Elevated urinary excretion of immunoglobulins in nonproteinuric patients with type 1 diabetes

Tomohito Gohda,1,2,3 William H. Walker,1 Pawel Wolkow,1,2,4 Jung Eun Lee,1,2,5 James H. Warram,1,2 Andrzej S. Krolewski,1,2 and Monika A. Niewczas1,2

1Research Division, Joslin Diabetes Center, Harvard Medical School, Boston, Massachusetts; 2Department of Medicine, Harvard Medical School, Boston, Massachusetts; 3Division of Nephrology, Department of Internal Medicine, Juntendo University School of Medicine, Japan; 4Department of Pharmacology, Faculty of Medicine, Jagiellonian University, Krakow, Poland; and 5Department of Medicine, Samsung Medical Centre, Sungkyunkwan University School of Medicine, Seoul, Korea

Submitted 8 August 2011; accepted in final form 10 April 2012

Gohda T, Walker WH, Wolkow P, Lee JE, Warram JH, Krolewski AS, Niewczas MA. Elevated urinary excretion of immunoglobulins in nonproteinuric patients with type 1 diabetes. Am J Physiol Renal Physiol 303: F157–F162, 2012. First published April 18, 2012; doi:10.1152/ajprenal.00443.2011.—Increased albuminuria is a hallmark of early diabetic nephropathy, whereas the role of immunoglobulins (Igs), such as IgG (its 1–4 subtypes), IgA, and IgM, different in charge and size, has not been examined in early nephropathy in the past due to a lack of a sensitive and reliable method. Our study group consisted of subjects with type 1 diabetes (T1D) and normoalbuminuria (n = 78), microalbuminuria (n = 78), and of 75 healthy subjects (HS). A Luminex-based immunoassay (1,000 time more sensitive than nephelometry-based method) was validated for the urine matrix and used for the measurements of IgG1–4, IgA, and IgM in our study groups. The Luminex-based assay detected Igs in 87% of HS subjects and in 100% of T1D subjects. Recovery of known amounts of Igs added to urine was 92–118%. In the normoalbuminuria group, urinary concentrations of albumin, IgG2, IgA, and IgM were significantly higher than in HS, whereas in the microalbuminuria, further elevation of IgG2, IgG4, and IgA was the most pronounced. In all three groups, fractional excretion of Igs was at least 100 times lower than that of albumin. Fractional excretion of IgG2 was the highest among all Igs. We validated a sensitive method for measuring Igs in urine using Luminex. We found that elevated concentrations of Igs, particularly in IgG2 and IgA, is present in subjects with T1D and no proteinuria. Elevation of those particular Ig subtypes suggests a contribution of novel mechanisms in early diabetic nephropathy, different from charge and size barrier impairment.

IN A TRADITIONAL MODEL of a diabetic nephropathy course, an increase in excretion of anionic albumin precedes a rise in excretion of immunoglobulins (Igs), the second abundant serum fraction, larger in size and of different charge, however. A small number of studies examined urinary excretion of total IgG and/or its cationic subtype IgG4 in subjects with diabetes (9, 10, 12, 33). The detailed urinary excretion profile of all Ig subclasses and IgG subtypes has not been studied in subjects with diabetes and no proteinuria so far, due to the lack of a sensitive and reliable method. Moreover, a profile of Ig excretion in healthy subjects (HS) has not been studied either. An excretion pattern of these proteins will allow us to gain knowledge on the role of impairment of the glomerular size and charge barrier selectivity in the diabetic nephropathy course.

Therefore, the goal of this study was to validate a newly developed sensitive Luminex-based immunoassay for measuring urinary concentrations of those proteins and to determine their urinary excretion profile in subjects with type 1 diabetes (T1D) and no proteinuria to compare with healthy individuals.

PATIENTS AND METHODS

Study protocol and informed consent procedures were approved by the Committee on Human Subjects of the Joslin Diabetes Center.

Patient rosters. For this study, we selected 75 HS from among nondiabetic, unrelated participants in our family study on genetics of type 2 diabetes and diabetic nephropathy. The characteristics of families and study protocols of the study were published previously (24).

