Significance of Urinary Full-Length and Ectodomain Forms of Megalin in Patients With Type 2 Diabetes

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OBJECTIVE—Megalin, an endocytic receptor in proximal tubule cells, is involved in the mechanisms of albuminuria in diabetic nephropathy (DN). To develop efficient novel biomarkers associated with the pathogenesis of DN, we investigated urinary megalin excretion in type 2 diabetes.

RESEARCH DESIGN AND METHODS—Sandwich enzyme-linked immunosorbent assay systems were established with monoclonal antibodies against the NH_2 (amino [A]-megalin assay) and COOH (C-megalin assay) termini of megalin to analyze urinary forms of megalin in 68 patients with type 2 diabetes.

RESULTS—The A-megalin assay mainly detected a megalin ectodomain form in the soluble urinary fraction, whereas the C-megalin assay identified a full-length form in both soluble and insoluble fractions. Urinary C-megalin levels were significantly high in patients with normoalbuminuria, were elevated in line with increased albuminuria, and showed a better association with estimated glomerular filtration rate (eGFR) (<60 mL/min/1.73 m^2) than did urinary albumin. In contrast, urinary A-megalin levels were increased in patients with normo- and microalbuminuria but not in those with macroalbuminuria. Urinary C-megalin levels were also positively associated with plasma inorganic phosphate and negatively with hemoglobin levels in those showing no features of bleeding and not taking vitamin D analogs, phosphate binders, or erythropoiesis-stimulating agents.

CONCLUSIONS—Urinary full-length megalin excretion as measured by the C-megalin assay is well associated with reduced eGFR and linked to the severity of DN, phosphate dysregulation, and anemia, whereas urinary excretion of megalin ectodomain as measured by the A-megalin assay may be associated with distinctive mechanisms of earlier DN in type 2 diabetes.

D iabetic nephropathy (DN) is a leading cause of chronic kidney disease (CKD) and end-stage kidney disease and is highly associated with the development of cardiovascular disease (CVD) (1). Albuminuria has been generally used as a biomarker for DN, but its clinical relevance as a surrogate outcome in CKD has not been confirmed (2).

Phenotypic changes in proximal tubule cells (PTCs) are initial signs of DN (3,4), and consequent tubulointerstitial damage plays a central role in the progression to end-stage kidney disease (5). Furthermore, PTC dysfunction is likely to be involved in the development of CVD in diabetes (6). However, efficient biomarkers associated with the mechanism of PTC dysfunction in diabetes have not been established.

Megalin is a large (~600 kDa) glycoprotein member of the LDL receptor family (7,8) that is expressed at the apical membranes of PTCs (9). Its extracellular region consists of 4,398 amino acids (in humans) and consists of four clusters of cysteine-rich repeats, the so-called ligand-binding domains (LBDs) 1–4. The extracellular region is followed by a single transmembrane segment and a cytoplasmic tail (CT) of 209 amino acids. Megalin plays a critical role in the reabsorption of glomerular-filtered substances, including albumin and low-molecular-weight proteins (9). In addition, megalin may be involved in signal transduction via regulated intramembrane proteolysis (RIP) in PTCs (10,11).

Impairment of megalin function has been suggested even in the early stages of diabetes in animal models (12,13) and patients (14–16). Therefore, dysfunction of megalin is likely to be associated with the development of proteinuria/albuminuria in diabetic patients.

Megalin is known to be shed into urine, as was first shown by immunoblotting of human urine samples (17). Increased urinary megalin excretion, evaluated by gel-based liquid chromatography–mass spectrometry, was suggested in microalbuminuric patients with type 1 diabetes (18). However, more qualitative and quantitative analyses of urinary megalin excretion are required to clarify its association with the pathogenesis of DN and its usefulness as a biomarker.

In this study, we therefore developed enzyme-linked immunosorbent assay (ELISA) systems to investigate urinary megalin forms and quantities and evaluate their clinical significance as novel biomarkers for DN and related disorders in patients with type 2 diabetes.

