Absence of Increased $\alpha_1$-Microglobulin in IgA Nephropathy Proteinuria*

Hiroyuki Yokota‡§, Masashi Hiramoto‡, Hirokazu Okada¶, Yoshihiko Kanno¶, Masatoshi Yuri‡, Shuji Morita‡, Masanori Naitou‡, Atsushi Ichikawa‡, Masao Katoh‡, and Hiromichi Suzuki¶

To search for biomarkers of IgA nephropathy, protein profiles of urine samples from patients with IgA nephropathy and normal volunteers were compared using two-dimensional DIGE. Most of the 172 spots identified in the urine were serum proteins, and their amounts in IgA nephropathy urine were much higher than those in normal urine; this can be explained as proteinuria caused by glomerular dysfunction. However, only $\alpha_1$-microglobulin, also one of the major serum proteins, in IgA nephropathy urine was not higher in amount than that in normal urine. We confirmed using ELISA analysis that the amounts of transferrin and albumin in IgA nephropathy and diabetic nephropathy urine were much higher than those in normal urine, whereas the amount of $\alpha_1$-microglobulin in IgA nephropathy urine was not higher than that in normal urine and was much lower than that in diabetic nephropathy urine. Approximately 50% of $\alpha_1$-microglobulin forms a complex with IgA in serum. These results suggest that $\alpha_1$-microglobulin in IgA nephropathy urine is a characteristic protein and might be a biomarker for IgA nephropathy and that $\alpha_1$-microglobulin might have a relationship with IgA nephropathy pathology. *Molecular & Cellular Proteomics 6:738–744, 2007.

IgA nephropathy is the most common form of glomerular nephritis in the world. IgA nephropathy is characterized by the presence of glomerular IgA deposits in the mesangial region, but the cause of primary IgA nephropathy is unknown. A definitive diagnosis of IgA nephropathy can be made only by renal biopsy with immunohistological examination of IgA deposits (1). Simple and easy diagnosis methods such as using diagnostic markers in body fluids are more desirable than renal biopsy.

The proteomics approach is promising for the discovery of biomarkers in body fluids, and urine is an attractive sample because its collection is easy and non-invasive, and in addition, a urine protein profile directly reflects glomerular disease such as IgA nephropathy. However, there are few reports on urinary proteome analysis for glomerular disease (2), particularly for IgA nephropathy (3, 4). There are no known reports on practical and specific biomarkers for IgA nephropathy diagnosis.

One of the most promising proteomics techniques is two-dimensional polyacrylamide gel electrophoresis, which has been used in many types of protein expression profiling (3, 5). In particular, two-dimensional (2D) DIGE is a reproducible method, enabling a reliable and precise comparison of urinary protein profiles (6, 7). To search for urinary biomarkers of IgA nephropathy, we compared the urinary protein profiles of IgA nephropathy with normal using 2D DIGE in conjunction with mass spectrometry-based protein identification procedures. The representative proteins picked up in 2D DIGE analysis were also measured using ELISA in normal urine, IgA nephropathy, and diabetic nephropathy urine.

EXPERIMENTAL PROCEDURES

Materials and Reagents—IPG strip gels, 1-(5-carboxypentyl)-1'-propylindocarboxyanine halide (Cy3) N-hydroxysuccinimidyl ester, 1-(5-carboxypentyl)-1'-methylindocarboxyanine halide (Cy5) N-hydroxysuccinimidyl ester, and other 2D DIGE-related materials were purchased from GE Healthcare. Peroxidase-conjugated goat anti-mouse immunoglobulin antibody (AM4404) was from Invitrogen. Primary sheep anti-human $\alpha_1$-microglobulin antibody (CR3021SP) and human $\alpha_1$-microglobulin standard were from Cortex Biochem (San Leandro, CA). Secondary mouse anti-human/rat $\alpha_1$-microglobulin antibody was from AgriSera (AS01-013; Vännäs, Sweden). Primary goat anti-human transferrin antibody (AB0-128A), secondary peroxidase-conjugated goat anti-human transferrin antibody (AB0-128P), human transferrin standard, primary goat anti-human albumin antibody (AB0-129A), secondary peroxidase-conjugated goat anti-human albumin antibody (AB0-129P), and human albumin standard were from Bethyl, Inc. (Montgomery, TX). Sequencing grade modified trypsin was from Promega Corp. (Madison, WI). Tetramethylbenzidine (TMB) Soluble Reagent and TMB Stop Buffer were from ScyTek Laboratories, Inc. (West Logan, UT). Block Ace reagent was from Dainippon Sumitomo Pharma. All other reagents were of analytical grade.

