Development and Evaluation of a Practical ELISA for Human Urinary Lipocalin-Type Prostaglandin D Synthase

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Background: Urinary excretion of lipocalin-type prostaglandin D synthase (L-PGDS) is significantly increased in patients with chronic renal failure, but its diagnostic potential in less advanced stages of renal diseases remains to be elucidated.

Methods: Six mouse monoclonal antibodies (MAbs) were raised against recombinant human L-PGDS. We constructed a sandwich ELISA with two MAbs that recognized different epitopes with high affinities and assessed its assay performance and clinical utility with urine samples from healthy controls, diabetic patients, and patients with various renal diseases.

Results: Western blot analyses with NH2-terminus-truncated L-PGDS mapped the epitopes to Ala23–Val28 (MAb-7F5 and -10A3), Ser52–Ala73 (MAb-9A6), Tyr107–Val120 (MAb-1B7 and -6F5), and Gly140–Pro155 (MAb-6B9). A sandwich ELISA was constructed with MAb-1B7 and -7F5, the Kd values of which were 3.6 and 3.9 nmol/L, respectively, for native L-PGDS. Recoveries were 91–111%, and intra- and interassay CVs were <6% and <9%, respectively. The ELISA showed parallelism of standard and urine samples and no significant interference by a variety of urinary constituents. Urinary L-PGDS excretion was significantly increased in patients with diabetic nephropathy, IgA nephropathy, and chronic glomerulonephritis even when serum creatinine was not increased. In patients with renal diseases, urinary L-PGDS was correlated with urinary albumin (r = 0.64; P < 0.0001), N-acetyl-β-D-glucosaminidase (r = 0.43; P < 0.001), and serum creatinine (r = 0.66; P < 0.0001). At a cutoff value of 284 mg/mol creatinine, the assay had sensitivities of 74% for diabetic nephropathy and 83% for chronic glomerulonephritis and a specificity of 93%.

Conclusions: This ELISA system is suitable for measurement of urinary L-PGDS in a routine clinical assay and may be useful to detect less advanced stages of renal diseases.

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Lipocalin-type prostaglandin (PG) D synthase (1) (EC 5.3.99.2; L-PGDS) is a secretory glycoprotein with a molecular mass of ~26 kDa. L-PGDS catalyzes the isomerization of PGH2, a common precursor of various prostanooids, to produce PGD2. This prostaglandin has various physiologic actions, including sleep induction, nociception, inhibition of platelet aggregation and nitric oxide release, and induction of vasodilation (1, 2). The cDNA of human L-PGDS encodes 190-amino acid residues with an N-terminal signal peptide of 22 amino acid residues. A homology search in databases of protein primary structure and comparison of the gene structure revealed that L-PGDS is a member of the lipocalin superfamily (3), which is composed of various secretory lipid-transporter proteins. L-PGDS is the first reported enzyme among members of the lipocalin family (4), and interestingly, it still maintains the ability to bind lipophilic ligands such as retinoids, thyroid hormones, and bile pigments (5, 6).

Recently, L-PGDS was identified as β-trace (7–9), which was originally discovered as a major protein of human cerebrospinal fluid (CSF) (10). β-Trace was also detected in human serum and urine (11, 12). Because

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\(\beta\)-trace is a low-molecular-mass protein, it may be filtered through the glomerular basement membrane. Thus, both serum and urinary \(\beta\)-trace (L-PGDS) have been investigated as indicators of renal dysfunction. The serum \(\beta\)-trace (L-PGDS) concentration was reported to be significantly higher in the patients with renal failure than in healthy individuals or non-renal failure patients (13, 14). Furthermore, serum \(\beta\)-trace was shown to be suitable as an indicator of a reduced glomerular filtration rate even in the creatinine (Cr)-blind range, as shown by use of a nephelometric assay (15). Urinary \(\beta\)-trace excretion was also reported to be significantly higher in the patients with chronic renal failure than in healthy individuals, as judged from data obtained by the single radial immunodiffusion technique (16). However, a possible change in urinary L-PGDS excretion in less advanced stages of renal diseases has not been demonstrated, because no highly sensitive and specific assay method for urinary L-PGDS has been available until recently.