RESEARCH DESIGN AND METHODS

Preparation of monoclonal antibodies against the LBD1 and CT of human megalin

The 867–base pair (bp) fragment of human megalin cDNA encoding the NH_2 terminus of the LBD1 (Gln26–Ala314) was amplified by PCR with the forward primer 5′-CGAGGATCCCAAGAATGTGACAGTGCGCA-3′ and the reverse primer 5′-GGTCTCGAGCTAGGCAGAGCACAGAGTCAT-3′. The 640-bp fragment of human megalin cDNA encoding the CT (His4447–Val4655) was also amplified with the forward primer 5′-GTTGGATCCCACTATAGAAGGACCGGCT-3′ and the reverse primer 5′-GGTCTCGAGGCTGGTATAGCTATACTTCAG-3′. The PCR products, digested with *Bam*HI and *Xho*I, were subcloned into the corresponding sites of a pGEX-6P-1 expression vector (GE Healthcare UK Ltd., Buckinghamshire, U.K.) with a glutathione S-transferase (GST) tag at the 5′ end. GST-fusion proteins were expressed in the *Escherichia coli* BL21 strain, and recombinant megalin LBD1 and CT proteins were released by cleavage with PreScission protease (GE Healthcare UK Ltd.) according to the manufacturer's instructions. These recombinant proteins were used as antigens to raise monoclonal antibodies (mAbs), as described previously (19). The specificity of the mAbs toward human megalin was determined by immunoblotting as described previously (20).

Preparation of human megalin LBD1-CT fusion proteins

The 867-bp fragment of the human megalin cDNA encoding the LBD1 was amplified by PCR with the forward primer described above and the reverse primer 5′-GGTGGATTCCTAGGCAGAGCACAGAGTCAT-3′. The PCR products, digested with *Bam*HI, were subcloned into the corresponding site of the aforementioned pGEX-6P-1 into which CT cDNA had been subcloned. Preparation of GST-LBD1-CT fusion proteins and purification of LBD1-CT proteins were carried out as described above. The LBD1-CT proteins were used as the reference samples for calibration of the ELISA.

Measurement of human megalin in urine by ELISA

The capture mAbs (5 μg/mL) were immobilized on the ELISA plates (LumiNunc F16 Maxisorp Surface plate; Thermo Fisher Scientific, Inc., New York, NY) (100 μL/well in 50 mmol/L carbonate buffer, pH 9.5) at 4°C overnight. The plates were washed with Tris-buffered saline (25 mmol/L Tris-HCl, 137 mmol/L NaCl, 2.68 mmol/L KCl, pH 7.4), blocked with 1% casein and 0.05% Tween 20 (200 μL/well) in Tris-buffered saline containing 0.1% NaN_3 at 4°C overnight, and stored at 2–8°C. The tracer mAbs were digested by pepsin to prepare $F(ab')_2$ fragments that were reduced to Fab′ fragments by 2-mercaptoethylamine. Reduced Fab′ fragments were conjugated to alkaline phosphatase (Roche Diagnostics GmbH, Mannheim, Germany) as described previously (21). Samples of urine (90 μl) were mixed with 10 μL of solution A (2 mol/L Tris-HCl, 0.2 mol/L EDTA, 10% [volume for volume] Triton X-100, pH 8.0), incubated for 1 min at room temperature for the COOH (C)-megalin assay or for 3 h at 50°C for the NH_2 (amino [A]- and full-length (F)–megalin assays, and reacted with alkaline phosphatase–labeled tracer mAbs in the ELISA plates. A chemiluminescent immunoassay detection system with CDP-Star substrate and Emerald-II enhancer (Applied Biosystems, Carlsbad, CA) was used according to the manufacturer's instructions (ELISA-Light System; Applied Biosystems). Urinary megalin concentrations were standardized by adjustment to urinary creatinine concentrations.