Patients and Controls—At Saitama Medical University Hospital, 17 patients with IgA nephropathy diagnosed by renal biopsy, 10 healthy volunteers without any kidney or related diseases, and 16 diabetic nephropathy patients with overt proteinuria diagnosed as type 2 diabetes according to the criteria of the Japanese Diabetes Society

The abbreviations used are: 2D, two-dimensional; TMB, tetramethylbenzidine; GFR, glomerular filtration rate.

From ‡Astellas Pharm Inc., 21 Miyukigaoka, Tsukuba-shi 305-8585, Japan and the ¶Department of Nephrology and Medical Education Center, School of Medicine, Faculty of Medicine, Saitama Medical University, 38 Morohongo, Moroyama, Iruma, Saitama 350-0495, Japan
Received, August 31, 2006, and in revised form, January 10, 2007 Published, MCP Papers in Press, January 21, 2007, DOI 10.1074/mcp.M600336-MCP200

© 2007 by The American Society for Biochemistry and Molecular Biology, Inc.
This paper is available on line at http://www.mcponline.org
(8) were recruited. All patients and healthy volunteers were informed of the use of their urine samples for the study, a procedure conducted in accordance with the research plan approved by the ethics committees of both Saitama Medical University (Number 194) and Astellas Pharma Inc.

Morphological Examination—Sections (4 mm) cut from paraffin blocks were processed for hematoxylin and eosin staining and Masson’s trichrome staining. Glomerular changes or tubulointerstitial changes were quantitatively determined with Mac SCOPE software (Version 2.5; Mitani Corp., Fukui, Japan) in 10 glomeruli and in five high power (≥200) cortical fields in tissue sections, respectively. The glomerular proliferation and sclerosis scores were expressed as the mean percent area with cell proliferation per one glomerulus in hematoxylin and eosin-stained sections and as that in blue per one glomerulus in Masson’s trichrome-stained sections, respectively. The tubulointerstitial change score was also expressed as the mean percent area in blue and with tubular atrophy as well as cell infiltration per one cortical field in the Masson’s trichrome-stained sections (9).

Urine Samples—Morning midstream spot urine samples were collected and stored below −20 °C. Just before use, the urine samples were thawed and centrifuged at 3000 × g for 10 min at 4 °C to remove insoluble material. For 2D DIGE analysis, the centrifuged urine (10 ml) was concentrated by centrifugation at 3500 × g for 45 min at 4 °C using a centrifugal filter device (Amicon Ultra 15; molecular mass cutoff, 10 kDa; Millipore). Ice-cold Milli Q water (10 ml) was added into the retentate and centrifuged at 3500 × g for 45 min at 4 °C; this was repeated three times. The desalted concentrate in the Amicon Ultra 15 was dissolved and recovered with 400 μl of 8% urea, 4% CHAPS, and 50 mM Tris/His (pH 8.0). Protein concentrations were determined using the BCA method (Pierce).

2D DIGE Analysis—2D DIGE was performed according to the manufacturer’s instructions (GE Healthcare). Briefly each normal (n = 10) and IgA nephropathy (n = 17) urinary protein sample (50 μg) was labeled with Cy5. To prepare a pooled reference sample, the normal and the IgA nephropathy urinary protein samples were mixed together (100 μg each), and the mixture (50 μg) was labeled with Cy3 as an internal control of each 2D gel. The two labeled samples were mixed together and co-separated in the same gel. The first dimensional IEF was performed with pH 3–10 non-linear 18-cm IPG gels using an IPGphor apparatus, and the second dimensional SDS-PAGE was performed with a 12.5% isocratic SDS-PAGE gel between low fluorescence glass plates using the SE-600 system. The gels were then scanned with a 2D MasterImager. The spot volumes on the images were quantified with Difference In-gel Analysis software, and spot matching between separate gels was conducted visually with special care. The normalized spot ratio was calculated by dividing the volume of the Cy5 spot by that of the corresponding Cy3 spot on each gel.