Recently, we raised mouse monoclonal antibodies (MAb-1B7 and -10A3) against recombinant human L-PGDS and used them to develop sensitive ELISA (17) and immunofluorometric (18) assays for the measurement of L-PGDS in various body fluids. We later raised additional MAb (MAb-6B9, -7F5, and -9A6), modified this ELISA system to use MAb-7F5 instead of MAb-10A3, and thereby demonstrated that urinary L-PGDS excretion may predict the progression of renal injuries in diabetic nephropathy (19). However, the antigenic epitopes and specificities of these MAb had not been fully characterized. Furthermore, because the ELISA system requires many incubation steps and the immunofluorometric assay system requires a particular assay device, these systems have not been used widely. Moreover, the diagnostic validity of urinary L-PGDS for diabetic nephropathy and other renal diseases in less advanced stages remained unclear. Therefore, in the present study we characterized these MAb against human L-PGDS and improved the previous ELISA system to be specifically useful for routine analysis of urinary L-PGDS. We also demonstrated the dynamic range, imprecision, linearity, absence of interference, and clinical performance of this L-PGDS ELISA format.

**Materials and Methods**

**Preparation of anti-L-PGDS MAb**

MAb against L-PGDS were obtained from BALB/c mice immunized with the purified recombinant protein expressed in *Escherichia coli*, as reported previously (17). Isotypes of MAb were determined with an isotyping reagent set for mouse assay MAb (Pierce). The dissociation constants \(K_d\) of the MAb were determined with the recombinant L-PGDS and the native protein purified from CSF by the ELISA technique, as reported by Friguet et al. (20).

**Imunoaffinity Purification of the Native L-PGDS**

Native L-PGDS was purified by MAb-conjugated immunoaffinity column chromatography from human CSF or urine, as reported previously (21). Purified L-PGDS was subjected to gel filtration with prep-grade HiLoad Superdex 200 (Amersham Pharmacia Biotech) previously equilibrated with phosphate-buffered saline (10 mmol/L sodium phosphate, pH 7.4, containing 154 mmol/L NaCl).

**Western Blot Analysis**

The samples were solubilized with 10 g/L sodium dodecyl sulfate (SDS) in the presence of 50 mL/L \(\beta\)-mercaptoethanol and electrophoresed in 14% polyacrylamide gels. After electrophoresis, the proteins separated in the gel were stained with a silver stain reagent set (Wako Pure Chemicals) or electrophoretically transferred to a polyvinylidene difluoride membrane (Millipore). The membrane was then immunostained with mouse MAb or rabbit polyclonal antibody (22) against L-PGDS followed by alkaline phosphatase-conjugated goat anti-mouse or anti-rabbit IgG (Organon Teknika).

**Epitope Mapping of MAb**

By PCR, we produced the cDNAs for NH2-terminus-truncated mutants of L-PGDS at intervals of approximately several to 20 amino acid residues. The glutathione S-transferase (GST) fusion proteins with the NH2-terminus-truncated L-PGDS were obtained from 4 mL of culture liquid from *E. coli* that had been transfected with the constructed plasmid. The cell extract was mixed with 0.1 mL of glutathione-Sepharose 4B (Amersham Pharmacia Biotech) at 4°C for 1 h to recover the fusion protein. After the resin had been washed with phosphate-buffered saline containing 5 mL/L Triton X-100, the fusion protein was extracted from the resin by incubation with 10 g/L SDS at 100°C for 5 min. The location of the epitope was determined by Western blotting with the GST fusion proteins constructed with a series of NH2-terminus-truncated L-PGDS.

**Sandwich ELISA**

Poly styrene 96-well microtiter plates (Costar 3590; Corning) were coated with 100 \(\mu\)L/well MAb-7F5 (10 mg/L in 50 mmol/L sodium carbonate, pH 9.6) for 18 h at 4°C. After being washed three times with Tris-buffered saline (TBS; 20 mmol/L Tris-HCl, pH 7.4, containing 154 mmol/L NaCl), the plates were blocked for 18 h at 4°C with TBS containing 10 g/L bovine serum albumin, 50 g/L sucrose, and 0.1 g/L thimerosal. After the blocking solution had been aspirated, the plates were dried in a vacuum desiccator, sealed in an aluminum-coated pack with drying agent (I.D. SHEET DESICCANT; I.D.), and stored at 4°C until used.