Participants and sample collection

Urine samples and clinical data were collected from adult residents who had participated in public medical examinations at Tagami-machi (Niigata-ken, Japan) from 2007 to 2009 and volunteers at Denka Seiken Co., Ltd. (Tokyo, Japan) in 2004. Normal control individuals ($n = 160$; 20–76 years of age; male/female = 76/84) who satisfied standard medical criteria as defined in Supplementary Table 1 were selected from these populations. The criteria included normal values for estimated glomerular filtration rate (eGFR) (60–104 mL/min/1.73 m^2) and urinary albumin creatinine ratio (<10 mg/g), which had been found in the general population with a mortality hazard ratio of ≤ 1.02 (22). Patients with type 2 diabetes ($n = 68$), including 43 men and 25 women (33–83 years of age), in Niigata, Tsukuba, and Juntendo university hospitals were also recruited into this study. The first- or second-void midstream urine samples were collected from the participants, on the basis of the results of a pilot study that had shown no significant differences between the first-void (preprandial) and the second-void (postprandial) urinary levels of A- and C-megalin in 19 randomly selected patients with type 2 diabetes (Supplementary Fig. 1). In addition, other pilot studies have indicated that urinary A- and C-megalin levels were stable during a 12-h incubation at 37°C (Supplementary Fig. 2) or after long-term (2–4 years) storage at −80°C (Supplementary Fig. 3). Urine samples (45 mL) were mixed with 633 μL of protease inhibitor mixture (120 μL of 10% [weight for volume] NaN_3, 45 μL of 5 mg/mL aprotinin, 450 μL of 500 mmol/L benzamidine, 13.5 μL of 11 mg/mL pepstatin A, and 4.5 μL of 50 mg/mL leupeptin), frozen at −80°C, and thawed within 1 year, just before analysis. Collection of urine samples and evaluation of clinical data evaluation were approved by the ethical committees of each university in accordance with the principles embodied in the Declaration of Helsinki, and all participants provided written informed consent.

Centrifugal fractionation of urine specimens

Urine specimens from patients with type 2 diabetes ($n = 10$) and normal control subjects ($n = 10$) were centrifuged at $17,000 \times g$ for 15 min at 4°C to precipitate urinary sediment, including whole cells, large membrane fragments, and other debris. Aliquots of the supernatants were further centrifuged at $200,000 \times g$ for 1 h at 4°C to precipitate exosomes (23) or other membrane vesicles (24). All the fractions of precipitants and supernatants were assayed by megalin sandwich ELISAs. The concentrations in the precipitants were standardized to the original sample volumes.

Preparation of normal human renal cortex lysates

Normal human renal cortex tissue was obtained at a site distant from renal cell carcinoma in a nephrectomized kidney. This procedure was approved by the Ethical Committee of Niigata University Graduate School of Medical and Dental Sciences, and the tissue donor provided written informed consent. The tissue was homogenized in 200 mmol/L Tris-HCl buffer (pH 8.0) containing 1% Triton X-100, 20 mmol/L EDTA, 10 mmol/L benzamidine, 5% aprotinin, and 1 mmol/L phenylmethylsulfonyl fluoride. After incubation for 60 min at 4°C, the suspension was centrifuged for 60 min at $100,000 \times g$ to obtain the supernatant lysates.

Gel filtration analysis of urinary and kidney megalin

Specimens of urine (900 μl) were mixed with 100 μL of solution A and subjected to gel filtration on a 160 × 1.5-cm Sephacryl S-300 column (GE Healthcare UK Ltd.) in 200 mmol/L Tris-HCl buffer (pH 8.0) containing 0.1% Triton X-100, 20 mmol/L EDTA, and 150 mmol/L NaCl. The fractionation was carried out under conditions of 1 mL/tube recovery at 10 cm/h of liner flow rate. Lysates (10 μg) of normal human renal cortex were also applied to the column for fractionation. All the fractions were assayed by megalin sandwich ELISA. Molecular weights of the fractionated samples were determined by using gel filtration calibration kits (GE Healthcare UK Ltd.).

