Determination of Urinary Lysozyme for Potential Detection of Tubular Dysfunction in Diabetic Nephropathy

Kenji Shima,† Meisei Hirota,† Masahiro Fukuda,‡ and Akira Tanaka‡

Seeking to study whether measurement of lysozyme (EC 3.2.1.17) in urine by a reliable radioimmunoassay can provide a suitable index of renal tubular function and how lysozymuria develops in temporal relation to proteinuria in diabetic nephropathy, we have compared the urinary excretion of lysozyme and β2-microglobulin with the 15-min excretion rate of phenolsulfonphthalein in 39 patients with Type 2 (non-insulin-dependent) diabetes and investigated the temporal relation between the onset of lysozymuria and proteinuria in 15 patients with Type 1 (insulin-dependent) diabetes. The concentrations of lysozyme and β2-microglobulin in urine increased in proportion to the decrease in the rate of excretion of phenolsulfonphthalein in these patients. The coefficient of correlation between lysozyme concentration and the 15-min excretion rate of phenolsulfonphthalein (r = −0.70) was higher than that between β2-microglobulin concentration and the 15-min excretion rate of phenolsulfonphthalein (r = −0.46). Abnormally high lysozymuria, suggesting the existence of tubular dysfunction, was demonstrated in six of the patients with Type 1 diabetes who showed no proteinuria or only a slight increase in urinary protein excretion. Lysozymuria may thus be added to a list of the indicators for diabetic nephropathy.

Additional Keyphrases: β2-microglobulin • phenolsulfonphthalein test • creatinine clearance • diabetes mellitus • hemoglobin A1c • plasma glucose during fasting

There is good evidence that the excretion of proteins of low molecular mass increases when renal tubular function is impaired (1). Several kinds of such proteins—e.g., β2-microglobulin, retinol-binding protein, lysozyme (EC 3.2.1.17), and ribonuclease (EC 3.1.27.5)—have been measured in urine and serum for estimation of tubular damage (2, 3). Excretion of these proteins in urine of patients with renal diseases is generally closely related to the extent of their renal impairment (2, 4). Among these proteins, β2-microglobulin is the most widely used marker because of the commercial availability of an RIA kit for it. However, the rapid degradation of this protein at 37 °C, especially at pH <6, is well documented (3, 5). Lysozyme, on the other hand, is stable for at least 24 h in urine at 37 °C and at a pH between 3 to 8 (6), thus making it potentially a more suitable marker for detecting tubular proteinuria.

Various methods have been described for estimating lysozyme (7–11), including measuring its lytic action on Micrococcus lysodeikcticus (12, 13). Such methods are popular, but the sensitivity is not satisfactory. Moreover, their accuracy and precision are greatly affected by several factors, including choice of standard, pH, ionic strength (14), agarose variability (15), and inhibition or activation (16). Thus far, only radioimmunoassay is sensitive enough for determination of lysozyme in normal urine and in urine from patients with minimally impaired renal function. However, we still have little information (9) as to whether lysozyme, measured radioimmunologically in urine, can be a suitable marker for impaired tubular function. Because of the limited use of this assay method, this issue has not been thoroughly investigated.

Recently, many have attempted to predict the development of diabetic nephropathy (17–20), primarily by examining by immun assay a minor increase in albumin excretion, sometimes called (incorrectly) "microalbuminuria," in individuals with Type 1 diabetes. However, few studies (21) on tubular dysfunction in Type 1 diabetics have been performed, especially with the aim of evaluating the disorder as a predictor of the development of diabetic nephropathy. To elucidate these problems, we have investigated the relationship between various indices of renal functions, including creatinine clearance rate, urinary protein, β2-microglobulin, and excretion rate of phenolsulfonphthalein, and urinary lysozyme measured with a highly sensitive radioimmunoassay in Type 2 (non-insulin-dependent) diabetics with various degrees of renal dysfunction. Using urinary lysozyme as an indication for tubular dysfunction, we further studied the temporal relation between the onset of an increased excretion of urinary protein and lysozyme in Type 1 (insulin-dependent) diabetics who had shown an abrupt onset of the disease. This permitted us to estimate a precise correlation between the duration of diabetes and the onset of glomerular and (or) tubular dysfunction in this subset of patients.