Patients with T1D were selected from among the participants of the 2nd Joslin Kidney Study. The Joslin study of a prospective design includes 667 subjects with T1D and no proteinuria, whose diabetic nephropathy progression is studied over time. The study design and description of this cohort have already been published (20, 27).

For this study, we selected 78 random patients with normoalbuminuria (NA) [urinary albumin-to-creatinine ratio (ACR) <17 μg/g creatinine for men and <25 μg/g creatinine for women] and 78 patients with microalbuminuria (MA) (ACR in the range 17–250 μg/g creatinine for men and 25–355 μg/g creatinine for women). Albuminuria status was defined based on the multiple measurements of ACR in 2 yr preceding the baseline examination. Only subjects who had all albuminuria measurements in 2-yr recruitment period falling consistently into either NA or MA category were considered for this study group selection. Creatinine-based glomerular filtration rate (GFR) was estimated by Modification of Diet in Renal Disease (MDRD) formula (MDRD-GFR) (18).

All relevant clinical and laboratory data from the previous studies were used in the present study. All samples for both the pilot and main studies were handled in the same way. Spot urine specimens were collected from patients into sterile cups (Vacutainer Urine Collection Cup; Cardinal Health, Dublin, OH) during the daytime, then centrifuged at 10,000 g for 5 min in the Centrifuge 5415D (Eppendorf, Hauppauge, NY), aliquoted in a smaller amount, and frozen without preservatives at −80°C until further analysis (up to 5 yr).

Measurements of concentrations of urinary albumin and Igs using high-sensitivity assays. All markers were measured by immunoassay using Luminex 100S (Luminex, Austin, TX). To measure urinary concentrations of isotypes of Igs (IgA, IgM) and subtypes of IgG1–4, we used Human Immunoglobulin Isotyping Panel (HGAM-301; Millipore, Billerica, MA). Albumin was measured with Human Skin Panel (SKIN-50K; Millipore). The assays were performed according to the manufacturer’s instruction.
Briefly, Ig assays were performed on a 96-well microtiter filter plate (Millipore). Samples were thawed at room temperature before assay. Subsequently, 25 μl of sample were added in dilution 1:2 with assay buffer to control for the matrix effect (16). The samples were incubated with the capturing antibody-coated beads (targeting heavy chain of the respective iso- or subtype) and subsequently with streptavidin-phycocerythrin-tagged anti-human κ- and λ-light chain. The plate was analyzed on a Luminex 100S, and the median fluorescent intensities data were interpolated through five-parameter curve-fitting algorithm.

Since this assay has not been designed primarily for urinary application, first, we validated the assay in the urines of the population of our interest. We spiked 10 randomly selected samples from each strata (NA, MA, and HS) with the known amount of the respective Ig sub/isotype or albumin. All samples were run in duplicate. Specifically, we added 25 μl of Human Multi-Immunoglobulin Standard (HGAM-8301, Millipore) diluted in the assay buffer with the respective concentrations: IgG1, 124 ng/ml; IgG2, 370 ng/ml; IgG3, 1.9 ng/ml; IgG4, 3.7 ng/ml; IgA, 18.5 ng/ml; IgM, 30.9 ng/ml, equal point three of the seven-point standard curve. Recovery was calculated according to the formula:

\[
\text{Recovery (\%)} = \left( \frac{\text{spiked sample} - \text{unspiked sample}}{\text{expected}} \right) \times 100
\]

To evaluate the matrix effect, we performed a pilot experiment, where we measured Igs in 40 samples from subjects with NA and 40 with MA in parallel with pH measurements with the usage of pHmeter S20FB (Mettler Toledo, Columbus, OH). Median pH (range) for this group was 5.86 (4.96–9.03). There was no correlation between pH with MA in parallel with pH measurements with the usage of pHmeter where we measured Igs in 40 samples from subjects with NA and 40 subjects with MA.