Measurement of other markers

Plasma concentrations of creatinine were measured by an enzymatic method in the clinical laboratories in Niigata, Tsukuba, and Juntendo university hospitals. Urinary concentrations of creatinine, albumin, *N*-acetyl-β-D-glucosaminidase (NAG), α_1-microglobulin, and β_2-microglobulin were measured by an automated instrument (7170S; Hitachi High-Technologies Corp., Tokyo, Japan) with reagent kits CRE-S (Denka Seiken Co., Ltd.), ALB-TIA ×1 (Denka Seiken Co., Ltd.), N-assay L NAG NITTOBO (Nittobo Medical Co., Ltd., Tokyo, Japan), αMi-Latex (Denka Seiken Co., Ltd.), and BMG-Latex (Denka Seiken Co., Ltd.), respectively. Concentrations of each urinary marker were normalized to those of urinary creatinine. The eGFR was calculated with an equation validated for the Japanese population (25).

Determination of cutoff levels

In urine samples collected from normal control individuals (*n* = 160), A-megalin, C-megalin, albumin, NAG, α_1-microglobulin, and β_2-microglobulin were measured as described above. Normalization transformation of the values was carried out by the Box-Cox transformation method. Outliers were rejected using the Grubbs-Smirnov test; the upper value of the 95% confidence interval was defined as the normal cutoff level for each marker.

Statistical analyses

Comparisons of three or more groups were performed by one-way ANOVA followed by the Tukey test (for the same distribution) and the Kruskal-Wallis H-test followed by the Dunn test (for different distribution). Spearman rank correlation test was used for single regression analysis in which dummy variables, 1 and 0, were assigned for male and female, respectively.

RESULTS

Establishment of sandwich ELISA systems to detect human megalin

We raised mAbs against recombinant proteins encoding the NH_2-terminal LBD1 (mAbs A5 and A12) and the COOH-terminal CT (mAbs C25 and C37) of human megalin. The specificity of the mAbs for human megalin was confirmed by immunoblotting (Supplementary Fig. 4). Three types of megalin sandwich ELISA systems were developed to measure LBD1 (the A-megalin assay), CT (the C-megalin assay), and F-megalin in human urine. mAbs A12 and A5 (for the A-megalin assay), C25 and C37 (for the urinary C-megalin assay), and A5 and C37 (for the F-megalin assay) were used as capture and tracer antibodies, respectively. Calibration standard curves for these ELISA systems using recombinant megalin protein as a reference are shown in Supplementary Fig. 5. The accuracies of these systems were all within a 10% coefficient of variation.

Identification of full-length and ectodomain forms of megalin excreted in human urine

To identify the forms of megalin in human urine, we used urine protein lysates fractionated by gel filtration. As representatively shown in Fig. 1*A*, C- and F-megalin assays detected the same high-molecular-weight fraction with a single peak at ∼600 kDa that corresponds to the full-length form of megalin in urine samples from patients with type 2 diabetes. However, the A-megalin assay detected not only the high-molecular-weight fraction but also another smaller but more abundant fraction with a peak at ∼550 kDa lacking the CT. In normal urine (Fig. 1*B*), the high-molecular-weight peak fraction was rarely detected by the C- and F-megalin assays, whereas the smaller fraction was detected by the A-megalin assay. These data demonstrate that there are primarily two forms of megalin in human urine: full-length and ectodomain forms. The full-length form appears to be less abundant in normal

individuals but increased in patients with type 2 diabetes.

Identification of full-length and COOH-terminal fragment forms of megalin in human kidney

Next, we used normal human kidney–derived protein lysates similarly fractionated. As shown in Fig. 1*C*, the A- and F-megalin assays detected the same fraction with a single peak at the high-molecular-weight region, which is likely to be the full-length form of megalin. In contrast, the C-megalin assay detected not only the same high-molecular-weight peak fraction but also smaller fractions with three peaks at ∼20, 40, and 100 kDa. Collectively, normal human kidney contains smaller COOH-terminal megalin fragments in addition to its full-length form.