Subjects and Methods

Subjects: We studied 39 non-insulin-dependent diabetics (Table 1) and 15 insulin-dependent diabetics (Table 2). None had apparent urinary tract infection, congenital renal disease, or hematological disorder. After an overnight fast, the 39 non-insulin-dependent diabetics took an ordinary test for 15-min excretion of phenolsulfonphthalein. Within the next week, they also were tested for endogenous creatinine clearance. The urinary specimens provided for creatinine clearance were also used for determinations of lysozyme, β2-microglobulin, and total protein. We also determined lysozyme in untimed urine specimens collected from 15 insulin-dependent diabetics and from 32 ostensibly healthy persons between the ages of 20 and 40 years.

Lysozyme antigen and antibody. Specific antiserum to lysozyme was obtained from a rabbit after multiple injections of purified human-milk lysozyme mixed with Freund's

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complete adjuvant into the hind-foot pads and femoral hypoderms.

Human-milk lysozyme was iodinated by the lactoperoxidase method (22). The iodinated products were purified by gel chromatography on a 1.0 x 50 cm column of Sephadex G-50. The specific activity of $^{125}$I-labeled human-milk lysozyme thus obtained was 30 Ci/g. The human-milk lysozyme (a gift from Dr. Kimura, National Institute of Industrial Health, Kawasaki, Japan) used for the assay was purified as reported previously (23). In brief, defatted human milk was mixed with carboxymethylcellulose, stirred overnight, then washed sequentially with phosphate buffer (0.02 mol/L, pH 6.8) and phosphate buffer containing NaCl (0.1 mol/L). Lysozyme was eluted from the carboxymethylcellulose with phosphate buffer containing 0.5 mol of NaCl per liter, and the active fractions were pooled and lyophilized. The lyophilized material was purified further on a column of Sephadex G-50, the active fractions being combined and desalted on a column of Bio-Gel P-2. The salt-free lysozyme was lyophilized.

Assay procedure. Lysozyme was measured by a modification of a radioimmunological method described by Yuzuriha et al. (24). Dilute rabbit antiserum to human-milk lysozyme 20 000-fold in phosphate buffer (50 mmol/L, pH 7.5) containing 10 mL of normal rabbit serum per liter. Incubate 0.2 mL of this diluted antiserum at 25 °C with 0.1 mL of human-milk lysozyme standard or unknown and 0.1 mL of labeled lysozyme solution. After 3 h, add 0.1 mL of goat antiserum to rabbit IgG to each sample. Continue the incubation at 25 °C for an additional 30 min, then centrifuge at 1800 $\times g$ for 30 min. Aspirate the supernate and count the radioactivity of the precipitate in a gamma counter. Use the amount of the lysozyme antiserum capable of binding about 70% of the available $^{125}$I-labeled lysozyme (ca. 50 pg).

We used undiluted urine specimens for the initial measurement; specimens in which the lysozyme concentration exceeded 80 $\mu$g/L were diluted with the phosphate buffer containing 10 g of bovine serum albumin per liter and reassayed. Figure 1 shows the standard curve. Under these conditions, the lowest detectable concentration of lysozyme was 0.5 $\mu$g/L, as estimated from the concentration that produced a response of 2 SD from the zero-dose response.

Precision: We used urine samples with low and high concentrations of lysozyme in this evaluation (Table 3). Two urine samples were measured 10 times, in duplicate, during a single analytical run to determine within-run precision. For evaluating between-run precision, we measured the same samples in duplicate in successive analytical runs.

Analytical recovery: Three urine samples with lysozyme concentrations ranging from 0.85 to 11.15 $\mu$g/L were measured with and without addition of various amounts of lysozyme. Mean recovery, calculated as the percent of added lysozyme recovered in the supplemented urines, was 101.2% (range 92.2-105.6%) (Table 4). When three urine samples with low and high concentration of lysozyme were diluted two- and fourfold with the phosphate buffer containing 10 g

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**Table 1. Clinical Characteristics of 39 Non-Insulin-Dependent Diabetic Subjects Studied**

<table>
<thead>
<tr>
<th>Case no.</th>
<th>Age, yr</th>
<th>Duration of diabetes, yr</th>
<th>Hemoglobin A$_{1c}$, %</th>
<th>Protein, mg/L</th>
<th>Lysozyme, $\mu$g/g Cr</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-5</td>
<td>0.2</td>
<td>0.3</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
</tr>
<tr>
<td>6-10</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
</tr>
</tbody>
</table>

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**Table 2. Clinical Characteristics, Urinary Protein, and Lysozyme in Insulin-Dependent Diabetic Subjects**

