Presence of Protein Fragments in Urine of Critically Ill Patients with Acute Renal Failure: A Nephrologic Enigma, Massimo Gai,1* Vincenzo Cantaluppi,2 Chiara Fenocchio,2 Daria Motta,2 Sergio Masini,1 Alfonso Pacitti,2 and Giacomo Lanfranco1 (1 Laboratory of Nephrology and 2 Chair of Nephrology, University of Torino, Torino, Italy; * address correspondence to this author at: San Giovanni Battista di Torino, S.C.U. Nefrologia, Dialisi e Trapianto, Laboratory of Nephrology, Corso Bramante 88, 10126 Torino, Italy; fax 39-011-6963158, e-mail massimogai@katamail.com)

Predicting complications in intensive care units (ICUs) is an important step in the care of critically ill patients. Intensive care specialists have developed numerous prognostication tools for patients admitted to the ICU; however, although useful, many of these tools are not applicable in a clinical setting, and multiple severity-of-illness scores often underestimate hospital mortality in several conditions (1, 2).

Many acute pathologic states, such as burns, trauma, bleeding, and sepsis, are associated with the induction of a “systemic inflammatory response”, which is characterized by the release of pro-inflammatory mediators and the activation of different types of cellular elements (3–6). This response primarily involves endothelial cells and leukocytes (7–9). It is possible to use renal function as an early marker for systemic illness because kidney involvement is a recognized complication of several systemic diseases. Acute renal failure (ARF), usually attributable to intrarenal hemodynamic changes, often complicates the clinical course of critically ill patients (10–12). Microalbuminuria has been proposed as a marker of capillary leak severity in the ICU (13, 14). It has previously been demonstrated that urinary albumin degrades into multiple fragments in meningococcal sepsis and that the quantity of degraded albumin is associated with severity (15).

Since April 2003, we have analyzed untimed urine samples from critically ill patients with ARF in ICUs: dipstick test and urine sediment analysis were performed on all samples, and none showed massive leukocyturia. The proteinuria is typed by quantitative (nephelometry) (16, 17) and qualitative (immunofixation) (18) immunologic techniques and by sodium dodecyl sulfate–agarose gel electrophoresis (SDS-AGE) (19), with retinol-binding protein (21 kDa) and α1-microglobulin (31 kDa) considered as markers of tubular damage; albumin (67 kDa), transferrin (80 kDa), and IgG (150 kDa) as markers of glomerular injury; and α2-macroglobulin (725 kDa) as a marker of postrenal proteinuria. In addition, we measure total urinary proteins by the biuret (20) and pyrogallol red assays. All samples were stored at 4°C after centrifugation (400g for 5 min) and were analyzed within 3–24 h. Storage in a refrigerator (4°C) did not change the results.

Here we report on 10 cases (9 males and 1 female; age range, 40–79 years; 18.1% of all screened patients) with ARF in whom we found evidence of urinary protein degradation. All patients had a prerenal cause of ARF; only one patient was not oliguric and did not need renal replacement therapy. Five patients died, and five had renal function recovery.

SDS-AGE performed at the time of the nephrologic visit and diagnosis revealed a smeared band of proteins and protein fragments, covering the molecular mass range between 10 and 300 kDa, and an absence of the typical bands for the single proteins.

The SDS-AGE migration patterns of one of these patients on 3 consecutive days are shown in Fig. 1; after 24 and 48 h, the bands for albumin, α1-microglobulin, and retinol-binding protein became visible. The patient was oliguric, and the urine volume and urine creatinine were superimposable on the 3 days.

In all 10 patients with evidence of protein degradation, the direct biuret technique, which can measure protein fragments, detected markedly higher concentrations of total proteins (range, 1520–23 280 mg/L) than were detected by the pyrogallol red method (range, 140–5030 mg/L), which underestimates protein fragments and low-molecular-weight proteins (21). The immunofixation and nephelometric results were mostly negative, but these immunologic techniques usually do not detect protein fragments (21, 22). The proteinuria dipstick tests were either negative or revealed traces of albuminuria.

Shown in Table 1 are protein patterns for urine samples

Fig. 1. SDS-AGE of spot urines from a critically ill patient with ARF on 3 consecutive days.

The urine sample obtained at admission (day 0; lane A) shows a smeared protein band. Progressive definition of the bands for albumin (arrow 1), α1-microglobulin (arrow 2), and retinol-binding protein (arrow 3) is evident after 24 h (day 1; lane B) and 48 h (day 2; lane C).
Table 1. Urinary protein patterns (biuret assay, pyrogallol red assay, nephelometry) in the same critically ill patient on 3 consecutive days.