MS Analysis—Protein spots of interest were excised from the gel and then subjected to in-gel trypsin digestion (10). The digested peptide mixture was concentrated on an on-line trap column (0.3 × 5 mm, PepMap C18, 5 μm, 100 Å; LC-Packings, Amsterdam, The Netherlands) and then separated with a reverse-phase microcapillary column (0.075 × 150-mm PepMap C18, 5 μm, 100 Å; LC-Packings). The separation was performed using a Nanoscale SI-1 pump (Shiseido) coupled with an Accurate microsplitter (AC-100-VAR; LC-Packings) or CapLC instrument (Waters, Milford, MA) at a flow rate of 200 nl/min with buffers of 2% acetonitrile, 0.1% formic acid (Buffer A) and 90% acetonitrile, 0.1% formic acid (Buffer B) using a 60-min gradient (4–50% B for 35 min, 90% B for 10 min, and 4% B for 15 min). The LC effluent was directly interfaced with the nanoelectrospray ion source on a Q-TOF Ultima API mass spectrometer (Waters) or on an LCQ ion trap mass spectrometer (Thermo Electron Corp., Waltham, MA). An MS survey scan was obtained for the m/z range of 400–1500, and MS/MS spectra were acquired for the two most intense ions from the survey scan. Dynamic exclusion of 2-min duration was used to acquire MS/MS spectra from low intensity ions.

Protein identification was performed using MASCOT software (Version 1.9.01, Matrix Science Inc., Boston, MA). An in-house customized database based on the National Center for Biotechnology Information (NCBI) non-redundant protein sequence database (ftp.ncbi.nih.gov/blast/db/Fasta/) with species limitation (only human, mouse, rat, and bovine proteins can pass) and with locus redundancy removal by NCBI EntrezGene (ftp.ncbi.nlm.nih.gov/ gene/) was used. For the LCQ data, DTA files (Bioworks 3.0) for each MS/MS spectrum were generated from the raw data for a peptide mass range of 400–3500 with a minimum ion count of 30. For the Q-TOF data, PKL files (ProteinLynx) were generated from the raw data, combining sequential scans with the same precursor, smoothing the spectrum with Savitzky-Golay smoothing, and measuring the peak top with a centroid top of 80%. The MASCOT search parameters were as follows. For LCQ, peptide tolerance was 2.0 Da, and MS/MS tolerance was 0.8 Da (average mass). For Q-TOF, peptide tolerance was 0.45 Da, and MS/MS tolerance was 0.15 Da (monoisotopic mass). For both instruments, fixed modification of propionamide (Cys) and variable modifications of oxidation (Met), acetylation (N-terminal), and pyro-Glu (Glu and Gln) were selected, and up to four missed trypsin cleavages were allowed. A protein score of 40 and each peptide score of 20 were the minimum identification criteria, and manual examination was conducted for all proteins identified with less than 80 points on the protein score or less than four unique peptides. The criteria used for manual validation included (a) peptide fragment ions being clearly above base-line noise, (b) intense y or b ions corresponding to the N-terminal cleavage of proline or glycine and absence of C-terminal cleavage of proline or glycine (except where proline follows), (c) the five major fragment ions being interpretable, and (d) not matching a common contaminant (such as silicone or polyethylene glycol) or trypsin-derived signals. In addition, each MS/MS spectrum of proteins identified with a small number (one or two) of peptides was compared with the same spectrum of the same proteins (if its existed) identifying using a large set (>10) of peptides for further manual confirmation.

