Apolipoprotein(a) Is Present in Urine and Its Excretion Is Decreased in Patients with Renal Failure

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To investigate the relation between renal function and concentrations of lipoprotein(a) [Lp(a)] in serum, we measured Lp(a) in samples of serum and urine from patients with diabetes mellitus and in samples sent to a laboratory center for measurements of creatinine clearance. Serum Lp(a) concentrations were significantly increased in subjects with obvious renal dysfunction (serum creatinine ≥176.8 μmol/L) compared with normal control subjects. Urinary Lp(a) excretion was decreased in subjects with obvious renal dysfunction compared with subjects without obvious renal dysfunction (serum creatinine ≥88.4 μmol/L) and was negatively and positively correlated with serum creatinine and creatinine clearance, respectively. More than 80% of urinary Lp(a) was recovered in the d >1.21 kg/L fraction. At least six bands for apolipoprotein(a) [apo(a)] fragments, which were smaller than native apo(a) in serum, were observed in urine by immunoblotting, and some of these were also detected in serum. Degraded apo(a) fragments are probably present in urine, and their excretion decreases in parallel with decreases in the glomerular filtration rate.

Additional Keyphrases: lipoproteins - immunoblotting

Since its description in the early 1960s by Berg (1), lipoprotein(a) [Lp(a)] has been shown to be associated with coronary atherosclerosis (2–5). Lp(a) is composed of one LDL particle to which one molecule of apo(a) is covalently linked to the apolipoprotein (apo) B moiety. Apo(a) shares considerable homology with plasminogen, and the gene for apo(a) is located adjacent to that of plasminogen (6, 7).

Plasma concentrations of Lp(a) and the molecular mass of apo(a) vary with the individual and appear to be largely genetically determined, in part because of increased copies of Kringle IV-like domains within the apo(a) gene (8, 9). However, the apo(a) gene accounts for only 40% of plasma Lp(a) concentrations (10).

Little is known about the physiological role of Lp(a). The liver is the primary and perhaps the only site of Lp(a) synthesis (11). The factors regulating the synthesis, secretion, and catabolism of Lp(a) remain largely unknown (2).

The possible role of the kidney in the regulation or modulation of Lp(a) metabolism was first suggested by Parra et al. (12), who found an increased serum Lp(a) concentration in chronically hemodialyzed patients. Karidi et al. (13) reported that plasma Lp(a) was increased in patients with membranoproliferative glomerulonephritis, primary amyloidosis, and nephrotic syndrome but not in patients with diseases causing minimal kidney changes. Increased plasma Lp(a) was also found in patients with diabetic nephropathy (14–16), which may account, in part, for the finding of increased plasma Lp(a) in diabetic patients.

Here we investigated urinary excretion of apo(a) and its correlation to renal function. We found that degraded polypeptides of apo(a) are present in human urine and that the urinary excretion of apo(a) is decreased in patients with renal dysfunction. Decreased urinary loss of apo(a) may account in part for the increased plasma Lp(a) concentrations seen in renal failure.

Materials and Methods

Subjects. We studied blood samples from 43 subjects with non-insulin-dependent diabetes mellitus (NIDDM) who were admitted to our hospital and 111 samples sent to a clinical laboratory center (BML Co. Ltd., Tokyo, Japan) from various hospitals for measurement of creatinine clearance (Table 1). No clinical information except for body weight and height was available for the latter samples. The samples were separated into two groups: samples from subjects with obvious renal dysfunction, whose serum creatinine concentrations were ≥176.8 μmol/L and samples from subjects without obvious renal dysfunction, whose serum creatinine concentrations were ≤88.4 μmol/L.

Control subjects. Two hundred thirty-five healthy volunteers were used as normal control subjects for assessing serum Lp(a) concentrations. They were recruited from employees of Obama Hospital in Fukui, Japan.