<table>
<thead>
<tr>
<th></th>
<th>Sample</th>
<th>Reference interval, mg/L</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A (day 0)</td>
<td>B (day 1)</td>
</tr>
<tr>
<td>Total proteinuria, mg/L</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Biuret assay</td>
<td>9490</td>
<td>4240</td>
</tr>
<tr>
<td>Pyrogallol red assay</td>
<td>3850</td>
<td>1120</td>
</tr>
<tr>
<td>Nephelometry&lt;sup&gt;a&lt;/sup&gt;</td>
<td>145 (3.7%)</td>
<td>254 (22.6%)</td>
</tr>
<tr>
<td>Nephelometric results for individual proteins, mg/L</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Retinol-binding protein</td>
<td>&lt;1</td>
<td>&lt;1</td>
</tr>
<tr>
<td>α2-Microglobulin</td>
<td>61</td>
<td>123</td>
</tr>
<tr>
<td>Albumin</td>
<td>80</td>
<td>127</td>
</tr>
<tr>
<td>Transferrin</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>IgG</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>α2-Macroglobulin</td>
<td>&lt;2</td>
<td>&lt;2</td>
</tr>
<tr>
<td>Urine creatinine</td>
<td>1040</td>
<td>1340</td>
</tr>
</tbody>
</table>

* Total of the single nephelometric determinations (retinol-binding protein, α2-microglobulin, albumin, transferrin, IgG, α2-macroglobulin). Ratio of nephelometric to pyrogallol red proteinuria is shown in parentheses.

obtained on the same days from the same patient shown in Fig. 1, as measured by the biuret assay, the pyrogallol red assay, and nephelometry. The nephelometric concentration of total proteinuria is the sum of the single nephelometric concentrations of the principal urinary proteins (retinol-binding protein, α2-microglobulin, albumin, transferrin, IgG, α2-macroglobulin). On “day 0” (day of admission), the ratio of nephelometric proteinuria to pyrogallol red proteinuria was 3.7%, probably because of the high presence of protein fragments; on the following days, the ratio was 22.6% and 82.2%, which correlated with the decrease in protein degradation and the appearance of the typical bands on SDS-AGE.

Currently, we do not have a complete explanation for this medical mystery. A possible cause could be “glomerulotubular” failure of ischemic origin, occurring after major surgery or acute damage. We cannot exclude Tamm Horsfall protein as a contributor as we did not measure it, but we think that this mucoprotein is not the principal explanation for the phenomenon seen in SDS gels. Electrophoretic testing for collagenses (Novex Zymogram Gel) and measurement of urine elastase activity produced results within the reference intervals (data not shown).

These data clearly demonstrate the presence of a large amount of protein fragments in the urine of some critically ill patients with ARF. No fragmented proteins were detected in serum (serum electrophoresis). The phenomenon was transient in all of these patients (1–2 days); it may relapse at different times, and we found no correlation between recovery of patients and the disappearance of the smeared protein bands on electrophoresis. Nevertheless, understanding this enigma could help advance our knowledge of protein handling in the kidney and could be a useful tool in predicting the severity of renal involvement (23), as well as the possible presence of a systemic inflammatory response in critically ill patients.

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References
18. Gai M, Motta D, Bertinetti F, Mezza E, Jeanett A, Cantaluppi V, Piccoli GB,
Method for Measurement of Peroxisomal Very-Long-Chain Fatty Acid β-Oxidation in Human Skin Fibroblasts Using Stable-Isotope-Labeled Tetracosanoic Acid, Stephan Kemp,* Fredoen Valianpour, Petra A.W. Mooyer, Willem Kulik, and Ronald J.A. Wanders (University of Amsterdam, Medical Academic Center, Department of Pediatrics/Emma Children's Hospital, and Clinical Chemistry, Laboratory for Genetic Metabolic Diseases, Amsterdam, The Netherlands; * address correspondence to this author at: Laboratory for Genetic Metabolic Diseases, Room F0-224, Academic Medical Center, Meibergdreef 9, 1105 AZ, Amsterdam, The Netherlands; fax 31-20-6962596, e-mail s.kemp@amc.uva.nl)