ELISA—The amounts of α1-microglobulin in urine were measured using ELISA. The primary anti-human α1-microglobulin antibody (Cortec Biochem CR3021SP) diluted 1:5000 in 0.1 M borate buffer (pH 9.5) was added into the wells of a 96-well microplate and incubated overnight at 4 °C. After washing the wells with PBS-Tween buffer (8.1 mM Na2HPO4, 1.5 mM KH2PO4, 137 mM NaCl, 2.7 mM KCl, and 0.05% Tween 20; Block Ace reagent was added for postcoating and incubated for 1 h at 37 °C. After washing the wells with the PBS-Tween buffer, centrifuged and diluted urine samples and α1-microglobulin standard were added into the wells and incubated for 1 h at 37 °C. After washing the wells, the secondary mouse anti-human α1-microglobulin antibody (AgriSera AS01-013) diluted 1:5000 in the PBS-Tween buffer was added and incubated for 1 h at 37 °C. After washing the wells, peroxidase-conjugated anti-mouse immunoglobulin antibody (Invitrogen AM4404) was added and incubated for 1 h at 37 °C. After washing the wells, TMB substrate solution was added and incubated for 30 min at ambient temperature. The reaction was stopped with TMB Stop Buffer, and then absorbance was measured at 450 nm. The amounts of transferrin and albumin in urine were also measured with ELISA in the same way as described above using the primary anti-human transferrin antibody (1:100 dilution, Bethyl, Inc. A80-128A), the secondary peroxidase-conjugated anti-human transferrin antibody (1:100,000 dilution, Bethyl, Inc. A80-126P), the primary anti-human albumin antibody (1:100 dilution, Bethyl, Inc. A80-129A), and the secondary peroxidase-conjugated anti-human albumin antibody (1:200,000 dilution, Bethyl, Inc. A80-129P). The data were analyzed using SAS software (SAS Institute Inc., Cary, NC).
α1-Microglobulin as a Urinary Marker of IgA Nephropathy

RESULTS

The clinical profiles of the patients with IgA nephropathy and diabetic nephropathy are provided in Tables I and II. Renal function evaluated by estimated GFR in the patients with IgA nephropathy mostly remained in the normal range, and most of the patients in both groups were classified as having early stage chronic kidney disease (11, 12).

Some of the patients with IgA nephropathy lacked a pathological score because of the shortage of samples. However, IgA nephropathy was diagnosed by positive immunofluorescence staining of glomerular IgA deposition. In addition, renal biopsy was not carried out for the patients with diabetic nephropathy in our unit because it is usually diagnosed by blood test and urinalysis without pathological findings.

None of the patients with IgA nephropathy received treatment at the sampling because we determined how to treat the patients with IgA nephropathy based on the pathological findings. On the other hand, all the patients with diabetic nephropathy were treated with angiotensin-converting enzyme inhibitors with the expectation of decreasing the amount of proteinuria. Before the sampling, treatment with angiotensin-converting enzyme inhibitors was interrupted for a week to avoid influence on urinary protein excretion.

The urine samples of normal (n = 10) and IgA nephropathy (n = 17) were analyzed with 2D DIGE. Twenty-seven gels were prepared, each containing a Cy5 image of the individual normal or IgA nephropathy urine sample and a Cy3 image of an internal control. Typical Cy5 images of normal and IgA nephropathy urine are shown in Fig. 1. The protein profile of IgA nephropathy urine was very different from that in normal urine (Fig. 1), although the patterns of individual samples of normal or IgA nephropathy were relatively similar, respectively (data not shown). The 174 representative spots on the gel were picked up and identified with LC/MS/MS. Furthermore, to compare the relative amounts of the proteins in individual normal and IgA nephropathy urine, the spots were matched between all the gels, and their amounts were quantified and normalized between all the gels, and their amounts were quantified and normalized.