To measure serum concentrations of isotypes of Igs and IgG subtypes, we used Human Immunoglobulin Isotyping Panel (HGAM-301) on a BN ProSpec instrument (Siemens Healthcare Diagnostics). The protein/serum protein

To validate the assay in the urines of the population of our interest, we spiked 10 randomly selected samples from each strata (NA, MA, and HS) with the known amount of the respective Ig sub/isotype or albumin. All samples were run in duplicate. Specifically, we added 25 μl of Human Multi-Immunoglobulin Standard (HGAM-8301, Millipore) diluted in the assay buffer with the respective concentrations: IgG1, 124 ng/ml; IgG2, 370 ng/ml; IgG3, 1.9 ng/ml; IgG4, 3.7 ng/ml; IgA, 18.5 ng/ml; IgM, 30.9 ng/ml, equal point three of the seven-point standard curve. Recovery was calculated according to the formula:

\[
\text{Recovery (\%)} = \left( \frac{\text{spiked sample} - \text{unspiked sample}}{\text{expected}} \right) \times 100
\]

To evaluate the matrix effect, we performed a pilot experiment, where we measured Igs in 40 samples from subjects with NA and 40 with MA in parallel with pH measurements with the usage of pHmeter S20FB (Mettler Toledo, Columbus, OH). Median pH (range) for this group was 5.86 (4.96–9.03). There was no correlation between pH with MA in parallel with pH measurements with the usage of pHmeter where we measured Igs in 40 samples from subjects with NA and 40 subjects with MA.

To measure serum concentrations of isotypes of Igs and IgG subtypes, we used Human Immunoglobulin Isotyping Panel (HGAM-301) on a BN ProSpec instrument (Siemens Healthcare Diagnostics). The protein/serum protein

### Table 1. Clinical characteristics of the study groups

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>HS</th>
<th>NA</th>
<th>MA</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>75</td>
<td>78</td>
<td>78</td>
</tr>
<tr>
<td>Age, yr</td>
<td>35 ± 11</td>
<td>40 ± 12</td>
<td>42 ± 10</td>
</tr>
<tr>
<td>Male, no. (%)</td>
<td>36 (48)</td>
<td>38 (49)</td>
<td>48 (62)</td>
</tr>
<tr>
<td>BMI, kg/m²</td>
<td>25.1 ± 4.0</td>
<td>26.3 ± 4.0</td>
<td>27.8 ± 5.4</td>
</tr>
<tr>
<td>SBP, mmHg</td>
<td>122 ± 14</td>
<td>118 ± 14</td>
<td>126 ± 15</td>
</tr>
<tr>
<td>DBP, mmHg</td>
<td>76 ± 9</td>
<td>71 ± 7</td>
<td>74 ± 7</td>
</tr>
<tr>
<td>HbA1C, %</td>
<td>5.2 ± 0.5</td>
<td>8.1 ± 1.2</td>
<td>8.7 ± 1.4</td>
</tr>
<tr>
<td>Duration of diabetes, yr</td>
<td>21 ± 9</td>
<td>23 ± 9</td>
<td>23 ± 9</td>
</tr>
<tr>
<td>MDRD-based GFR, ml·min⁻¹·1.73 m⁻²</td>
<td>90 ± 25</td>
<td>94 ± 24</td>
<td>87 ± 25</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>P Value</th>
<th>NA vs. HS</th>
<th>MA vs. NA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>0.02</td>
<td>1.0</td>
</tr>
<tr>
<td>Male</td>
<td>0.84</td>
<td>0.20</td>
</tr>
<tr>
<td>BMI</td>
<td>0.27</td>
<td>0.11</td>
</tr>
<tr>
<td>SBP</td>
<td>0.51</td>
<td>0.906</td>
</tr>
<tr>
<td>DBP</td>
<td>0.004</td>
<td>0.20</td>
</tr>
<tr>
<td>HbA1C</td>
<td>&lt;0.0001</td>
<td>0.0006</td>
</tr>
<tr>
<td>Duration of diabetes, yr</td>
<td>0.09</td>
<td>0.17</td>
</tr>
<tr>
<td>MDRD-based GFR, ml·min⁻¹·1.73 m⁻²</td>
<td>0.7</td>
<td>0.17</td>
</tr>
</tbody>
</table>

Values are means ± SD or proportion, respectively; n, no. of subjects. Differences between groups were assessed using χ² tests for categorical variables or by analysis of variance for continuous variables. Bonferroni correction was applied. BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; HbA1C, hemoglobin A1C; MDRD, modification of diet in renal disease formula; GFR, glomerular filtration rate; HS, healthy subjects; NA, patients with normoalbuminuria; MA, patients with microalbuminuria.

