Severely modified lipoprotein properties without a change in cholesteryl ester transfer protein activity in patients with acute renal failure secondary to Hantaan virus infection

Jihoe Kim1*, Hyun Ho Park1, Inho Choi1, Young Ok Kim2 & Kyung-Hyun Cho1,*

1School of Biotechnology, Yeungnam University, Gyeongsan 712-749, 2Department of Internal Medicine, College of Medicine, The Catholic University of Korea, Seoul, Korea

Patients with hemorrhagic fever with renal syndrome (HFRS) often exhibit altered serum lipid and lipoprotein profile during the oliguric phase of the disease. Serum lipid and lipoprotein profiles were assessed during the oliguric and recovery phases in six male patients with HFRS. In the oliguric phase of HFRS, the apolipoprotein (apo) C-III content in high-density lipoproteins (HDL) was elevated, whereas the apoA-I content was lowered. The level of expression and activity of antioxidant enzymes were severely reduced during the oliguric phase, while the cholesteryl ester transfer protein activity and protein level were unchanged between the phases. In the oliguric phase, electromobility of HDL2 and HDL3 was faster than in the recovery phase. Low-density lipoprotein (LDL) particle size was smaller and the distribution was less homogeneous. Patients with HFRS in the oliguric phase had severely modified lipoproteins in composition and metabolism. [BMB reports 2010; 43(8): 535-540]

INTRODUCTION

Hemorrhagic fever with renal syndrome (HFRS) is caused by infection with the Hantaan virus (Family Bunyaviridae), which is broadly distributed throughout Europe, Russia, and Asia (1). The principal symptoms of HFRS include fever and hemorrhagic manifestations, such as petechiae and acute renal impairment. Patients with HFRS develop acute renal failure complicated by proteinuria, hematuria, hyponatremia, and hyperphosphatemia (2, 3). Acute renal failure, as well as chronic kidney disease, is associated with hypertriglyceridemia and a reduction in high density lipoprotein-cholesterol (HDL-C) (4). The lipid metabolism disorders are accompanied by significant and transient changes in the levels of expression of apolipoprotein (apo), specifically apoC-I and apoC-III (5). Changes in the levels of lipoproteins/apolipoproteins are linked to the incidence of cardiovascular disease (CVD). Adults with chronic kidney disease also have a high burden of CVD (6). Indeed, CVD is a frequent cause of death in patients on chronic hemodialysis (7).

Thus, we have determined the changes in lipoprotein metabolism occurring in patients in the oliguric phase of HFRS. In the current study, the lipoprotein profiles of six male patients with HFRS who were admitted to the Nephrology Unit of Uijeongbu St. Mary’s Hospital (Uijeongbu, Korea) were analyzed. We determined the lipoprotein metabolism parameters from the sera of patients with HFRS in the oliguric phase, as well as sera from the same patients after recovery. Four normolipidemic serum samples from age- and gender-matched healthy persons were also analyzed and compared to the serum samples from the patients with HFRS.

RESULTS

Hypertriglyceridemic serum lipid profile in the oliguric phase

In the oliguric phase, blood urea nitrogen (BUN) and creatinine (Cr) levels were increased by 5.4- and 7-fold, respectively, as compared to the recovery phase levels, as shown in Table 1. The calculated glomerular filtration rate (GFR) was decreased in the oliguric phase (12.5 ± 3.7 ml/min), but increased (71.7 ± 23.1 ml/min) in the recovery phase.