| Case | Sex | Age | eGFR | Uprot | Ubld | Proliferation | Sclerosis | TI change |
|------|-----|-----|------|-------|------|---------------|-----------|-----------|
| 1    | F   | 53  | 50.9 | 0.22  | (-)  | 0             | 0         | 7         |
| 2    | M   | 31  | 156.9| 0.37  | (+-) | 4             | 0         | 0         |
| 3    | F   | 60  | 107.4| 0.42  | 2+   | 30            | 3         | 0         |
| 4    | F   | 19  | 160.7| 0.11  | 4+   | 14            | 2         | 3         |
| 5    | M   | 36  | 101.7| 0.35  | (-)  | NA            | NA        | NA        |
| 6    | F   | 60  | 58.2 | 3.11  | 1+   | 21            | 12        | 0         |
| 7    | F   | 64  | 51.4 | 0.67  | 4+   | 14            | 10        | 12        |
| 8    | M   | 53  | 104.1| 0.24  | (+-) | 11            | 21        | 5         |
| 9    | M   | 34  | 143.2| 0.06  | 3+   | 0             | 17        | 17        |
| 10   | M   | 51  | 101.5| 0.39  | 1+   | 28            | 8         | 3         |
| 11   | M   | 19  | 120.5| 0.07  | 2+   | 14            | 0         | 10        |
| 12   | M   | 10  | 76.6 | 1.32  | 4+   | NA            | NA        | NA        |
| 13   | M   | 19  | 127.0| 0.17  | 4+   | 28            | 3         | 3         |
| 14   | F   | 51  | 57.8 | 1.02  | 2+   | 25            | 35        | 9         |
| 15   | M   | 26  | 104.9| 0.12  | 4+   | 9             | 0         | 0         |
| 16   | F   | 13  | 137.2| 0.24  | 4+   | 8             | 0         | 0         |
| 17   | M   | 34  | 115.1| 0.20  | 3+   | 17            | 11        | 0         |

| Case | Sex | Age | eGFR | Uprot | Ubld | HbA1c |
|------|-----|-----|------|-------|------|-------|
| 1    | F   | 58  | 69.1 | 0.23  | 1+   | 5.6   |
| 2    | F   | 50  | 85.0 | 0.57  | 2+   | 6.6   |
| 3    | M   | 56  | 78.8 | 1.03  | 1+   | 7.1   |
| 4    | M   | 69  | 61.6 | 0.54  | 2+   | 7.2   |
| 5    | F   | 60  | 50.4 | 2.45  | (-)  | 6.2   |
| 6    | M   | 46  | 94.0 | 1.69  | 1+   | 5.8   |
| 7    | M   | 61  | 85.3 | 0.16  | 2+   | 6.8   |
| 8    | M   | 48  | 83.6 | 1.68  | (+-) | 7.1   |
| 9    | F   | 59  | 89.3 | 1.70  | 1+   | 6.5   |
| 10   | M   | 39  | 88.4 | 1.24  | (-)  | 8     |
| 11   | M   | 45  | 128.2| 0.70  | (+-) | 8.2   |
| 12   | F   | 44  | 68.0 | 0.19  | 2+   | 6.2   |
| 13   | F   | 56  | 69.4 | 1.21  | (-)  | 8     |
| 14   | F   | 66  | 54.6 | 0.91  | 2+   | 6.5   |
| 15   | F   | 59  | 64.5 | 0.87  | (-)  | 6.9   |
| 16   | F   | 53  | 71.9 | 0.56  | 1+   | 7.4   |

The clinical data of patients with diabetic nephropathy

eGFR, estimated GFR; Uprot, urine protein; Ubld, urine occult blood; (-), negative; (+), just positive. In detail, (-) means less than 0.015 mg hemoglobin/dl of urine, (+) means 0.015–0.062 mg hemoglobin/dl (5–20 red blood cells/μl) of urine, which is considered as the normal upper border in the guideline proposed by the Japanese Society of Nephrology. F, female; M, male.

