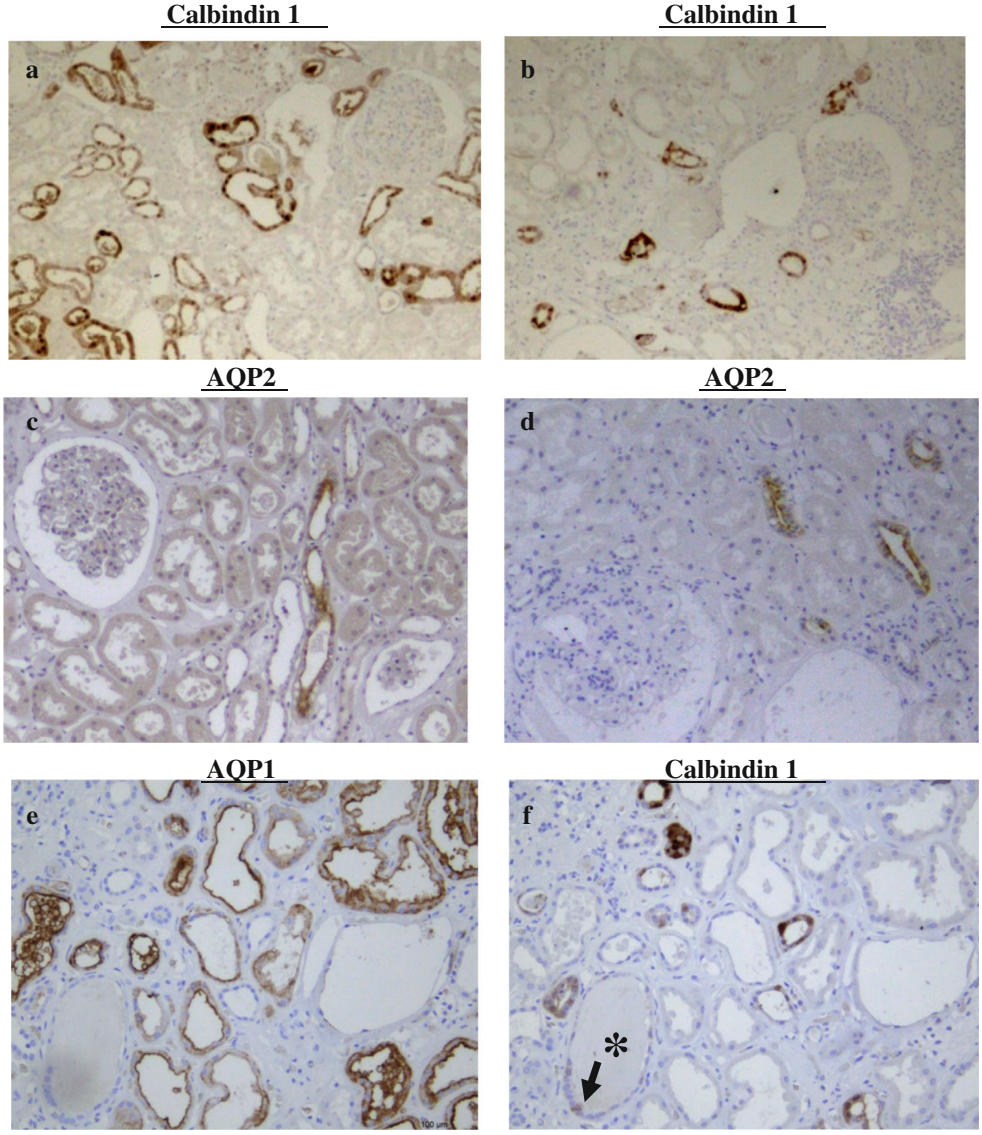


Fig. 4 Calbindin 1 protein expression was decreased in the dilated distal tubules of human glomerulonephritis kidney cortices. In an immunohistochemical examination, calbindin 1 protein was clearly detected in the distal tubule cells of the normal kidney cortices (**a**), whereas its expression was decreased in the kidney cortices of the glomerulonephritis patients (**b**). The protein expressions of AQP2 (collecting tubule cells marker) were not obviously decreased in the kidney cortices of IgAN patients (**d**) compared with normal human kidney cortices (**c**). The sequential serial sections of the kidneys of IgAN patients for AQP1 (**e,** proximal tubule cells marker) and for calbindin 1 (**f**) demonstrated that dilated tubules with protein casts (*asterisk*) were not AQP1-positive proximal tubules. This should be called a distal tubule as only a faint staining signal of calbindin 1 was observed (*arrow*). Original magnification: **a, b**: ×80; **c–f**: ×150