The ectodomain form of megalin detected in urine samples was not found in kidney lysates, and small COOH-terminal megalin fragments detected in kidney lysates were not found in urine samples. These data suggest that the ectodomain form may be shed into urine by cleavage of the full-length form in PTC membranes, and the small COOH-terminal megalin fragments found in kidney lysates may also be derived from these cleavage reactions.

In the following analyses, we chose the C-megalin assay to evaluate the full-length form of megalin in urine, as this assay virtually detects only this form in urine and does not require heat activation. In addition, we used the A-megalin assay to evaluate primarily the ectodomain form of megalin in urine, because the ectodomain form was highly abundant compared with the full-length form in urine.

The ectodomain form of megalin is present in the soluble urinary fraction but the full-length form is in both soluble and insoluble fractions

To investigate the origins or mechanisms of shedding of the full-length and ectodomain forms of human megalin into urine, we used urine samples, fractionated by centrifugation, from normal control individuals and patients with type 2 diabetes. As shown in Supplementary Fig. 6*A*, the A-megalin assay detected its targets predominantly in the soluble urinary fraction but less in the insoluble fractions. In contrast, the C-megalin assay detected its targets in both the soluble and insoluble fractions (Supplementary Figure 6*B*). In addition, the targets of the A-megalin

assay were stoichiometrically correlated with C-megalin assay targets in the insoluble fractions precipitated by low-speed centrifugation and ultracentrifugation, respectively (Supplementary Fig. 6*C* and *D*). These data indicate that the ectodomain form of megalin is primarily in the soluble fraction, whereas the full-length form is present both in the soluble and insoluble urinary fractions.

Determination of normal upper cutoff levels of A- and C-megalin assays and other urinary biomarkers

The urinary profiles of 160 normal control individuals are shown in Supplementary Table 2. The normal upper cutoff levels for urinary A-megalin, C-megalin, albumin, NAG, α_1-microglobulin, and β_2-microglobulin are 211.3 pmol/g creatinine, 523.5 fmol/g creatinine, 9.2 mg/g creatinine, 5.3 IU/g creatinine, 9.0 mg/g creatinine, and 207.9 μg/g creatinine, respectively. In the normal control individuals, urinary C-megalin levels were weakly associated with age ($r_s = 0.33$; $P < 0.001$). When the normal control individuals were stratified into two groups at the median age (42 years), the cutoff levels of the C-megalin assay were 434.7 and 608.0 (fmol/g creatinine) for the younger (20–41 years; $n = 78$) and the older group (42–76 years; $n = 82$), respectively, although the levels were much lower than those of patients with type 2 diabetes. Urinary A-megalin levels were not associated with age.

Relationship of urinary A- and C-megalin levels to albuminuria and reduced eGFR in patients with type 2 diabetes

We then investigated the relationship between urinary megalin excretion and albuminuria stratification in patients with type 2 diabetes ($n = 68$). Urinary C-megalin levels tended to increase with increasing albuminuria in these patients (Fig. 2*A* and Supplementary Table 3). Notably, the levels in patients with normoalbuminuria were significantly higher than those in normal control individuals; 39% of normoalbuminuric patients (15 of 39) showed urinary C-megalin levels over the normal cutoff level.