| Case | Sex | Age | eGFR | Uprot | Ubld | HbA1c |
|------|-----|-----|------|-------|------|-------|
| 1    | F   | 58  | 69.1 | 0.23  | 1+   | 5.6   |
| 2    | F   | 50  | 85.0 | 0.57  | 2+   | 6.6   |
| 3    | M   | 56  | 78.8 | 1.03  | 1+   | 7.1   |
| 4    | M   | 69  | 61.6 | 0.54  | 2+   | 7.2   |
| 5    | F   | 60  | 50.4 | 2.45  | (-)  | 6.2   |
| 6    | M   | 46  | 94.0 | 1.69  | 1+   | 5.8   |
| 7    | M   | 61  | 85.3 | 0.16  | 2+   | 6.8   |
| 8    | M   | 48  | 83.6 | 1.68  | (+-) | 7.1   |
| 9    | F   | 59  | 89.3 | 1.70  | 1+   | 6.5   |
| 10   | M   | 39  | 88.4 | 1.24  | (-)  | 8     |
| 11   | M   | 45  | 128.2| 0.70  | (+-) | 8.2   |
| 12   | F   | 44  | 68.0 | 0.19  | 2+   | 6.2   |
| 13   | F   | 56  | 69.4 | 1.21  | (-)  | 8     |
| 14   | F   | 66  | 54.6 | 0.91  | 2+   | 6.5   |
| 15   | F   | 59  | 64.5 | 0.87  | (-)  | 6.9   |
| 16   | F   | 53  | 71.9 | 0.56  | 1+   | 7.4   |
compared. The results are shown in Table III. More than 80% of the identified proteins were degradation products of albumin, judging from their spot migration position on the 2D gels (Fig. 1). The other protein spots were also major serum proteins such as transferrin, H9251 1-microglobulin, H9251 1-antitrypsin, and H9252 β-globin. All the identified proteins, except for H9251 1-microglobulin, were increased in IgA nephropathy urine (Table III). The increase of serum proteins in urine can be explained as proteinuria caused by glomerular dysfunction in IgA nephropathy.

The amounts in individual urine are shown for several representative proteins in Fig. 2. The amounts of transferrin, retinol-binding protein 4, and H9252 β-globin in individual IgA nephropathy urine were much higher than those in normal. However, the amount of H9251 1-microglobulin was much lower than that in normal, suggesting that a decrease in H9251 1-microglobulin in urine is a possible marker for IgA nephropathy.

To compare the absolute amounts of H9251 1-microglobulin in the normal and the IgA nephropathy urine more precisely, we performed ELISA analysis of H9251 1-microglobulin, transferrin, and albumin. In addition, to compare another renal disease with IgA nephropathy, we also measured the amounts in diabetic nephropathy urine. The results are shown in Fig. 3. The amounts of transferrin and albumin were much higher in IgA nephropathy and diabetic nephropathy urine than those in normal urine (p < 0.0001). However, the amount of H9251 1-microglobulin in IgA nephropathy urine was not higher than that in normal urine, whereas the amount in diabetic nephropathy urine was much higher than that in normal urine (p < 0.0001). These results also indicate that H9251 1-microglobulin is a characteristic protein among other serum proteins in IgA nephropathy urine.

**DISCUSSION**

H9251 1-Microglobulin, also called protein HC, is a lipocalin carrying unknown heterogeneous chromophores (13). It is one of

**TABLE III**

The identified proteins of representative spots on 2D gels

The data were obtained from 27 gels, each containing a Cy5 image of the individual normal (n = 10) or IgA nephropathy urine sample (n = 17) and a Cy3 image of an internal control.

| Accession no. | Protein name a | Spots b | Normalized spot volume ratio median (range) |
|---------------|----------------|--------|-------------------------------------------|
| Increased     |                |        |                                           |
| NP_000468     | Albumin        | 122    | 4.3 (1.5–32.6)                            |
| NP_001054     | Transferrin    | 15     | 7.2 (3.9–10.3)                            |
| NP_000286     | H9251 1-Antitrypsin | 8        | 3.1 (2.0–5.7)                            |
| NP_000509     | β-Globin       | 7      | 5.7 (2.4–7.5)                            |
| NP_000508     | α2-Globin      | 3      | 10.7 (5.2–11.7)                           |
| NP_001729     | Carbonic anhydrase I | 3        | 3.3 (1.8–4.4)                            |
| NP_000090     | Cystatin C     | 1      | 5.4                                        |
| NP_006735     | Retinol-binding protein 4 | 1        | 4.2                                        |
| NP_001176     | α2'-Glycoprotein 1 | 1       | 4.2                                        |
| Decreased     |                |        |                                           |
| NP_001624     | H9251 1-Microglobulin | 11    | 0.16 (0.12–0.26)                         |

*a* The top scoring protein identified with the MASCOT program from each spot is listed in this table.