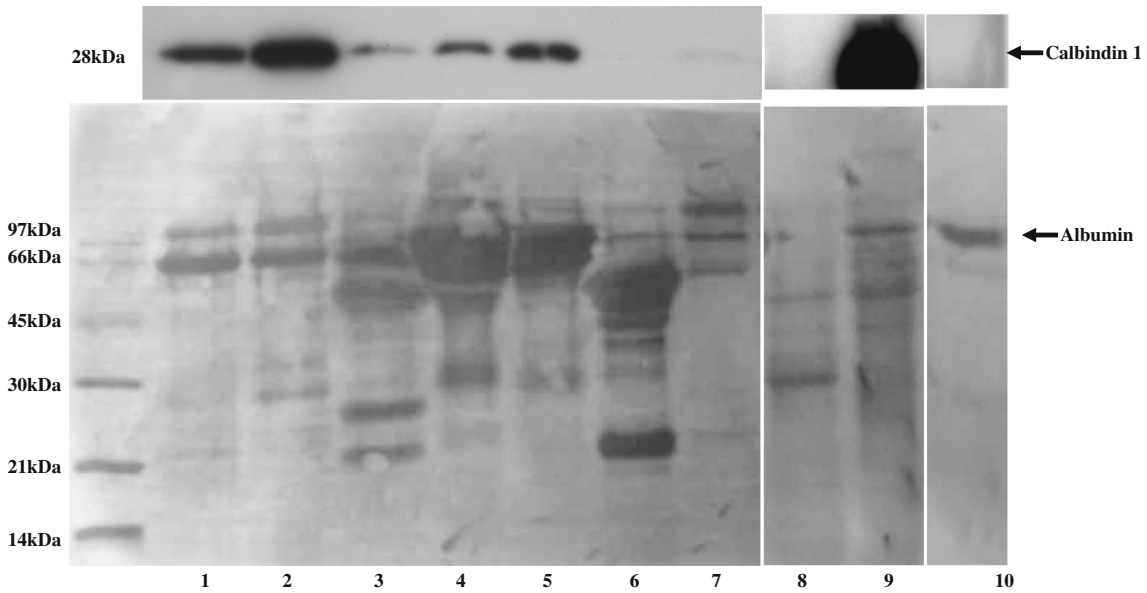


Fig. 5 Calbindin 1 protein expression in human urine samples. Western blotting demonstrated that the urinary calbindin 1 protein levels (*arrow*, 28 kDa) of IgAN patients were not significantly lower than those of the normal subjects. However, some IgAN patients displayed low urinary calbindin 1 protein excretion compared with the normal subjects, even though their urinary albumin levels (*arrow*, 67 kDa) were increased (*lane 6*). In one patient, the amount of excreted calbindin 1 protein (densitometry/urinary creatinine) was higher (*lane 5*) than the mean value for normal human urine, which was different from the impression of Western blotting gel band image. *Upper photograph*: Western blotting, *lower photograph*: Coomassie blue staining. *Lanes 1, 2*: urine from human subjects with normal kidney histology. *Lanes 3–7*: urine from IgAN patients. *Lane 8*: normal human kidney glomerulus. *Lane 9*: normal human kidney cortex. *Lane 10*: normal human serum samples. The approximate amount of loading protein: *lane 1, 2*: 50 μg, *lane 3–6*: 200 μg, *lane 7*: 50 μg, *lane 8* 10 μg, *lane 9*: 10 μg and *lane 10*: 100 μg. Degraded albumin bands were detected in the urine of some of the IgAN patients (*lanes 3, 6, 7*). As a control, calbindin 1 protein was strongly detected in normal human kidney cortex protein samples (*lane 9*), but not in normal human kidney glomerular protein samples (*lane 8*) or serum samples (*lane 10*)

Urinary calbindin 1 protein expression in normal humans and IgAN patients

In Western blotting, some of the IgAN patients displayed lower urinary calbindin 1 protein levels than the normal humans; however, the overall difference between the groups was not significant (Fig. 5) [mean expression values (densitometry/urinary creatinine)—1.16 ± 0.10 (normal) and 0.86 ± 0.32 (IgAN) (ns)]. Intense calbindin 1 protein expression was detected in the normal kidney cortical protein samples as a positive control, whereas no bands were detected in the normal glomerular protein samples or the serum protein samples. Furthermore, the urinary calcium excretion (/creatinine) was not significantly enhanced in the IgAN patients compared with the normal humans [0.16 ± 0.04 (normal) and 0.18 ± 0.10 (IgAN) (ns)].