L-PGDS was purified from CSF and pooled urine as the primary and secondary standards, respectively, as described above. Urine samples were diluted 1:500 to 1:1000 (2 \(\mu\)L of sample in 998 or 1998 mL of diluent) with
TBS containing 1 g/L bovine serum albumin and 0.1 g/L thimerosal. The diluted sample or calibrator (100 μL) was added in duplicate to coated wells, and the plate was then incubated for 90 min at 25 °C. After the plate had been washed four times with TBS containing 0.5 mL/L Tween 20, horseradish peroxidase-conjugated MAb-1B7 (100 μL) was added to each well, and the plate was incubated for 90 min at 25 °C. After another four washes with TBS containing 0.5 mL/L Tween 20, 100 μL of substrate solution containing 3,3′,5,5′-tetramethylbenzidine (BM blue-POD substrate; Roche Diagnostics) was added to each well, and the plate was incubated at 25 °C for 30 min. The reaction was stopped by the addition of 1 mol/L sulfuric acid (100 μL/well). The plate was then read on a SPECTRAmax 250 microplate reader (Molecular Devices) at a wavelength of 450 nm.

CLINICAL SAMPLES

Human CSF was supplied from the Department of Neurosurgery, Faculty of Medicine, University of Kanazawa (Kanazawa, Japan). Human urine samples were collected from patients with various renal diseases and from patients with type 2 diabetes at the Department of Medicine, The University of Tokyo (Tokyo, Japan); the Department of Nephrology, Kanto Medical Center NTT EC (Tokyo, Japan); the Department of Medicine, Tokyo Police Hospital (Tokyo, Japan); and the Department of Medicine III, Okayama University Medical School (Okayama, Japan). Control samples were collected at the Health Service Center, Mitsui Memorial Hospital (Tokyo, Japan). Criteria for the controls included (a) absence of a history of diabetes mellitus, renal diseases, or coronary heart diseases; (b) serum Cr within reference values (<106 μmol/L), and (c) urinary albumin within reference values [normal albuminuria; <3.4 g/mol Cr (<30 mg/g Cr)]. All samples were collected after informed consent had been obtained from the patients as part of the routine diagnostic procedures. All samples were stored at −70 °C until used for analysis.

The urinary albumin concentration was measured by a turbidimetric immunoassay (Eiken Alb-II). Urinary N-acetyl-β-D-glucosaminidase (NAG) activity was determined spectrophotometrically with a commercial assay (NAG test Shionogi). Urinary concentrations of albumin, NAG, and L-PGDS were divided by the urinary Cr concentration to correct for the influence of the urinary volume.

STATISTICAL ANALYSIS

Differences were examined by the Student t-test or the Mann–Whitney U-test. Male/female ratios and diagnostic sensitivities were compared by χ² analysis. The correlation between different analytes was assessed by use of the Pearson correlation coefficients. A linear relationship between two analytes was evaluated by a linear regression model. P <0.05 was considered significant.

Results

PREPARATION AND CHARACTERIZATION OF ANTI-L-PGDS MABS

Specificity of MAbs against native human L-PGDS. We isolated six positive hybridoma clones (Table 1). Clone 1B7 came from a P3-X63-Ag8.653/spleen cell hybridoma, and the other clones were from P3-X63-Ag8-U1/spleen cell hybridomas. The subclasses of immunoglobulin (light chain) were identified as IgG1 (λ) for MAb-1B7 and -6F5; IgG1 (κ) for MAb-7F5, -10A3, and -6B9; and IgG2a (κ) for MAb-9A6.

The apparent Kd values of the MAbs were calculated for the native and recombinant L-PGDS (Table 1). The Kd values of MAb-1B7, -7F5, -10A3, and -6F5 for the native L-PGDS were 3.6, 3.9, 5.5, and 10.3 nmol/L, respectively. Those of MAb-1B7 and -6F5 for the recombinant protein were 1.3- to 1.5-fold higher, 5.4 and 13.2 nmol/L, respectively. On the other hand, the Kd values of MAb-7F5 and -10A3 for the recombinant L-PGDS were 10–15% lower than those for the native protein, i.e., 0.5 and 0.8 nmol/L, respectively. MAb-9A6 hardly cross-reacted with the native L-PGDS, although the Kd value of this antibody was calculated to be 7.4 nmol/L for the recombinant L-PGDS. Although MAb-6B9 bound to the immobilized recombinant L-PGDS used in the direct ELISA at the step of hybridoma screening, it barely bound to the soluble form of native and recombinant L-PGDS used in the ELISA for calculating the Kd values.

The specificities of the MAbs were also assessed by Western blot analysis after SDS-polyacrylamide gel electrophoresis of human urine. A single, broad immunoreactive band was observed with all of the MAbs except for MAb-9A6 at the same position as that of L-PGDS purified.