**RESULTS**

**Characteristics of the study groups.** Clinical characteristics of T1D subjects with NA and MA and of HS are summarized in Table 1. T1D subjects were older, had higher body mass index and higher hemoglobin A1c compared with the HS group. Subjects with T1D and increased albumin excretion (MA) did not differ in regards to age, sex distribution, or MDRD-GFR, but had higher body mass index, hemoglobin A1c, and blood pressure values compared to subjects with T1D and albuminuria in the normal range (NA). Hyperfiltration was present in 8% of the subjects with T1D (8 with NA and 4 with MA, respectively) (11).

**Detectability of albumin and Igs in urine.** To validate the Luminex-based assay to measure albumin and Igs in urine matrix, we conducted two pilot studies. First, we evaluated detectability in 30 individuals (10 randomly selected subjects from each group: HS, NA, and MA). The lowest limit of quantification derives from the five-parameter logistic standard curve and is calculated with the usage of Milliplex Analyst software (Millipore). In-house experiments based on three independent runs showed that median CV (25th and 75th percentile) for all standard points of all Igs measured and run in replicates was 5% (2–9%) (16).

Second, in the same urine subset, we measured the recovery of the specific proteins after spiking the specimens with the known quantities of the respective proteins. The results are shown in Table 2. In this pilot experiment, we were able to detect albumin in 100% of urine samples, whereas Ig detection varied by iso- and subtypes, with 87% being the lowest, and...
95% being the average. Urinary albumin was measured by a Luminex-based assay and, in addition, by nephelometry, with the latter method having been approved for a clinical use. Both methods were able to detect albumin in 100% samples. Correlation of the albumin concentrations obtained with the two methods was very strong (Spearman correlation coefficient \( r = 0.93, P < 0.001 \)). Nevertheless, nephelometry is a not sensitive enough assay to detect Igs in the urine of subjects without overt proteinuria (the detection limit is 1,000 times higher than that of the Luminex-based assay).

Furthermore, the performance of spike recoveries was very satisfactory for each protein measured, as evaluated in three groups, HS, NA, MA, separately (between 92 and 118%).

Comparison of urinary concentrations of albumin and Igs among the study groups. Table 3 shows urinary concentrations of albumin and Igs adjusted for urinary creatinine in the three study groups, HS, NA, and MA, respectively, and FE of those proteins in diabetic subjects with NA and MA. In all groups, albumin was the most abundant protein in the urine, with IgG2 being the second. The least abundant Igs in urine were IgG3 and IgG4. In all groups, FE of Igs was at least 100 times lower than that of albumin. FE of IgG2 in subjects was more pronouncedly elevated than FE of IgG1 or IgG3. FE of IgG4 was higher than of IgG1 and IgG3; comparable to FE of IgA and IgM and lower than FE of IgG2. Serum concentrations of Igs did not differ between subjects with NA and MA.

Subsequently, the obtained data were expressed as fold-changes across albuminuria strata and are illustrated in Fig. 1. Figure 1A shows concentrations of urinary proteins in diabetic subjects, with NA divided by concentration of the same urinary proteins in HS. Urinary concentrations of IgG2, IgA, and IgM were elevated 2.5 times or more in NA compared with HS, whereas urinary albumin concentrations differed by only 1.8-fold. Urinary concentrations of IgG1, IgG3, and IgG4 in the NA group did not statistically differ from those of the HS group. Figure 1B shows concentrations of urinary proteins in diabetic subjects with MA divided by concentration of the same urinary proteins in HS. The fold-changes in the MA group were much more pronounced. The highest fold-changes were for albumin and IgA (fold-change for each, 17-fold); for IgG2 13-fold and for IgG4 11-fold. Lower pronounced elevation was observed for IgG1, IgG3, and IgM (fold-change in the range four- to sevenfold). Information on size and charge of the protein and their serum concentrations are summarized together in Table 4. There was no relationship between elevated excretion of the specific protein and either its respective charge, size, or its abundance in serum.