Discussion

Calbindin 1, which plays a role in renal tubular Ca^{2+} reabsorption, is a cytosolic vitamin D-dependent Ca^{2+}-binding protein [9]. Three processes of the following (1)–(3) are involved in renal tubular Ca^{2+} reabsorption—(1) the entry of Ca^{2+} into the cells across the luminal membrane via the calcium channels TRPV5 and TRPV6, (2) the transportation of Ca^{2+} through the cytoplasm via calbindin 1, and (3) its extrusion across the basolateral membrane via the PMCA1b calcium pump and the NCX1 exchanger [10]. Calbindin 1 facilitates Ca^{2+} diffusion from the luminal entry site to the basolateral extrusion site and protects cells against toxically high Ca^{2+} levels by buffering cytosolic Ca^{2+} levels [10]. As reported in rodents [4], it has been published that calbindin 1 in normal humans is localized in the distal tubule cells and the proximal parts of the collecting duct cells (http://www.proteinatlas.org/), and it was also noted that significant amounts of calbindin 1 protein are excreted in human urine (http://www.hkupp.org/) [11]. Calbindin 1 was demonstrated to increase calcium uptake in the distal tubules in vitro [12], and the urinary calcium/creatinine ratios of calbindin 1 knockout mice were three-fold higher than those of the wild-type mice [13]. Increased renal calbindin 1 expression was reported in diabetic mice, which was considered to be a compensatory response to

diabetes-associated hypercalciuria [14]. In contrast, decreased renal calbindin 1 expression was reported in rats/mice that had been treated with calcineurin inhibitors (cyclosporine A or FK-506) [13, 15–17] due to impairment of the vitamin D activation pathway [17, 18]. The present study aims to examine renal and/or urinary calbindin 1 levels in the early stages of primary kidney diseases involving tubulointerstitial injury, such as hydronephrosis and immune-mediated glomerulonephritis.

In the rat UUO model, calbindin 1 protein expression was markedly decreased in the dilated distal nephron segments, and this result was supported by the detection of significantly decreased mRNA expression levels in the hydronephrotic kidney cortices. In mice UUO ipsilateral kidneys, significant reduction of calbindin 1 mRNA has already been reported [19], but the protein expression and localization in the kidneys have not been investigated. It was reported that in UUO, the distal nephron segment tends to dilate more rapidly than the proximal nephron segment, and the dilated tubular epithelial cells tend to suffer apoptosis [20]. The distal tubule preferential injury in the kidney cortices of UUO rats was confirmed in the present study by the marked decrease of calbindin 1 mRNA, in contrast to the moderate decreases of AQP1 (proximal tubule), AQP2 (connecting tubule–collecting duct) and AQP3 (collecting duct). The decreased expression of calbindin 1 in the distal nephron can be explained by morphological alterations in renal architecture caused by pressure increases, such as dilation of the tubular lumen, and the flattening and atrophy of epithelial cells. The decreased AQP2 expression was also observed in the collecting duct of the obstructive kidneys [21, 22]. Impaired calbindin 1 synthesis can be explained by a general disturbance of cellular function in the distal nephron segment. In the healthy state, calbindin 1 might be excreted in urine in a similar manner to the way in which AQP2 is disposed of by exocytosis from the luminal side of the cell membrane [23]. Although calbindin 1 contributes to cytoplasmic Ca^{2+} transport, it was reported to be predominantly localized along the luminal side of renal cells, where the calcium channel TRPV-5 is expressed [10]. Since urine sample collection was not possible from the hydronephrotic rat kidneys, as the connecting ureters had been ligated, another rat model with tubulointerstitial injury was studied in order to clarify whether decreased urinary excretion of calbindin 1 is indicative of decreased renal calbindin 1 expression.

In the rat anti-GBM GN model, calbindin 1 mRNA expression in the kidney cortices was found to be significantly decreased on day 14 and was reduced even further on day 21, although the difference in protein expression between days 14 and 21 was not significant according to our immunohistochemical examination. In Western blotting, urinary calbindin 1 excretion was demonstrated to gradually decrease from the levels observed in the normal rats to those observed at 21 days after the administration of the anti-GBM antibody, although the difference between days 14 and 21 was not significant. This might have been because calbindin 1 protein remained in the dilated distal tubule cells after its transcription was impaired by cell injury or because of the difficulty of evaluating small differences in protein expression (between days 14 and 21) using antibody. Urinary calcium excretion was also shown to be significantly enhanced during the course of this model, which is consistent with the notion that urinary calbindin 1 plays a role in renal tubular Ca^{2+} reabsorption.

It may be reasonable that more tubular specific injury models are employed in order to study tubular protein expression. However, with regard to calbindin 1 protein, the localization has already been established exclusively in the distal tubule cells and the proximal parts of the collecting duct cells. Moreover, no glomerular protein expression of calbindin 1 was confirmed in the present study by immunostaining and Western blotting. It is presumed that the decreased expressions of calbindin 1 in anti-GBM GN kidney and urine were due to distal tubule cell injury, rather than glomerular cell injury, although the anti-GBM GN is a model of primary glomerulonephritis with extensive tubulointerstitial injury.