*b* Two spots that did not satisfy our minimum identification criteria described in the under “Experimental Procedures” were excluded from this table.
the major serum proteins, and its exact function is not known (13). α₁-Microglobulin in urine is reported to be a marker of glomerular and renal tubular dysfunction, and its amounts are greatly increased in many types of renal disease (14–18). However, the amount of α₁-microglobulin in IgA nephropathy urine was lower than that in normal urine in our 2D DIGE analysis, whereas the amounts of other serum proteins identified in IgA nephropathy urine were much higher than those in normal urine (Table III). There was no significant correlation between the amounts of urinary α₁-microglobulin of IgA nephropathy patients and their tubulointerstitial change scores (data not shown), also supporting that this phenomenon is not due to the difference of severity of tubular dysfunction. Park et al. (3) have reported that many kinds of urinary protein differed in amount between normal and IgA nephropathy using two-dimensional electrophoresis and silver staining. The data in the literature (3) include decreased amounts of α₁-microglobulin in IgA nephropathy urine compared with normal urine, consistent with our 2D DIGE results. On the other hand, our ELISA analysis showed that the amount of α₁-microglobulin in IgA nephropathy urine was almost equal to that in normal urine. The apparent difference between the results of 2D DIGE and ELISA can be explained by the fact that absolute quantification of each protein is difficult using 2D DIGE because 2D DIGE quantification is based on the relative ratio of the protein spots among all the protein spots on the 2D gels where constant amounts of total urinary proteins are applied. The observed difference in urinary α₁-microglobulin amounts between IgA nephropathy and diabetic nephropathy (Fig. 3) was also supported by the data in the previous report (14), which showed that the amount of α₁-microglobulin in IgA nephropathy urine was lower than that in diabetic nephropathy and chronic glomerulonephritis urine. IgA nephropathy and diabetic nephropathy are renal diseases with glomerular dysfunction, and the amounts of albumin and transferrin in the urine, which are also urinary markers of glomerular dysfunction, are much higher than those in normal urine (Fig. 3). These results suggest that α₁-microglobulin is a characteristic protein among other serum proteins in IgA nephropathy urine and that the relative amount of α₁-microglobulin among other serum proteins in urine is a useful indicator in IgA nephropathy diagnosis. Approximately 50% of α₁-microglobulin forms a complex
with IgA in serum by a reduction-resistant bond (13); this may have a close relationship with these results. Itoh and co-workers (15) have reported that 1-microglobulin was stained by immunofluorescence in the mesangial regions in IgA nephropathy renal biopsy. The most characteristic pathological finding in IgA nephropathy is the mesangial region where IgA deposits are predominantly found (1, 15). To elucidate the pathology of IgA nephropathy, much attention has been paid to IgA itself, such as the abnormality of the IgA carbohydrate chain (1), but the importance of 1-microglobulin may have been overlooked. Our results suggest that 1-microglobulin might have a close relationship with IgA nephropathy, although the specificity of this 1-microglobulin phenomenon for IgA nephropathy also remains to be further confirmed by studying other related diseases.

1-Microglobulin is one of the major lipocalins and also binds to unidentified small substances (13). The Cy5 image of normal urine (Fig. 1) where 1-microglobulin is prominent is different from other two-dimensional gel electrophoresis images of normal urine reported by previous studies using silver staining without Cy dye where 1-microglobulin is not so dominant (3). The reason for the apparent difference may be that 1-microglobulin easily binds to Cy dyes because of its lipocalin character. One hypothesis is that abnormal forms of 1-microglobulin or 1-microglobulin bound to pathogenic substances in serum makes a complex with IgA that might be deposited in renal mesangial regions, causing IgA nephropathy.

Acknowledgments—We thank Takeyuki Yatsu, Sadao Kuromitsu, Masato Kobori, Hitoshi Matsushime, and Kiyoshi Furuichi for support and many helpful discussions. We also thank Mari Masumoto and Rika Hashimoto for technical assistance.