**DISCUSSION**

In this study, we demonstrated that Luminex-based assays are sensitive and reliable methods for measuring albumin and iso- and subtypes of Igs in the urine of nonproteinuric subjects. We found that healthy individuals excrete into urine, in addition to albumin, a noticeable amount of proteins of larger size and of different charge, such as IgG2, IgA, and even IgM. Urinary excretion of these proteins was significantly higher in subjects with T1D and NA and was elevated further in the MA range.

We report here an expanded urinary profile of isotypes of IgG, IgA, and IgM, together with IgG subtypes measured in its intact forms, in subjects without proteinuria, with and without diabetes. Until now, nephelometry was the only method available for measurements of the entire profile of iso- and subtypes of Igs, but it was not sensitive enough to detect those molecules in the urine. Regarding the method principle, it shares some similarities with the Luminex-based method introduced here, in terms of conducting antigen-antibody reaction in solution (three-dimensional exposure). Nevertheless, nephelometry measures an intensity of scattering light from antigen-antibody complexes (aggregation), whereas Luminex reads fluorescence intensity of aggregated enzyme-labeled antibody with flow cytometry. It results in an enhanced ability of Luminex-based platform to detect 1,000 times smaller amount of the proteins than nephelometry is capable of. Nephelometry is a very clinically useful tool for the measurements of Igs in serum though. Urinary albumin was measured by those two methods, and it was very reproducible in our study.

Intact Ig consists of two heavy chains (determining its isotype) and two light chains (\( \kappa \) and \( \lambda \)). Increased excretion of light chains correlating, also to a certain degree with albuminuria, has been reported in diabetic nephropathy (13). Nevertheless, the principle of our assay, which requires ultimate binding to both heavy and light chains, ascertainsthat our findings represent elevated excretion only of intact form of Igs.

The glomerular filtration barrier consists of three components: endothelium, negatively charged basement membrane, and the podocyte slit diaphragm, functioning together as a size-
and charge-selective barrier. Various theories exist (8, 13), but the most accepted one speculates on the presence in healthy individuals of some number of the small pores that allow albumin leakage, a minimal number of large pores that could potentially allow the leakage of larger proteins as IgG, and hardly any shunts that would allow IgM excretion (8, 21). By demonstrating the presence of IgG as well as IgM in the urine of our study subjects, we support the theory of an existence of

Fig. 1. Ratio of urinary excretion of albumin and immunoglobulins (Igs; their iso- and subtypes) in comparison between subjects with type 1 diabetes and normoalbuminuria (NA) vs. healthy subjects (HS; A), and between microalbuminuria (MA) vs. HS (B). Solid bars, proteins anionic in charge; shaded bars, cationic proteins. Significance between the groups was tested by analysis of variance (SAS command, proc glm/lsmeans) with urinary Igs adjusted by creatinine and transformed to its logarithms (base 10) as a dependent variable, whereas groups HS, NA, and MA were considered as independent class variables in the model. Bonferroni correction (n = 3) was applied. *P value < 0.05.

Table 4. Characteristics of albumin and Ig iso/subtypes

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Molecular Mass, kDa</th>
<th>Stoke Radius, nm</th>
<th>Isoelectric Point</th>
<th>Normal Value in Serum, g/l</th>
</tr>
</thead>
<tbody>
<tr>
<td>Albumin</td>
<td>69</td>
<td>3.6</td>
<td>4.7–5.5</td>
<td>35–50</td>
</tr>
<tr>
<td>IgG1</td>
<td>156</td>
<td>5.5</td>
<td>7.0–9.5</td>
<td>5.9 ± 1.6</td>
</tr>
<tr>
<td>IgG2</td>
<td>156</td>
<td>5.5</td>
<td>7.0–9.5</td>
<td>3.6 ± 1.1</td>
</tr>
<tr>
<td>IgG3</td>
<td>156–170</td>
<td>5.5</td>
<td>8.0–9.0</td>
<td>0.6 ± 0.2</td>
</tr>
<tr>
<td>IgG4</td>
<td>156</td>
<td>5.5</td>
<td>5.5–6.0</td>
<td>0.6 ± 0.4</td>
</tr>
<tr>
<td>IgA</td>
<td>160</td>
<td>6.1</td>
<td>4.0–7.1 (4.7–5.9)</td>
<td>2.3 ± 0.8</td>
</tr>
<tr>
<td>IgM</td>
<td>900</td>
<td>12.0</td>
<td>4.0–9.1 (5.5–6.7)</td>
<td>1.6 ± 0.6</td>
</tr>
</tbody>
</table>