Lipids, Lp(a), and other measurements. Blood was drawn after an overnight fast, and urine was collected for 24 h during the day before blood sampling. The serum and urine samples were stored in screw-cap vials at −30°C until being measured. Total cholesterol, triglycerides, and high-density lipoprotein (HDL) cholesterol were determined as previously described (17). Serum Lp(a) concentrations were determined at two dilutions with a commercially available enzyme-linked immunosorbent assay [Tint Elize Lp(a) kit; Biopool AB, Umeå, Sweden]. Urinary immunoreactive substances to

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1 Nonstandard abbreviations: Lp(a), lipoprotein(a); apo, apolipoprotein; NIDDM, non-insulin-dependent diabetes mellitus; HDL, high-density lipoprotein; LDL, low-density lipoprotein; and IDDM, insulin-dependent diabetes mellitus.

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Serum creatinine: 88.4 μmol/L = 10 mg/L.
an anti-apo(a) antibody in the assay, which we tentatively refer to as urinary Lp(a), were measured in 1 10-fold dilutions of urine. Values of urinary Lp(a) were expressed as micrograms of total Lp(a) per day, determined by using the standard sample provided in the kit.

Urinary albumin was determined by radioimmunoassay (Albumin RIA; Pharmacia Diagnostics AB, Upsala, Sweden). Urinary plasminogen was measured in a separate experiment by single radial immunodiffusion (M-Partigen Plasminogen; Behringwerke AG, Marburg, Germany), with an assay range of 15–220 mg/L for plasminogen. All assays were performed within 1 month of sample collection.

**Immunoblotting of apo(a).** Urine samples were dialyzed against saline containing 1 mmol/L EDTA, pH 7.4, and then concentrated ~80-fold by placing a packed dialysis bag in dry Aquacide II powder (Calbiochem, La Jolla, CA) at 4 °C. The samples were prepared by mixing 2 μL of serum or 20 μL of concentrated urine with 20 μL of buffer (0.5 mol/L Tris buffer, pH 6.8, containing, per liter, 100 mL of glycerol, 100 mL of sodium dodecyl sulfate, 50 mL of β-mercaptoethanol, and 0.5 mL of bromphenol blue). The well-mixed solutions were boiled for 5 min in a water bath and then were applied to the sample wells of a precast 4–12% polyacrylamide gradient gel (SDS-PAGE mini; TEF Corp., Nagano, Japan). In addition to urine and serum, plasminogen (Sigma Chemical Co., St. Louis, MO; dissolved in phosphate-buffered saline and stored in screw-cap vials at ~30 °C until use) and low-density lipoprotein (LDL; 1.019 < d < 1.063 kg/L; stored at 4 °C for 1 month) were applied. The Pharmacia HMW-SDS calibration kit (Pharmacia; mixture of myosin (200 kDa), α,β-macroglobulin (170 kDa), β-galactosidase (116 kDa), transferrin (76 kDa), and glutamic dehydrogenase (63 kDa)) was used as a negative control for proteins, and the Biotinylated SDS-PAGE standards, high range (Bio-Rad Labs, Richmond, CA; mixture of myosin (200 kDa), β-galactosidase (116 kDa), phosphorylase B (97 kDa), bovine serum albumin (66 kDa), and hen egg white ovalbumin (45 kDa)) was used for molecular mass determination on the nitrocellulose membrane. Electrophoresis was performed with an EASY-7M (Funakoshi, Tokyo, Japan) cooled to 10 °C.