Table 1. Characterization of six MAbs raised against recombinant L-PGDS.

<table>
<thead>
<tr>
<th>MAb</th>
<th>Myeloma strain</th>
<th>Subclass of immunoglobulin</th>
<th>Kd, nmol/L</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>(light chain)</td>
<td>Native</td>
</tr>
<tr>
<td>1B7</td>
<td>P3-X63-Ag8.653</td>
<td>IgG1 (λ)</td>
<td>3.6</td>
</tr>
<tr>
<td>7F5</td>
<td>P3-X63-Ag8-U1</td>
<td>IgG1 (κ)</td>
<td>3.9</td>
</tr>
<tr>
<td>10A3</td>
<td>P3-X63-Ag8-U1</td>
<td>IgG1 (κ)</td>
<td>5.5</td>
</tr>
<tr>
<td>6F5</td>
<td>P3-X63-Ag8-U1</td>
<td>IgG1 (λ)</td>
<td>10.3</td>
</tr>
<tr>
<td>9A6</td>
<td>P3-X63-Ag8-U1</td>
<td>IgG2a (κ)</td>
<td>&gt;1000</td>
</tr>
<tr>
<td>6B9</td>
<td>P3-X63-Ag8-U1</td>
<td>IgG1 (κ)</td>
<td>&gt;1000</td>
</tr>
</tbody>
</table>
from human urine (26 kDa; data not shown). MAb-9A6 did not cross-react with the native enzyme, but showed a single immunoreactive band after deglycosylation of the purified native protein at the same position as that of the recombinant L-PGDS without glycosylation (20 kDa). Although MAb-6B9 hardly bound to the soluble L-PGDS, this antibody bound to the immobilized native and recombinant proteins as examined by Western blot analysis.

Mapping of antigenic epitopes of L-PGDS for MAbs. The locations of the epitopes were determined with the GST fusion proteins containing a series of NH₂-terminus-truncated mutants of L-PGDS (Fig. 1A). All of these fused proteins were expressed in the soluble fraction of E. coli and purified by glutathione affinity chromatography (Fig. 1B, Silverstain). In Western blot analysis with polyclonal anti-L-PGDS antibody, immunoreactivity was observed with fusion proteins having an N-terminal deletion up to 155 residues and disappeared after deletion of 173 residues (Fig. 1B, Polyclonal), indicating that the C-terminal region between Glu₁⁷⁴ and Glu₁⁹⁰ of L-PGDS had poor antigenicity. The immunoreactivity disappeared after deletion of 28 N-terminal residues for MAb-7F5 and -10A3, 73 residues for MAb-9A6, 120 residues for MAb-1B7 and -6F5, and 155 residues for MAb-6B9. Thus, four antigenic epitopes were determined, located within Ala₂³–Val₂₈, Ser₅₂–Ala₇₃, Tyr₁₀₇–Val₁₂₀, and Gly₁₄₀–Pro₁₅₅.

We next constructed a model structure of L-PGDS, composed of a three-turn α-helix and nine β-strands (A to I), from the homology with other members of the lipocalin superfamily (3, 23) and mapped the antigenic epitopes for MAbs on this model (Fig. 1C). We hypothesized that the epitope for MAb-1B7 and -6F5 (Tyr¹⁰⁷–Val¹²₀) is located within the loop region between β-strands E (Ser¹⁰⁴–Arg¹⁰⁸) and F (Tyr¹₁⁶–Thr¹₂³) and that the epitope for MAb-9A6 is located within Ser⁶⁵–Ala⁷₃, in which two loop regions reside, one between β-strands A (Gly⁴⁰–Ala⁴⁹) and B (Cys⁶⁵–Ala⁷₂) and one between β-strands B and C (Gly⁷₆–Arg⁸₅). Because MAb-9A6 bound only to the deglycosylated L-PGDS (Table 1), we deduced that its antigenic epitope resides in the loop region between β-strands B and C, possibly near an N-glycosylation site (Asn⁷₈). The epitope for MAb-7F5 and -10A3 was mapped on the N-terminal 6 amino acid residues and that for MAb-6B9 was mapped on the region of β-strand H (Phe¹₄₃–Ser¹₅₀) and its surroundings.