In the oliguric phase, the serum cholesterol concentrations were significantly reduced by approximately 32%, and the serum triglyceride (TG) concentrations were increased by 5.4- and 7-fold, respectively, as compared to the recovery phase levels, as shown in Table 1. The calculated glomerular filtration rate (GFR) was decreased in the oliguric phase (12.5 ± 3.7 ml/min), but increased (71.7 ± 23.1 ml/min) in the recovery phase. In the oliguric phase, the serum cholesterol concentrations were significantly reduced by approximately 32%, and the serum triglyceride (TG) concentrations were increased significantly (up to approximately 203% of the recovery phase levels), indicating a change in the hypocholesterolemic and hypertriglyceridemic lipid profiles. The average HDL-C level was reduced in the oliguric phase. After recovery, the HDL-C reverted back to normal levels, similar to that of the reference levels. Interestingly, serum total protein and albumin concentrations were reduced by approximately 20% and 30% in the oliguric phase as compared to the recovery phase, respecti-

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Table 1. Serum parameters and enzyme activities obtained from patients with hemorrhagic fever and renal syndrome during oliguric and recovery phase as compared to healthy controls

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Oliguric (n = 6)</th>
<th>Recovery (n = 6)</th>
<th>Reference (n = 4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (yr)</td>
<td>40.8 ± 3.8</td>
<td>40.8 ± 3.8</td>
<td>42 ± 4.5</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>25.5 ± 0.8</td>
<td>25.8 ± 1.1</td>
<td>24.5 ± 1.0</td>
</tr>
<tr>
<td>TC (mg/dl)</td>
<td>146 ± 16</td>
<td>214 ± 39</td>
<td>189 ± 20</td>
</tr>
<tr>
<td>TG (mg/dl)</td>
<td>403 ± 167</td>
<td>39 ± 6</td>
<td>41 ± 4</td>
</tr>
<tr>
<td>HDL-C (mg/dl)</td>
<td>29 ± 2</td>
<td>15 ± 3</td>
<td>11 ± 5</td>
</tr>
<tr>
<td>BUN (mg/dl)</td>
<td>82 ± 23</td>
<td>0.53 ± 0.2</td>
<td>0.2-1.2</td>
</tr>
<tr>
<td>Total bilirubin (mg/dl)</td>
<td>0.38 ± 0.1</td>
<td>0.38 ± 0.1</td>
<td>0.2-1.2</td>
</tr>
<tr>
<td>Albumin (g/dl)</td>
<td>3.0 ± 0.2</td>
<td>4.3 ± 0.3</td>
<td>4.5 ± 0.4</td>
</tr>
<tr>
<td>GOT (U/L)</td>
<td>81 ± 34</td>
<td>23 ± 14</td>
<td>20 ± 8</td>
</tr>
<tr>
<td>GPT (U/L)</td>
<td>27 ± 9</td>
<td>10 ± 5</td>
<td>18 ± 3</td>
</tr>
<tr>
<td>LCAT (% CE-conversion/hr/50 μl of serum)</td>
<td>1.6 ± 0.26</td>
<td>8.5 ± 0.2</td>
<td>10.6 ± 1.5</td>
</tr>
<tr>
<td>PON (μU/L/10 ml of serum)</td>
<td>30 ± 5</td>
<td>73 ± 9</td>
<td>76 ± 6</td>
</tr>
<tr>
<td>CETP (% CE transfer/50 μl of serum)</td>
<td>27 ± 3</td>
<td>24 ± 2</td>
<td>21 ± 3</td>
</tr>
</tbody>
</table>

BMI: body mass index, BUN: blood urea nitrogen, CETP: cholesteryl ester transfer protein, GOT: glutamic oxaloacetic transaminase, GPT: gamma-glutamic pyruvic transaminase, HDL-C: high-density lipoprotein-cholesterol TG: triglyceride, *Data are expressed as the mean ± SD from three independent measurements, † Normal range from guidelines, ‡ P < 0.05 versus recovery phase (paired t-test) § P < 0.01 versus recovery phase (paired t-test).

Immunodetection
SDS-PAGE analysis with equal amounts of HDL₃ (5 g/lane) revealed that the composition of apolipoproteins were markedly changed between the phases (supplemental figure). Corresponding to the molecular weight, apoA-I (28 kDa) and apoA-II (17 kDa) are major proteins observed in both the oliguric and recovery phases. ApoC-III band (14 kDa) appeared distinctly in the oliguric phase as indicated by the rectangular box (supplemental figure). Immunodetection revealed that the apoC-III level was elevated only in the oliguric phase (Fig. 1). In the same period, the level of expression of apoA-I was reduced in the oliguric phase by approximately 2-3-fold, as compared to of the recovery phase samples based on immunodetection (Fig. 1). This result indicates a different expression of apolipoproteins in the oliguric phase of HFRS in that apoA-I was decreased and apoC-III was increased.