Cisplatin nephropathy was investigated as a tubular specific injury model. Urinary calbindin 1 protein levels were shown to be significantly decreased but only slightly on day 8; the slight decrease might be as a result of the slight pathological changes in the cortical distal tubules in this model. The protein levels were also shown not to be decreased on day 2, although serum creatinine levels were significantly elevated on day 2. The creatinine elevation on day 2 may be due to proximal dominant tubular injury. These results from anti-GBM GN and cisplatin nephropathy suggest that decreased urinary calbindin 1 protein levels are useful to indicate the portion of nephron segment injury as distal nephron, although the earlier decrease of urinary calbindin 1 protein levels in comparison to elevation of serum creatinine levels could not be demonstrated.

As shown by Coomassie blue staining (Fig. 3A), the overall amount of urinary protein, as indicated by the ~67 kDa band size representing albumin excretion, was markedly increased in anti-GBM GN rats compared with the normal rats, whereas their calbindin 1 protein excretion was decreased in Western blotting. The urinary protein contents of the normal and proteinuric rats differed, i.e., a protein with a lower molecular weight than albumin (<20 kDa), which was considered to be alpha-2u-globulin (α_{2U}-G), a close homolog of mouse major urinary protein (MUP) that accounts for 30–50 % of total excreted protein in adult male rat urine, was the main protein component of

normal rat urine [24]. The reason why densities of these low-molecular-weight proteins were very low in anti-GBM GN rats was not clear; nevertheless, a similar observation had been reported in mice urine where the intense bands of MUP diminished after induction of proteinuria and tubular injury [25]. The protein expression of α_{2U}-G or MUP in urine was observed only in rodents, whereas urinary calbindin 1 can be detected both in rats and humans [11]. Since decreased urinary calbindin 1 protein excretion was observed in the rat model, it is presumed that urinary calbindin 1 protein excretion might also be decreased in human renal disease, correlated with decreased renal calbindin 1 protein expression.

In humans, calbindin 1 protein was clearly detected in the distal tubular cells of normal subjects, whereas its expression was decreased in the kidneys of IgAN patients with tubulointerstitial injuries, especially in dilated distal tubules, as found in rat kidneys. Some of the IgAN patients exhibited quite low levels of urinary calbindin 1 protein excretion (lane 6) compared with the normal subjects, although the overall difference between the two groups was not significant. There were no significant correlations between the degree of tubular injury and urinary calbindin 1 protein levels. This may be because all 5 of the IgAN kidneys were shown to have mild tubulointerstitial injuries (tubular injury score 1–2). The reason for the remarkably decreased urinary calbindin 1 in one patient (lane 7) may be due to the smaller amount of loaded protein than the other patients (lanes 4 and 5), or the patient may have severe distal tubule injuries in areas of kidney cortices which were not involved in the biopsy specimen. The degree of urinary calbindin 1 excretion might change according to the period of time from disease onset, age, sex, serum calcium concentration, or food intake. In this study, young subjects (aged between 6 and 18 years) were enrolled, since younger individuals are considered to possess fewer chronic confounding variables such as smoking, obesity, diabetes, cardiovascular diseases, hypertension, and chronic medication use, than older people [26]. In addition, no subjects with hypercalcemia were enrolled in this study. Further studies with an increased sample size and patients with a higher tubular injury score are needed.

In conclusion, it was demonstrated that decreased urinary calbindin 1 levels are indicative of decreased calbindin 1 expression in the kidneys due to distal nephron segment injury in both rats and some humans. Recently, several proteins such as NGAL, L-FABP, KIM-1, cystatin C, and IL-18 have been proposed as urinary biomarkers of kidney injury, and their urinary concentrations are usually elevated in comparison with those of normal subjects. The urinary excretion of calbindin 1 was shown to be uniquely decreased in patients with distal kidney nephron segment injuries. Furthermore, urinary calbindin 1 meets some of the criteria for ideal biomarkers, i.e., it originates from injured distal nephron cells and plays a role in renal tubular Ca^{2+} reabsorption, although the earlier decrease of urinary calbindin 1 protein in comparison to elevation of serum creatinine levels could not be demonstrated. Calbindin 1 might be a useful urinary biomarker for identifying kidney distal nephron segment injury. Other urinary proteins of kidney distal tubule origin should be explored next, the expressions of which are altered earlier than elevation of serum creatinine levels.

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Conflict of interest All the authors have declared no competing interest.

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