CONSTRUCTION AND CHARACTERIZATION OF SANDWICH ELISA

Construction of sandwich ELISA for detection of L-PGDS in human urine. A sandwich ELISA was constructed with two MAbs, MAb-1B7 and -7F5, that recognized different epitopes with high affinities, as described above. Fig. 2A shows the typical calibration curve of the native L-PGDS purified from human urine in the ELISA, with MAb-7F5 used for coating and enzyme-labeled MAb-1B7 for detection.

Fig. 1. Mapping of antigenic epitopes of L-PGDS recognized by MAbs.

(A), scheme of the GST fusion proteins containing a series of NH₂-terminus-truncated L-PGDS. The N-terminal amino acid residues of each mutant L-PGDS are indicated on the left. The mature form of L-PGDS was obtained by the N-terminal deletion of 22 amino acid residues corresponding to the signal peptide (line 1). Asterisks indicate the N-glycosylation sites. (B), the recombinant NH₂-terminus-truncated L-PGDS mutants were subjected to SDS-polyacrylamide gel electrophoresis and Western blotting. Lanes 1–11 correspond to lines 1–11 in panel A. (C), the antigenic epitopes for MAbs were mapped on the homology model structure of L-PGDS. In this model, the β-strands comprise the residues Gly⁴⁰–Ala⁴⁹ (strand A), Cys⁶⁵–Ala⁷₂ (strand B), Gly⁷₆–Arg⁸₅ (strand C), Glu⁴⁰–Pro⁵₈ (strand D), Ser¹⁰⁴–Arg¹⁰⁸ (strand E), Tyr¹₁⁶–Thr¹₂³ (strand F), Val¹₂₈–Gly¹₃₅ (strand G), Phe¹₄₃–Ser¹₅₀ (strand H), and Ile¹₇₇–Phe¹₇₉ (strand I), and there is a three-turn α-helix, Ala¹₅₇–Ala¹₆₉.
Detection limit and working assay range of L-PGDS. The native L-PGDS purified from urine was serially diluted and used for testing the detection limit of L-PGDS, which was defined as the lowest L-PGDS concentration that could be differentiated from zero (assay blank) by the t-test. The detection limit was 0.08 μg/L of assay solution (n=5; P<0.001). The working range of the assay was established by calculating the CV of each calibrator in five independent calibration curves. The CV obtained for each calibrator at 0.3–20 μg/L of assay solution was <10% (Fig. 2A).

Imprecision, recoveries, and dilution analysis. The imprecision in this ELISA was assessed by use of urine samples that contained L-PGDS at three different concentrations (nearly 0.6 mg/L in the urine from a healthy control, 2.7 mg/L in the urine from a diabetic patient with urinary albumin of 9.4 g/mol Cr, and 4.7 mg/L in the urine from a diabetic nephropathy patient with urinary albumin of 45.4 g/mol Cr). Intra- and interassay CVs ranged from 3.2% to 5.8% (n=10) and from 7.6% to 8.3% (n=10), respectively (Table 2). To determine the analytical recovery, we mixed different amounts of the purified L-PGDS (0.4–3.0 mg/L of urine) with 15 urine samples containing 0.6–3.7 mg/L L-PGDS. Recoveries of L-PGDS ranged from 91% to 111%. When urine samples containing 0.9, 2.7, and 4.7 mg/L L-PGDS were serially diluted and assayed, each sample gave results close to linearity (r = 0.998–1.000; Fig. 2B), confirming parallelism between the calibrators and urine samples.

Interference. Urine samples containing a low (0.6 mg/L), medium (2.7 mg/L), or high (4.7 mg/L) concentration of L-PGDS were supplemented with potentially interfering agents at various concentrations. Although the data are not shown, there was no substantial interference from ascorbic acid (<56.8 mmol/L), hemoglobin (<1.0 g/L), albumin (<5.0 g/L), globulin (<0.1 g/L), Cr (<132.6 mmol/L), creatine (<38.1 mmol/L), bilirubin (<3.3 mmol/L), urea (<1.0 mol/L), uric acid (<59.5 mmol/L), glucose (<250 mmol/L), or sodium chloride (<0.5 mol/L), indicating that the assay was resistant to interference from a wide range of urinary constituents.

Table 2. Imprecision of the L-PGDS assay for urine samples with different protein concentrations.