Peroxisomes are present in virtually every eukaryotic cell type except the mature erythrocyte. In higher eukaryotes, one of the main functions of peroxisomes is the β-oxidation of very-long-chain fatty acids (VLCFAs; > 22 carbon atoms) (1). The importance of peroxisomal β-oxidation is emphasized by the existence of a variety of different diseases in which peroxisomal β-oxidation is impaired and VLCA concentrations are increased (1–4). Peroxisomal disorders can be categorized as (a) single peroxisomal enzyme deficiencies, including X-linked adrenoleukodystrophy (X-ALD) and disorders attributable to defects in one of the peroxisomal β-oxidation enzymes, such as acyl-CoA oxidase (AOX) deficiency and bifunctional protein (DBP) deficiency; and (b) disorders attributable to defects in peroxisome biogenesis. The peroxisome biogenesis disorders (PBDs) represent a continuum of clinical features ranging from the most severe form, Zellweger syndrome, through neonatal adrenoleukodystrophy to the least severe form, infantile Refsum disease.

Currently, measurement of the peroxisomal fatty acid β-oxidation activity is performed with 1-[14C]-radiolabeled VLCA substrates and one of two available methods: either in intact human skin fibroblasts cultured in monolayer (5); or in isolated fibroblasts permeabilized with digitonin (6). We investigated the feasibility of using deuterium-labeled tetracosanoic acid (D3-C24:0) as an alternative substrate to radiolabeled 1-[14C]-labeled C24:0 for the measurement of peroxisomal β-oxidation activity in cultured primary human skin fibroblasts.

Before use, the purity of 24,24,24-D3-C24:0 (Larodan Fine Chemicals AB) was determined. The D3-C24:0 substrate contained ∼6% deuterium-labeled octadecanoic acid (D2-C18:0). Acetone was used to purify D3-C24:0 according to the following procedure: 4 mL of acetone was added to 20 mg of D3-C24:0. The sample was vortex-mixed vigorously, left at room temperature for 30 min, and centrifuged at 1600g for 10 min; approximately 80% of the acetone was then removed, and 3 mL of fresh acetone was added. This procedure was repeated two more times. After three washing steps with acetone, ∼80% of the acetone was removed, and the remaining acetone was evaporated at room temperature under a constant stream of nitrogen. The residue was weighed, and a stock solution of 10 mmol/L D3-C24:0 in absolute ethanol was prepared. After purification, the purity of D3-C24:0 was analyzed, and the contribution of the D3-C18:0 contaminant was determined to be <0.2%.

Fibroblasts from healthy controls and patients with X-ALD were cultured in the absence or presence of 20 μmol/L D3-C24:0 in HAM-F10 tissue culture medium supplemented with 100 mL/L fetal calf serum, penicillin (100 IU/mL), streptomycin (100 IU/mL), and glutamine (2 mmol/L). Before usage, the D3-C24:0 stock solution was put in a water bath for 5 min, vortex-mixed, and diluted in HAM-F10 tissue culture medium to a final concentration of 20 μmol/L. Cells were used between passage numbers 6 and 18. For fatty acid analysis, cells were harvested with trypsin, washed twice with phosphate-buffered saline (PBS) and once with 9 g/L NaCl dissolved in 200 μL of deionized water, and sonicated, and the protein concentration was determined. The peroxisomal β-oxidation activity was calculated by measurement of the amount of intracellular deuterium-labeled hexadecanoic acid (D2-C16:0) present in nmol/mg of protein. In our method we chose D3-C16:0 as a marker for peroxisomal β-oxidation because of the availability of a D3-C16:0 internal standard, which enabled accurate calculation of the amount of D3-C16:0 present in the cells.

Fatty acids were analyzed by electrospray ionization mass spectrometry using a recently described isotopodilution method (7). For calculation of the amount of D3-C16:0, we constructed a five-point calibration curve. Of a calibration mixture containing D3-C16:0 (40 μmol/L), we added 0, 25, 50, 100, and 200 μL to 100 μL of internal standard containing deuterium-labeled behenic acid (D3-C22:0; 50.0 μmol/L), D3-C24:0 (50.0 μmol/L), and deuterium-labeled hexacosanoic acid (D3-C26:0; 1.0 μmol/L). Samples were extracted and analyzed as described previously (7). The input concentration of D3-C16:0 (in nmol) was plotted against the ratio of the peak height of D3-C16:0 to the peak height of the D3-C22:0 internal standard. The trend line and the intercept were used to calculate the D3-C16:0 concentration in the samples.

The effect of incubation time on the production of D3-C16:0 from D3-C24:0 in fibroblasts from healthy indi-