A constant current of 15 mA per gel was applied throughout the run. Afterward, the gel was transferred onto a nitrocellulose membrane (Bio-Rad Labs), and the proteins were electroblotted according to the method of Towbin et al. (18) by using a Mini Trans-Blot (Bio-Rad Labs). The blotting was performed for 1 h with a constant voltage of 100 V. The membrane was treated with Block Ace (Dainihon Seiyaku Co., Ltd., Tokyo, Japan) for 3 h at room temperature to saturate free protein-binding sites and then incubated overnight with two monoclonal anti-human apo(a) antibodies (5 mg/L of 2D1 and 4F3 antibodies; Organon Teknika Corp., Durham, NC). [Note: According to the manufacturer, these monoclonal anti-human apo(a) antibodies do not cross-react with plasminogen.] The blots were rinsed three times with phosphate-buffered saline, and Vectastain immunoperoxidase stain (Vectastain ABC kit; Vector Labs, Burlingame, CA) was used as a secondary antibody, according to the manufacturer’s protocol, to increase the sensitivity of the immunoblotting method (19). 4-Chloro-1-naphthol (4 CN Membrane Peroxidase Substrate System; Kirkegaard and Perry Labs Inc., Gaithersburg, MD) was used as a substrate for horseradish peroxidase.

**Ultracentrifugation of urine.** To examine whether Lp(a) in urine is present in the lipoprotein fraction d <1.21 kg/L, urine from eight subjects was ultracentrifuged at 60 000 rpm for 48 h at 8 °C in a Hitachi 70P-72 ultracentrifuge (Hitachi RP70T rotor; Hitachi Koki Co., Ltd., Tokyo, Japan), after which the density of urine was adjusted to 1.21 kg/L by adding KBr. After ultracentrifugation, each fraction was dialyzed against saline containing 1 mmol/L EDTA, pH 7.4, and then Lp(a) was measured.

**Statistics.** Results are expressed as mean ± SD (median). Analyses were made with the Stat View statistical program (Abacus Concept, Inc., CA). A Mann–Whitney test was used to compare data between groups. For bivariate tables, Spearman rank correlation coefficients were determined and tested for significance.

**Results**

The distribution of serum Lp(a) concentrations in the subjects with renal dysfunction (serum creatinine...
≥176.8 μmol/L was shifted from that for the normal control subjects (Figure 1). Serum Lp(a) for the subjects was significantly correlated with serum cholesterol, but not with serum creatinine, albumin, triglyceride, HDL cholesterol, or daily urinary albumin excretion.

Urinary Lp(a) was detectable with the kit used. Daily urinary Lp(a) excretion was less in the subjects with obvious renal dysfunction than in the subjects without obvious renal dysfunction (Figure 2); it was significantly correlated negatively with serum creatinine, and positively with creatinine clearance, but not with serum Lp(a), serum lipids, serum albumin, or daily urinary albumin excretion (Figure 3). Lp(a) in urine was recovered mostly (84 ± 8%, n = 8) in the d >1.21 kg/L fraction after one 24-h ultracentrifugation, which suggests that apo(a) is present as a lipid-free form.

To confirm the presence of apo(a) in urine, we performed Western blotting with monoclonal antibodies to apo(a) that do not cross-react with plasminogen (Figure 4). Intact 81 kDa plasminogen did not react with the monoclonal antibodies (lane 7), and the apparent molecular masses of some of the stained bands were larger than that of 81 kDa plasminogen. The immunoblot pattern closely resembled that of serum, indicating that bands were apo(a)s. The specificity of the immunoblotting was shown by the lack of blots with myosin, α2-macroglobulin, β-galactosidase, transferrin, and glumatic dehydrogenase (lane 2). Many bands between 44 and 200 kDa observed in urine were obviously smaller than the main bands in serum. The 172 and 128 kDa bands were also observed in the LDL fraction stored for ~1 month at 4°C. The 172, 148, and 128 kDa bands were also present faintly in fresh serum, indicating that these bands were degraded polypeptides of apo(a). Although the urine and serum samples used for the immunoblotting were as fresh as possible in an attempt to avoid degradation of apo(a) during storage, urine stored at ~30°C for 1 month revealed an essentially identical immunoblot pattern. In contrast, degraded apo(a) bands seemed to be increased in the stored serum. The immunoblot patterns from 12 different urine samples resembled each other.