<table>
<thead>
<tr>
<th>Protein Concentration</th>
<th>Mean, mg/L</th>
<th>SD, mg/L</th>
<th>CV, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intrassay (n=10)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Urine 1</td>
<td>0.64</td>
<td>0.03</td>
<td>3.9</td>
</tr>
<tr>
<td>Urine 2</td>
<td>2.65</td>
<td>0.15</td>
<td>5.8</td>
</tr>
<tr>
<td>Urine 3</td>
<td>4.71</td>
<td>0.15</td>
<td>3.2</td>
</tr>
<tr>
<td>Interassay (n=10)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Urine 1</td>
<td>0.68</td>
<td>0.05</td>
<td>7.6</td>
</tr>
<tr>
<td>Urine 2</td>
<td>2.80</td>
<td>0.23</td>
<td>8.3</td>
</tr>
<tr>
<td>Urine 3</td>
<td>4.70</td>
<td>0.38</td>
<td>8.0</td>
</tr>
</tbody>
</table>
Patients with diabetic nephropathy, IgA nephropathy, chronic glomerulonephritis, chronic renal failure; DM-normo, diabetes mellitus with normoalbuminuria.

**Table 3. Clinical characteristics of the healthy controls and patients with various renal diseases or normoalbuminuric diabetes mellitus.**

<table>
<thead>
<tr>
<th></th>
<th>M/F</th>
<th>Age, years</th>
<th>Median (range)</th>
<th>Serum Cr, μmol/L</th>
<th>Urinary albumin, g/mol Cr</th>
<th>Urinary NAG, kU/mol Cr</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls</td>
<td>15/14</td>
<td>53 (41–70)</td>
<td>80 (57–97)</td>
<td>0.7 (0.5–1.8)</td>
<td>0.38 (0.07–1.76)</td>
<td></td>
</tr>
<tr>
<td>DN*</td>
<td>16/7</td>
<td>66 (30–81)</td>
<td>97 (44–663)</td>
<td>92.2 (0.5–703)</td>
<td>1.48 (0.30–4.52)</td>
<td></td>
</tr>
<tr>
<td>IgA</td>
<td>6/5</td>
<td>39 (28–63)</td>
<td>79 (53–168)</td>
<td>24.2 (2.0–331)</td>
<td>0.45 (0.25–2.17)</td>
<td></td>
</tr>
<tr>
<td>MN</td>
<td>6/2</td>
<td>59 (50–70)</td>
<td>78 (54–99)</td>
<td>65.0 (1.9–586)</td>
<td>1.79 (0.34–3.72)</td>
<td></td>
</tr>
<tr>
<td>FGS</td>
<td>3/1</td>
<td>64 (50–65)</td>
<td>97 (53–106)</td>
<td>30.8 (6.7–150)</td>
<td>0.68 (0.41–0.90)</td>
<td></td>
</tr>
<tr>
<td>CGN</td>
<td>3/3</td>
<td>63 (40–73)</td>
<td>69 (62–115)</td>
<td>47.7 (1.9–461)</td>
<td>1.46 (0.36–3.72)</td>
<td></td>
</tr>
<tr>
<td>CRF</td>
<td>4/3</td>
<td>74 (57–81)</td>
<td>235 (109–663)</td>
<td>129 (10.5–512)</td>
<td>1.22 (0.50–5.17)</td>
<td></td>
</tr>
<tr>
<td>DM-normo</td>
<td>9/7</td>
<td>59 (47–69)</td>
<td>67 (49–99)</td>
<td>1.4 (0.5–2.7)</td>
<td>0.87 (0.42–2.83)</td>
<td></td>
</tr>
</tbody>
</table>

* a DN, diabetic nephropathy; IgA, IgA nephropathy; MN, membranous nephropathy; FGS, focal segmental glomerulosclerosis; CGN, chronic glomerulonephritis; CRF, chronic renal failure; DM-normo, diabetes mellitus with normoalbuminuria.

b–e Compared with healthy controls: *a P < 0.01; *b P < 0.001; *c P < 0.005; *d P < 0.05.