<table>
<thead>
<tr>
<th>Case no.</th>
<th>Urinary excretion (15 min)</th>
<th>Lysozyme, $\mu$g/g Cr</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-5</td>
<td>0.5</td>
<td>0.5</td>
<td></td>
</tr>
<tr>
<td>6-10</td>
<td>0.5</td>
<td>0.5</td>
<td></td>
</tr>
</tbody>
</table>

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**Table 3. Reproducibility of the Present Assay**

<table>
<thead>
<tr>
<th>Assay</th>
<th>Mean</th>
<th>SD</th>
<th>CV, %</th>
<th>Between-assay</th>
</tr>
</thead>
<tbody>
<tr>
<td>Within-assay</td>
<td>15.7</td>
<td>0.63</td>
<td>6.3</td>
<td>15.4</td>
</tr>
<tr>
<td>Between-assay</td>
<td>6.3</td>
<td>4.5</td>
<td>7.5</td>
<td>0.96</td>
</tr>
</tbody>
</table>
of bovine serum albumin per liter, the dilution-corrected lysozyme values were those expected (Figure 2).

Other analytical methods. β2-Microglobulin was estimated radioimmunologically, with a commercially available kit (Pharmacia, Bromma, Sweden). Total urinary protein was assayed by an automated Coomassie Blue B colorimetric method (25) and determined semiquantitatively with Multistick S. G.® ( Ames, Elkhart, IN). Creatinine was measured by the Jaffe method (26). The proportion of hemoglobin A1c was determined by a liquid chromatographic method (27).

Results

Urinary lysozyme excretion measured in 32 normal subjects was 0.56–11.55 µg/L (mean 3.85 µg/L) and 0.19–8.53 µg/g of creatinine (mean 3.25 µg/g), and 1–6193 µg/L (mean 414 µg/L) and 0.72–4381 µg/g of creatinine (mean 361.7 µg/g) in diabetics. As shown in Figure 3, there was no significant correlation between urinary lysozyme excretion and any indices of diabetes control. These data suggest that neither acute nor chronic disturbance of diabetes control to such degree as observed in our patients has anything to do with urinary lysozyme excretion. However, the amount of lysozyme excreted in urine was significantly correlated with the urinary excretion of protein and with creatinine clearance, which are considered to be indices of renal glomerular function (Figure 4). This shows that the population we studied comprised patients with a wide range of glomerular, tubular, or mixed-type proteinuria and also that tubular function may deteriorate in parallel with that of the glomerulus. To evaluate whether the determination of urinary lysozyme was as suitable as β2-microglobulin for the detection of tubular dysfunction, we compared the urinary excretion of both proteins with the 15-min excretion of phenolsulfonphthalein, which is considered to be removed from the kidney mainly via secretion by the proximal tubular cells. As shown in the upper panel of Figure 5, the urinary excretion of β2-microglobulin was inversely correlated with that of phenolsulfonphthalein. The same was true for lysozyme and phenolsulfonphthalein, but the correlation coefficient in the latter was much higher than that in the former. Thus, correlation between the decrease in excretion of phenolsulfonphthalein from the renal tubulus and the reabsorption of the lower-Mr proteins in that site was evident. Therefore, we conclude that lysozyme could be a more suitable index of proximal tubular function than is β2-microglobulin. If urinary lysozyme excretion >20 µg/g of creatinine is abnormal, based on the values determined for the healthy subjects, then the urinary excretion of lysozyme was abnormally high in six of the insulin-dependent diabetics (Table 2). Routine urinalysis by dipstick revealed only

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Table 4. Analytical Recovery of Lysozyme Added to Three Urine Samples*

<table>
<thead>
<tr>
<th>Measured Lysozyme concn, µg/L</th>
<th>Added</th>
<th>Recovered</th>
<th>Recovery, %</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Sample A</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.85</td>
<td>2.3</td>
<td>1.5</td>
<td>96.7</td>
</tr>
<tr>
<td>14.0</td>
<td>13.5</td>
<td>13.15</td>
<td>97.4</td>
</tr>
<tr>
<td>127.0</td>
<td>121.5</td>
<td>126.15</td>
<td>103.8</td>
</tr>
<tr>
<td><strong>Sample B</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3.15</td>
<td>7.9</td>
<td>4.5</td>
<td>105.6</td>
</tr>
<tr>
<td>16.8</td>
<td>13.5</td>
<td>13.65</td>
<td>101.1</td>
</tr>
<tr>
<td>44.5</td>
<td>40.5</td>
<td>41.35</td>
<td>103.7</td>
</tr>
<tr>
<td><strong>Sample C</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>11.15</td>
<td>15.3</td>
<td>4.5</td>
<td>92.2</td>
</tr>
<tr>
<td>25.3</td>
<td>13.5</td>
<td>14.25</td>
<td>105.6</td>
</tr>
<tr>
<td>53.6</td>
<td>40.5</td>
<td>42.45</td>
<td>104.8</td>
</tr>
</tbody>
</table>

*To 0.9-mL urine samples we added 0.1 mL of water or lysozyme solution.