Moreover, the urinary C-megalin assay was found to show a significant association with reduced eGFR (<60 mL/min/1.73 m^2), whereas traditional markers of PTC injury such as NAG, α_1-microglobulin, and β_2-microglobulin did not show such an association with eGFR (Table 1 and Supplementary Fig. 7). Of note, the Spearman rank correlation coefficient of the urinary
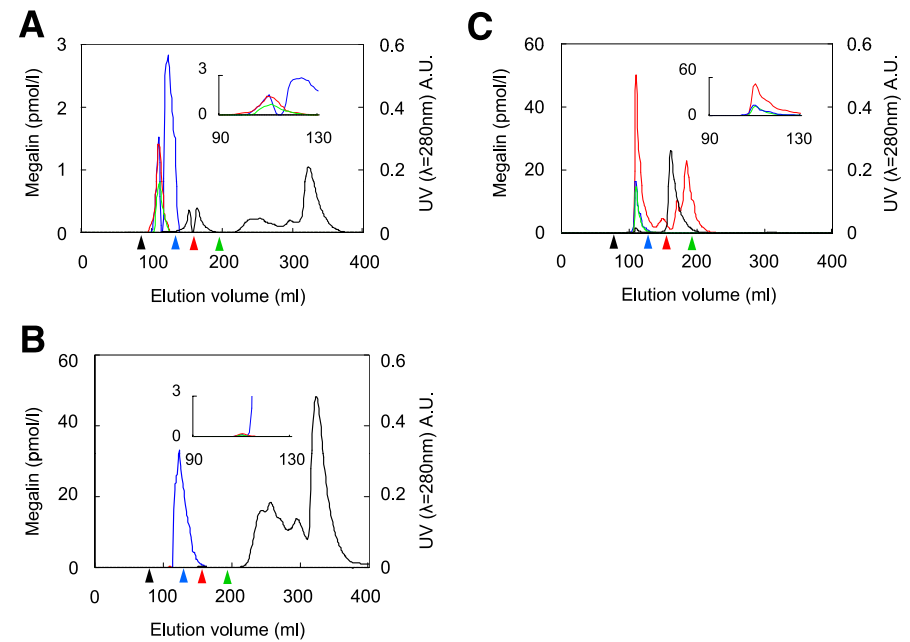
C-megalin assay against eGFR (<60 mL/min/1.73 m^2) was higher than that of urinary albumin, even in the normo- and microalbuminuric stages (Table 1).

On the other hand, urinary A-megalin levels were significantly higher in normo- and microalbuminuric patients than in normal control individuals but not in patients with macroalbuminuria (Fig. 2*B* and Supplementary Table 3). However, this is not because the A-megalin assay is extensively inhibited by other proteins,

such as albumin, in urine (Supplementary Fig. 8). Urinary A-megalin levels also showed no association with eGFR (<60 mL/min/1.73 m^2) (Table 1).

Association of urinary A- and C-megalin levels with other clinical parameters of patients with type 2 diabetes

The urinary A- and C-megalin assays were further evaluated for association with other clinical parameters in 52 of 68 patients with type 2 diabetes (Supplementary Table 4),
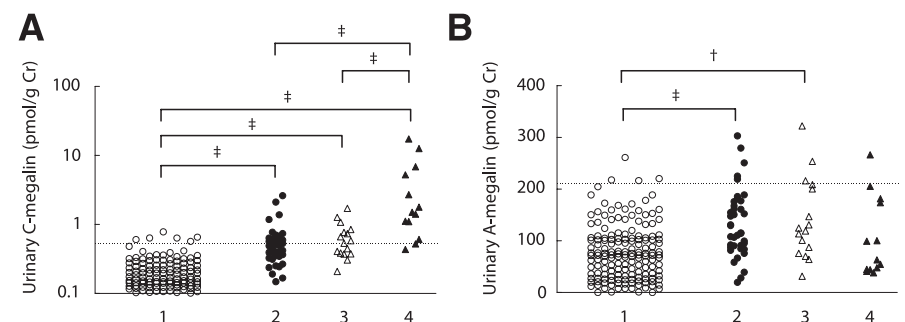

Figure 1—*Identification of the forms of megalin in human urine and kidney lysates. Urine samples of a patient with type 2 diabetes (A) and a normal control individual (B) and normal kidney lysates (C) were fractionated by gel chromatography and subjected to A- (blue), C- (red), and F-megalin (green) assays. Black lines show UV absorbance units (A.U.) at $\lambda = 280$ nm. Black arrowheads indicate the position of the void volume. Blue, red, and green arrowheads indicate the position of the calibration markers ferritin (440 kDa), conalbumin (75 kDa), and carbonic anhydrase (25 kDa), respectively. The initial peaks are also shown in the insets.*