* The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

§ To whom correspondence should be addressed. Tel.: 81-29-852-5111; Fax: 81-29-852-5444; E-mail: hiroyuki.yokota@jp.astellas.com.
REFERENCES

1. Donadio, J. V., and Grande, J. P. (2002) IgA Nephropathy. *N. Engl. J. Med.* 10, 738–748
2. Thongboonkerd, V., and Malasit, P. (2005) Renal and urinary proteomics: current applications and challenges. *Proteomics* 5, 1033–1042
3. Park, M. R., Wang, E. H., Jin, D. C., Cha, J. H., Lee, K. H., Yang, C. W., Kang, C. S., and Choi, Y. J. (2006) Establishment of a 2-D human urinary proteomic map in IgA nephropathy. *Proteomics* 6, 1066–1076
4. Haubitz, M., Wittke, S., Weissinger, E. M., Walden, M., Rupprecht, H. D., Floege, J., Haller, H., and Mischak, H. (2005) Urine protein patterns can serve as diagnostic tools in patients with IgA nephropathy. *Kidney Int.* 67, 2313–2320
5. Mori, Y., Kondo, T., Yamada, T., Tsuchida, A., Aoki, T., and Hirohashi, S. (2005) Two-dimensional electrophoresis database of fluorescence-labeled proteins of colon cancer cells. *J. Chromatogr. B Anal. Technol. Biomed. Life Sci.* 823, 82–97
6. Unlü, M., Morgan, M. E., and Minden, J. S. (1997) Difference gel electrophoresis: a single gel method for detecting changes in protein extracts. *Electrophoresis* 18, 2071–2077
7. Tonge, R., Shaw, J., Middleton, B., Rowlinson, R., Rayner, S., Young, J., Pognan, F., Hawkins, E., Currie, I., and Davison, M. (2001) Validation and development of fluorescence two-dimensional differential gel electrophoresis proteomics technology. *Proteomics* 1, 377–396
8. Kuzuya, T., Nakagawa, S., Satoh, J., Kanazawa, Y., Iwamoto Y., Kobayashi, M., Nanjo, K., Sasaki, A., Seino, Y., Ito, C., Shima, K., Nonsaka, K., and Kadowaki, T. (1999) Report of the Committee of Japan Diabetes Society on the classification and diagnostic criteria of diabetes mellitus. *J. Japan Diabetes Soc.* 42, 385–404
9. Okada, H., Inoue, T., Kikuta, T., Watanabe, Y., Kanno, Y., Ban, S., Sugaya, T., Horiuchi, M., and Suzuki, H. (2006) A possible anti-inflammatory role of angiotensin II type 2 receptor in immune-mediated glomerulonephritis during type 1 receptor blockade. *Am. J. Pathol.* 169, 1577–1589
10. Shevchenko, A., Wilm, M., Vorm, O., and Mann, M. (1996) Mass spectrometric sequencing of proteins silver-stained polyacrylamide gels. *Anal. Chem.* 68, 850–858
11. Taal, M. W., and Brenner, B. M. (2006) Predicting initiation and progression of chronic kidney disease: developing renal risk scores. *Kidney Int.* 70, 1694–1705
12. Cockcroft, D. W., and Gault, M. W. (1976) Prediction of creatinine clearance from serum creatinine. *Nephron* 16, 31–41
13. Åkerström, B., Lögdberg, L., Berggård, T., Osmark, P., and Lindqvist, A. (2000) α1-Microglobulin: a yellow-brown lipocalin. *Biochim. Biophys. Acta* 1482, 172–184
14. Woo, K. T., and Lau, Y. K. (1997) Pattern of proteinuria in tubular injury and glomerular hyperfiltration. *Ann. Acad. Med. Singapore* 26, 465–470
15. Murakami, T., Kawakami, H., Kobayashi, K., and Itoh, Y. (1989) Glomerular alpha-1-microglobulin in IgA nephropathies. *Am. J. Nephrol.* 9, 438–439
16. Itoh, Y., and Kawai, T. (1990) Human α1-microglobulin: its measurement and clinical significance. *J. Clin. Lab. Anal.* 4, 376–384
17. Bakoush, O., Grubb, A., Rippe, B., and Tencer, J. (2001) Urine excretion of protein HC in proteinuric glomerular diseases correlates to urine IgG but not to albuminuria. *Kidney Int.* 60, 1904–1909
18. Norden, A. G., Scheiman S. J., Deschiodt-Lanciman, M. M., Lapsley, M., Nortier J. L., Thakker, R. V., Unwin, R. J., and Wrong, O. (2000) Tubular proteinuria defined by a study of Dent’s (CLCN5 mutation) and other tubular diseases. *Kidney Int.* 57, 240–249