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Fig. 2. Linear relationship between measured lysozyme and urine samples with different concentrations of lysozyme (low O, middle □, high ○) diluted with 50 mmol/L phosphate buffer containing 10 g of bovine serum albumin per liter

Fig. 3. Correlation between urinary excretion of lysozyme and control of diabetes

FPG: fasting plasma glucose; HbA1c: hemoglobin A1c.
increase urinary concentration of lysozyme, though no correlation between lysozyme value in urine and the number of leukocytes has been observed (31, 32). Thus we can reasonably assume that, in our patients, increased urinary lysozyme concentration may well indicate tubular proteinuria and therefore impaired tubular function. This conclusion is also supported by the close inverse correlation between urinary lysozyme concentration and urinary excretion rate of phenolsulphonphthalein (Figure 5), believed to be one of the indices for renal tubular function.

In view of its greater stability in urine and the closer correlation of its urinary secretion with the phenolsulphonphthalein value, lysozyme appears to be a more practical and reliable index of proximal tubular function than is $\beta_2$-microglobulin.

In the present study four out of 15 patients with Type 1 diabetes without clinical proteinuria excreted lysozyme in an amount exceeding the normal range, and two patients showed a minor increase in urinary excretion of both protein and lysozyme. We believe this indicates a development of tubular dysfunction as early as the impairment of the glomerular function, which can be diagnosed by the presence of proteinuria. Reversible tubular dysfunction has been reported in diabetic ketoacidosis as well as in poorly controlled diabetes (21). The increased excretion of lysozyme observed in one patient (Table 2, No. 1) may be reversible, because she was convalescing from diabetic ketoacidosis, but is unlikely to be reversible in the other patients, judging from their diabetic condition.

The chemical method we used to quantify total urinary protein was not sensitive enough to detect a minor increase in urinary excretion of protein. Therefore, we cannot conclude which is an earlier index of clinical nephropathy in

Fig. 4. Correlation of urinary excretion of lysozyme with urinary protein and creatinine clearance in diabetics

minor proteinuria in two of these six patients. Four of the six patients with lysozymuria had had diabetes mellitus for more than 10 years.

Discussion

Several lysozyme assays exist (12, 13, 28, 29), but they do not permit reliable quantification of urinary lysozyme within physiological concentrations and may not be lysozyme specific (4). The radioimmunoassay we used is very reliable, as judged from the data on precision, analytical recovery, and linearity of dilution. The method is sensitive enough for quantifying lysozyme in "normal" urine, i.e., $>$1 $\mu$g/L, and can be completed within 4 h. Although heterogeneity of human lysozyme has been described (30), the dilution curves obtained were parallel to the standard curve.

The concentrations of urinary lysozyme we determined were considerably lower than those reported by Thomas et al. (9) and Brouwer et al. (6), who found mean values of 450 $\mu$g/L in 18 healthy subjects and 14.5 $\mu$g/L in 155 normals, respectively. This discrepancy is not easily explainable; however, one should compare the total daily urinary excretion or urinary concentration per gram of urinary creatinine because the urinary excretion rate of lysozyme might not be constant throughout the day. Expression of urinary lysozyme concentration in terms of creatinine excretion seems to be necessary in comparing the urinary lysozyme measured in dissimilar collection periods.

To evaluate urinary excretion of lysozyme as an index of renal tubular function, one must determine whether the normal renal threshold has been exceeded, because this is accompanied by overflow lysozymuria. No subjects suffering from diseases associated with increased concentrations of lysozyme in serum (e.g., monocytic leukemia or inflammatory bowel disorder) were included in this study. Furthermore, our patients showed no apparent pyuria, which might
diabetics—lysozymuria or albuminuria (and hence tubular or glomerular dysfunction). The relation between changes in the tubular system, developing in the early phase of diabetes, and the pathological changes in the glomeruli, occurring later, remain to be clarified.

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References