Figure 2—*The relationship between urinary megalin excretion and albuminuria stratification in patients with type 2 diabetes. Urinary C- (A) and A-megalin (B) assay levels are shown according to albuminuria stratification in patients with type 2 diabetes. Normal control individuals (1, ○; n = 160), patients with normoalbuminuria (2, ●; n = 35; urinary albumin <30 mg/g creatinine), microalbuminuria (3, △; n = 15; 30 mg/g≤ urinary albumin <300 mg/g creatinine), and macroalbuminuria (4, ▲; n = 13; 300 mg/g creatinine≤ urinary albumin). Dashed line denotes the upper normal cutoff level. Cr, urinary creatinine. ‡P < 0.001 and †P < 0.01.*

Table 1—Association with eGFR (<60 mL/min/1.73 m²)

| | Patients with type 2 diabetes | | | |
| | Normo- and microalbuminuria (n = 12) | | Total (n = 21) | |
Urinary markers	r_s	P value	r_s	P value
C-megalin	0.51	NS (<0.1)	0.76	<0.001
A-megalin	−0.06	NS	−0.29	NS
Albumin	0.24	NS	0.64	<0.01
NAG	0.15	NS	0.28	NS
α_1-Microglobulin	−0.31	NS	0.28	NS
β_2-Microglobulin	−0.32	NS	0.26	NS
1/Cr (reference)	0.19	NS	0.33	NS

Cr, urinary creatinine. Microalbuminuria, 30–300 mg/g Cr; normoalbuminuria, <30 mg/g Cr. r_s vs. 1/eGFR.

who were cared for in the same Niigata University Hospital with the same laboratory examination standards used. The patients showed no features of bleeding, and took no medicines such as vitamin D analogs, phosphate binders, or erythropoiesis-stimulating agents before and during the study. Single regression analysis, performed with the Spearman rank correlation method (Table 2), showed that urinary C-megalin levels were associated positively with urinary albumin, urinary NAG, and plasma inorganic phosphate (Pi) and negatively with blood hemoglobin. However, urinary C-megalin levels were not associated with age or sex. Moreover, plasma Pi levels were not associated with urinary albumin, NAG, α_1-microglobulin, or β_2-microglobulin levels. An association of blood hemoglobin levels was found with urinary NAG levels, possibly because blood hemoglobin and urinary NAG levels appear to be confounding factors for aging. In contrast, urinary A-megalin levels were negatively associated with fractional excretion of sodium (FENa), a renal sodium handling marker.

CONCLUSIONS—We identified full-length and ectodomain forms of megalin in human urine with newly established sandwich ELISA for C- and A-megalin, respectively. The urinary full-length form of megalin was increased in correlation with the severity of DN in patients with type 2 diabetes. Notably, the urine levels in normoalbuminuric patients were significantly higher than those in normal control individuals, suggesting that the urinary C-megalin assay is sensitive enough for early diagnosis or prediction of the development of DN. The assay also showed a significant association with eGFR <60 mL/min/1.73 m², whereas urinary NAG, α_1-microglobulin, and β_2-microglobulin did not show such an association with reduced eGFR. Of note, the C-megalin assay showed a better association with reduced eGFR than did albuminuria.

The molecular mechanisms by which full-length megalin is shed into urine may be complicated because it is found in both soluble and insoluble urinary fractions. It is not simply derived from PTCs detached into urine because COOH-terminal megalin fragments (~20, 40, and 100 kDa)

seen in the kidney tissue are not detected in urine samples. A part of the shed form in the insoluble urinary fractions may be derived from exosomes (23) or other membrane vesicles such as microparticles and microvesicles (24). The endocytic machinery is directly regulated by the oxygen-sensitive pathway (26). The CT of megalin interacts with many intracellular adaptor proteins that act as connectors to motor proteins (9). Therefore, PTC hypoxia may alter the molecular interactions and cause urinary shedding of full-length megalin.