Discussion

We show here that human urine contains apo(a) and that the apparent molecular mass of urinary apo(a) is smaller than that of serum apo(a). The origin of the apo(a) detected in urine is not known but is probably from circulating serum, because the liver is considered to be the sole site of production of Lp(a) (II) and several of the apo(a) bands found in urine were also observed in serum. Urinary Lp(a) seems to be present as lipid-free apo(a) because urinary Lp(a) was present mostly in the d >1.21 kg/L fraction and it is unlikely that all of the
observed six peptides contain sequences forming linkages with apo B-100. Furthermore, apo(a) itself is hydrophilic.

It is unlikely that the "urinary Lp(a)" measured by the Tint Elize Lp(a) kit was due to plasminogen present in urine, because plasminogen was not detectable in the 20 tested urine samples, in which urinary Lp(a) was 1.3 ± 0.6 g/L as determined by a kit for plasminogen that can detect >15 mg/L of plasminogen. According to the manufacturer's protocol for the Tint Elize Lp(a) kit, 10 mg/L of Lp(a) is measurable even if the sample contains 1 g/L of plasminogen, which means that a concentration of plasminogen 100-fold higher than Lp(a) does not interfere with the assay. If the measured urinary Lp(a) was due to plasminogen present in urine, the urinary plasminogen concentration should be ~130 mg/L (1.3 mg/L of urinary Lp(a) × 100 = 130 mg/L).

It is not clear to what extent urinary Lp(a) excretion contributes to the catabolism of serum Lp(a). The mechanisms of Lp(a) degradation remain controversial. Hoffmann et al. (20) showed that the low-density lipoprotein receptor is involved in the catabolism of Lp(a).

No significant negative correlation was observed between serum Lp(a) and urinary Lp(a). This may be due to wide, genetically determined variations in serum Lp(a) concentrations. The observation that urinary Lp(a) excretion was significantly correlated negatively with serum creatinine concentrations and positively with creatinine clearance strongly suggests that urinary Lp(a) excretion is regulated by the glomerular filtration rate, although the mechanisms involved are obscure. Karádi et al. (13) examined serum Lp(a) concentrations in patients with massive proteinuria of various etiologies and found discordant results between the patients with minimal disease changes and those with membranoproliferative glomerulonephritis. In the former patients, serum Lp(a) did not increase and correlated positively with total daily urinary protein loss. In contrast, serum Lp(a) in the latter patients was increased and correlated negatively with daily urinary protein loss. In our study, neither serum Lp(a) nor urinary Lp(a) excretion correlated with daily urinary albumin loss. We have no information about the underlying cause of renal failure in our subjects except for the patients with diabetic nephropathy.

Conflicting data have been reported concerning serum concentrations of Lp(a) in diabetic patients. The increased serum Lp(a) concentrations found in patients with insulin-dependent diabetes mellitus (IDDM) decreased when glycemic control improved (21, 22). A positive correlation between glycohemoglobin values and serum Lp(a) concentrations was also observed in white IDDM children (23). Thus, poor diabetic control appears to increase serum Lp(a) concentrations.
ever, the relationship between diabetic control and serum Lp(a) is not as clear in patients with NIDDM. Diabetic nephropathy in NIDDM seems to affect serum Lp(a) values (14, 15). Increased serum Lp(a) was also found in IDDM subjects with microalbuminuria (16). These discrepant results are probably due to the heterogeneity of diabetes mellitus, the relatively small number of subjects examined, and the wide genetically determined variations in serum Lp(a) concentrations. In our NIDDM subjects, serum Lp(a) was significantly greater than in the control subjects (P<0.01, Table 1). The subjects with heavy proteinuria had higher serum Lp(a) concentrations [371 ± 394 (204) mg/L, n = 8] than did the subjects with no albuminuria [199 ± 146 (179) mg/L, n = 26] or with microalbuminuria [139 ± 70 (118) mg/L, n = 9], but these differences were not statistically significant. Analysis of additional subjects who have the same apo(a) isof orm is necessary for clarifying whether serum Lp(a) concentration is affected by nephropathy and glycemic control.

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References
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