Values are fold change of urinary concentrations of those proteins in subjects with T1D and NA or MA in comparison with HS. *P value <0.05. Isoelectric point values are shown as range (peak) (25). Normal value in serum is shown as reference interval or mean ± SD (17).
all three types of pores in subjects without proteinuria. In our study, we also show that the urinary profile of Igs is not a simple fraction of the respective serum profile of the proteins. The most abundant Igs in serum are IgG1 (31), whereas in urine IgG2 was the most abundant Ig. IgG2 was also characterized by the highest FE among Igs, despite the fact that IgG1, -2, and -3 are similar in size and charge. A ratio of anionic IgG4 to the remaining cationic IgGs regarded as a selectivity index has been proposed to represent a charge barrier impairment (9, 10, 12). Interestingly, in our study, anionic in charge IgG4 was excreted in favor to the cationic IgG1 or IgG3, but its excretion was still lower than FE of the other cationic subtype G2.

In addition to physical characteristics (size and charge) of Igs, they differ in regards to other chemical and biological properties (like complement activation, ability to bind macrophages, and regulation by immune system). IgG3 has a significantly shorter half-life compared with the other IgG subtypes. On the other hand, IgG2 is the most resistant to the proteolytic enzymes. It has the least flexible hinge region and is capable of opsonization (15, 17, 32).

The glomerular barrier as a major determinant of the subsequent increase in protein excretion has also been recently called into question. A number of studies showed that tubular function is important for albumin handling. Impaired tubular uptake of albumin was shown to be a major determinant of albuminuria in streptozocin-induced mice (28). In addition, low concentrations of kidney injury molecule-1, a tubular injury marker, were recently shown to be associated with MA regression in the prospective study in humans (34). Numerous studies by the group of Comper et al. (22, 23, 29) supported the hypothesis that a nephrotic range of albumin is filtered via the glomerulus in healthy individuals, providing another piece of evidence for the importance of tubular processing. Protein charge impacts not only passage via the glomerulus, but also interaction with tubules (7). Cationic proteins bind more avidly to the negatively charged proximal tubules, but, again, impact of the charge in either glomerulus or tubules did not explain our findings.

Tubular uptake neither depends on the charge only, nor is it a high-affinity low-specificity process, as it was primarily anticipated. Albumin is transported by the megalin-cubulin system in proximal tubules. The neonatal Fc receptor present in podocytes, proximal tubule cells, and vascular endothelium is involved in albumin and IgG handling by the kidney, as described in the animal model (2, 30). Whether different iso- and subtypes of Igs have distinct receptors in the kidney remains unknown.

Our motivation to study Igs was led by a number of reports that pointed to the importance of proteins other than albumin as mediators of the toxic effect on the kidney and on mutual deposition of IgG and albumin in the diabetic kidney. Distinct toxic effects have been demonstrated in the in vitro study of proximal tubules exposed to albumin and to proteins derived from the proteinuric urine (4). Moreover, albumin was shown to be nontoxic to proximal tubules (5). Exposure of proximal tubules to IgG (but not to albumin) resulted in the increased inflammatory response in another study (26). In the diabetic kidney, infiltrates of IgG+ B cells and IgG deposition (colocalizing with C3 staining, macrophage accumulation, and chemokine expression) were demonstrated in diabetic nono-
IMMUNOGLOBULINURIA IN EARLY DIABETIC NEPHROPATHY


Christensen EI, Rennke HG, Carone FA. 13.


D'Amico G, Bazzi C, 10.

Osicka TM, MacIsaac RJ, Jerums G, Comper WD. 22.