age-related statistical differences in the urinary L-PGDS of the controls (Table 3). In addition, there were no statistical differences in the male/female ratio between the controls and each patient group. Therefore, male/female data were combined in each group in this study. Compared with that of the controls (median, 65 mg/mol Cr; range, 25–486 mg/mol Cr; Fig. 3), urinary L-PGDS excretion was significantly increased in the patients with diabetic nephropathy (median, 632 mg/mol Cr; range, 98–3250 mg/mol Cr; *P < 0.001), IgA nephropathy (median, 291 mg/mol Cr; range, 21–1393 mg/mol Cr; *P < 0.05), membranous nephropathy (median, 321 mg/mol Cr; range, 40–1531 mg/mol Cr; *P < 0.01), focal segmental glomerulosclerosis (median, 250 mg/mol Cr; range, 200–1988 mg/mol Cr; *P < 0.01), chronic glomerulonephritis (median, 389 mg/mol Cr; range, 40–616 mg/mol Cr; *P < 0.01), and chronic renal failure (median, 1008 mg/mol Cr; range, 204–3220 mg/mol Cr; *P < 0.001). Moreover, urinary L-PGDS excretion in the patients with renal diseases varied more widely than that in controls, indicating that urinary L-PGDS excretion might reflect the severity of the renal diseases. We therefore reanalyzed the data on urinary L-PGDS excretion from the patients with diabetic nephropathy, IgA nephropathy, and chronic glomerulonephritis with a serum Cr concentration within reference values (<106 μmol/L). Even in this case, urinary L-PGDS excretion was significantly higher in the patients with diabetic nephropathy (n = 12; median, 545 mg/mol Cr; range, 98–1713 mg/mol Cr; *P < 0.0001), IgA nephropathy (n = 8; median, 242 mg/mol Cr; range, 30–776 mg/mol Cr; *P < 0.05), or chronic glomerulonephritis (n = 5; median, 445 mg/mol Cr; range, 40–616 mg/mol Cr; *P < 0.05) than in the controls. All patients with membranous nephropathy and focal segmental glomerulosclerosis enrolled in this study showed a serum Cr concentration within reference values. Furthermore, urinary L-PGDS excretion was significantly higher in the diabetic patients with normoalbuminuria (median, 262 mg/mol Cr; range, 42–750 mg/mol Cr; *P < 0.0005) than in the controls.

Urinary L-PGDS excretion was only weak correlated or not correlated with urinary albumin excretion (*r = 0.47; P < 0.05), NAG excretion (*r = 0.38; P < 0.05), and serum Cr (*r = 0.04; P = 0.82) in the controls (n = 29). On the other hand, in the patients with various renal diseases (n = 59; Fig. 4), the excretion was significantly correlated with urinary albumin (*r = 0.64; *P < 0.0001) and NAG excretion (*r = 0.43; *P < 0.001) as well as with serum Cr (*r = 0.66; *P < 0.0001), indicating that urinary L-PGDS excretion reflects renal function.

We then preliminarily evaluated the diagnostic validity of urinary L-PGDS for each renal disease. When the cutoff value of urinary L-PGDS was set at 284 mg/mol Cr, which was the 95th percentile of the controls, the sensitivity for each renal disease was as follows: diabetic
nephropathy, 74%; IgA nephropathy, 55%; membranous nephropathy, 50%; focal segmental glomerulosclerosis, 50%; chronic glomerulonephritis, 83%; and chronic renal failure, 86%. The specificity was estimated to be 93%.

Urinary NAG excretion showed a sensitivity of 70% for diabetic nephropathy, 36% for IgA nephropathy, 63% for membranous nephropathy, 25% for focal segmental glomerulosclerosis, 50% for chronic glomerulonephritis, and 86% for chronic renal failure at a cutoff value of 0.9 kU/mol Cr

There was no statistical difference in sensitivity between urinary L-PGDS and NAG for each renal disease at these cutoff values.

Discussion

In this study, we constructed a practical assay system designed for routine analysis of urinary L-PGDS by use of MAbs against L-PGDS whose antigenic epitopes and affinities for the antigen had been characterized.

Using the NH$_2$-terminus-truncated mutants, we mapped four antigenic epitopes of L-PGDS on the model structure: the N-terminal 6-amino acid residues for MAb-7F5 and -10A3, the loop region between $\beta$-strands B and C for MAb-9A6, the loop region between $\beta$-strands E and F for MAb-1B7 and -6F5, and the region of $\beta$-strand H and its surroundings for MAb-6B9 (Fig. 1C). In this model, the N-terminal region and the loop region between $\beta$-strands E and F are exposed on the surface of the molecule. This observation is consistent with the cross-reactivities of MAbs against L-PGDS whose native L-PGDS or be different between the native and recombinant proteins, because the affinities of MAb-7F5 and -10A3 for the native L-PGDS were lower than those for the recombinant protein. Although the loop region between $\beta$-strands B and C is also exposed on the surface of the molecule, this region may be covered by a sugar chain as judged from the cross-reactivity of MAb-9A6. MAb-6B9 bound only to the denatured protein, not to the soluble form (Table 1), indicating that its antigenic epitope appears on the surface of the molecule only in the denatured protein. Therefore, we believe that the region Gly$_{140}^\text{N}-\text{Pro}_{155}^\text{N}$, i.e., $\beta$-strand H and its surroundings, is easily unfolded to be exposed at the surface after denaturation.