In contrast, urinary excretion of megalin ectodomain appears to be associated with distinctive mechanisms involved in early stages of DN. Megalin undergoes RIP similarly to the Notch and amyloidal precursor protein families (27,28). Megalin is subjected to metalloprotease-mediated ectodomain shedding that produces a 35–40-kDa, membrane-bound megalin COOH-terminal fragment (MCTF), and the MCTF is processed by γ-secretase activity to produce a soluble megalin intracellular domain (MICD) involved in signal transduction in vitro (10). In this study, we identified for the first time that the small CT fragments of megalin (~20 and 40 kDa), which may correspond to MICD and MCTF, respectively, are present in human kidney tissue. It is thus likely that RIP is a mechanism for urinary shedding of the megalin ectodomain. Decreased FENa in diabetic patients may compensatorily induce RIP of megalin, which increases the release of the ectodomain and the production of MICD that is known to suppress the expression of Na⁺/H⁺ exchanger isoform 3 in PTCs (29).

Low-molecular-weight protein markers of PTC injury such as α_1-microglobulin and β_2-microglobulin are filtered by glomeruli and reabsorbed by PTCs via megalin (30,31). In the early stage of PTC injury, megalin may be dissociated from the cell membranes and excreted into urine, but its endocytic function may still be preserved. In that stage, urinary excretion of megalin's endocytic ligands is not likely to be increased. In addition, in advanced stages with reduced GFR, their urinary excretion may not be increased because their glomerular filtration is decreased. In addition, the release of NAG, a lysosomal enzyme, into urine may not be as sensitive to PTC injury as megalin, which is abundantly expressed at the apical PTC membranes.

Similarly, albumin is excreted into urine by its increased glomerular leakage and/or decreased PTC reabsorption via

Table 2—Single regression analysis for the correlation of urinary markers and clinical parameters in the patients with type 2 diabetes

	C-meg	A-meg	Alb	NAG	α_1-MG	β_2-MG	Age	Hb	Pi
C-meg									
A-meg	−0.03								
Alb	0.38†	−0.06							
NAG	0.48‡	0.18	0.45‡						
α_1-MG	0.25	−0.12	0.58‡	0.57‡					
β_2-MG	0.26	0.13	0.41†	0.58‡	0.69‡				
Age	0.23	0.14	0.05	0.55‡	0.30*	0.31*			
Hb	−0.34†	−0.22	−0.12	−0.50‡	−0.15	−0.13	−0.45‡		
Pi	0.27*	0.13	−0.12	0.00	−0.25	−0.01	0.11	−0.37†	
FENa	0.42†	−0.34*	0.22	0.12	0.09	−0.03	0.08	−0.04	0.12

Alb, albumin; Hb, hemoglobin; meg, megalin; MG, microglobulin. *P < 0.05. †P < 0.01. ‡P < 0.001.

megalin and cubilin (9). In fact, a substantial number of diabetic patients are known to show normoalbuminuria with reduced GFR (32,33). In such patients, hypertension and dyslipidemia may be involved in the development of intrarenal arteriosclerosis or glomerular ischemia to reduce GFR and albuminuria (34,35), but concomitant PTC hypoxia may cause increased urinary megalin excretion.

Anemia is recognized to play an important role in the development of CVD in patients with CKD (36). Dysfunction of PTCs is known to impair the production of erythropoietin and is involved in the development of renal anemia (37,38). Because of the association of blood hemoglobin levels with urinary C-megalin levels, it is speculated that the C-megalin assay reflects the pathologic role of PTCs involved in the development of renal anemia. The positive association of plasma Pi levels with urinary C-megalin levels is also intriguing, because hyperphosphatemia is also associated with the development of CVD and high mortality in patients with CKD (39). This mechanism needs to be studied further because megalin is known to downregulate sodium-phosphate cotransporter IIa in PTCs (40).

Collectively, the urinary full-length form of megalin could be a novel biomarker associated with the severity of DN and related disorders in patients with type 2 diabetes. The urinary megalin ectodomain may be a distinctive RIP-related biomarker that appears to be involved in the mechanisms of early DN. These two urinary forms of megalin should be further investigated to confirm their clinical usefulness for DN by prospective studies.

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