The sandwich ELISA system constructed with MAb-1B7 and -7F5 exhibited excellent imprecision, analytical recovery, and parallelism and showed no significant interference by urinary constituents. In this assay, the L-PGDS concentration in all urine samples (0.2–16.0 mg/L of urine) was measured in 1:500 to 1:1000 dilutions, and that in all control urine samples (0.2–9.0 mg/L of urine) was measured in a single dilution (1:500). A coating step for the capture antibody (MAb-7F5) and a blocking step are not required for each assay with this ELISA format because the ELISA plates can be stored at 4 °C after these treatments. Furthermore, horseradish peroxidase-labeled secondary antibody was used for detection, rather than biotin-avidin complex. Therefore, this ELISA system is simpler than our original one (17). Moreover, urinary L-PGDS can be measured without being concentrated (25) or purified (13), as required for other assays. Considering all of these properties, this ELISA system meets the requirements for a routine clinical assay of urinary L-PGDS. There is also a nephelometric assay that uses latex particles coated with polyclonal antibody for routine clinical analysis of L-PGDS (15, 26), but this method has not been used to determine urinary L-PGDS. Our well-characterized MAbs would also be applicable to a nephelometric assay for the measurement of urinary L-PGDS.

Urinary L-PGDS ($\beta$-trace) excretion has been reported to be significantly higher in patients with chronic renal failure than in healthy individuals (16). However, urinary

Fig. 4. Relationship between urinary L-PGDS excretion and several markers for renal injuries.

Urinary L-PGDS excretion was significantly and positively correlated with urinary albumin excretion (A), NAG excretion (B), and serum Cr (C) in the patients with various renal diseases, as described in Table 3 (n = 59). Symbols are the same as in the legend for Fig. 3.
L-PGDS excretion has not been demonstrated in less advanced stages of renal disease until recently. With our original ELISA system, we demonstrated that urinary L-PGDS excretion may predict the progression of renal injuries in diabetic nephropathy (19). In accordance with that study, we observed in the present study that urinary L-PGDS excretion was significantly higher in another diabetic cohort, even in those without nephropathy (nephroalbuminuria), than in the healthy controls (Fig. 3). Furthermore, we found that urinary L-PGDS excretion was significantly higher not only in patients with diabetic nephropathy but also in those with other renal diseases, even in patients with a serum Cr concentration within reference values, than in the healthy controls. The range of urinary L-PGDS excretion in the patients with various renal diseases was broader than that for the controls. These observations suggest that urinary L-PGDS excretion may reflect the severity of renal disease. In fact, urinary L-PGDS excretion was significantly correlated with several markers of renal function (Fig. 4). In contrast to this observation, Whitsed and Penny (25) reported no definitive relationship between \( \beta \)-trace and any marker of renal function. They used a radial immunodiffusion method with polyclonal antiserum to measure urinary L-PGDS (\( \beta \)-trace); for that assay, all urine samples had to be concentrated more than 100-fold. Therefore, the discrepancy between their results and ours may partly be attributable to the assay method used in their study, which was semiquantitative and insensitive for determining the urinary L-PGDS concentration accurately.

Because low-molecular-mass proteins (<33 kDa) are readily filtered by the glomerulus (27), L-PGDS may be small enough to allow its passage through the glomerular basement membrane. Therefore, an increase in urinary L-PGDS would likely serve as a marker for tubular damage. Thus, we preliminarily evaluated the diagnostic validity of urinary L-PGDS and NAG, the latter of which is a marker for tubular damage. The sensitivities of urinary L-PGDS excretion for the renal diseases studied were almost comparable to those of urinary NAG. However, the correlation between urinary L-PGDS and NAG was relatively low (\( r = 0.38 \pm 0.43 \)). These results suggest that increased urinary L-PGDS excretion may reflect decreased reabsorption of this protein by renal tubules. At present, we have no comparative data on other low-molecular-mass proteins, such as \( \alpha_1 \)-microglobulin, \( \beta_2 \)-microglobulin, and retinol-binding protein. To demonstrate the exact diagnostic validity of urinary L-PGDS, we are planning future studies to compare L-PGDS with those low-molecular-mass proteins.

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