Electrophoretic migration of HDL
The purified HDL from each patient was compared via electrophoresis using agarose gel, as the migration ability of each lipoprotein is highly dependent on the intact charge and size. HDL₂ from the oliguric phase migrated faster than in the recovery phase, as shown by agarose gel electrophoresis with equal loads of protein (5 μg in each lane), thereby indicating that the HDL₂ of the oliguric phase may have smaller particles and a greater charge, and after recovery the size and charge are restored (Fig. 2A).

This result correlates well with our previous report (8) that HDL in the oliguric phase may have a decreased particle size and/or increased negative charge with down-regulated protein levels.

ELISA-based detection of apoC-III
Sandwich ELISA-based detection revealed that the serum apoC-III level from the oliguric phase was approximately 3-fold higher than the recovery phase (Table 2), which fell in the normal range (123±32 μg/ml). All lipoprotein fractions showed significantly elevated apoC-III levels in the oliguric phase. ApoC-III in very low density lipoprotein (VLDL) from the oliguric phase was 4.5-fold higher than the recovery phase. HDL₂ and HDL₃ from the oliguric phase were 3.6- and 3.1-fold higher than the apoC-III level of the recovery phase, respectively (Table 2).

Activities of HDL-associated enzymes
Lecithin:cholesterol acyltransferase (LCAT) activity was almost deprived in the oliguric phase, as shown in Table 1. The cholesteryl ester (CE)-conversion activity was 82% lower in the oliguric phase compared to the recovery phase. Immunodetection revealed that LCAT band was undetectable in the oliguric phase, and was restored back to normal levels (similar to the control sera), indicating that LCAT activity and expression were lost in the oliguric phase (Fig. 1).

Paraoxonase (PON) activity was reduced approximately 60% in the oliguric phase compared to the recovery phase.
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Fig. 1. Level of expression of apolipoproteins and enzymes in HDL$_3$ from representative patients. Equal amounts of HDL$_3$ (6 μg/lane) were loaded and immunodetected with anti-apoC-III (#AB821; Chemicon) anti-apoA-I (#ab7613; Abcam), anti-LCAT Abcam #ab786; Abcam), anti-PON 1 (ab24261; Abcam), and anti-CETP (abcam 19012; Abcam) antibodies. The lower number indicates the relative band intensity of apoA-I in the recovery phase as compared to each of the oliguric phase samples.

Table 1. The level of protein expression of PON in the oliguric phase was reduced, and increased back to control levels on recovery, as shown in Fig. 1. These results show that LCAT and PON, which are crucial in maintaining antioxidant activity of HDL, were transiently impaired with respect to the activity of expression in the oliguric phase.

CETP activity was not changed in the oliguric phase

Cholesteryl ester transfer protein (CETP) activity and the level of immunodetected protein were not changed between the oliguric and recovery phases, as shown in Table 1 and Fig. 1. The CE-transfer activity to low density lipoprotein (LDL) from [3H]-CE-HDL was 23 ± 3% in the oliguric phase and 24 ± 2% after recovery, while the control sera had levels of 21 ± 2%.

Change of LDL properties

The particle size of LDL and its distribution was determined via electron microscopy with the same amount of LDL (0.3 mg/ml of protein). Interestingly, LDL from the oliguric phase had a severely distorted morphology, fewer particle numbers and a smaller size (average range of diameter of 46-57 nm). In contrast, LDL from the recovery phase showed a more spherical shape and larger particle size (average diameter range of 65-73 nm; Fig. 2B). These results indicate that normal LDL was prone to change into smaller LDL in the oliguric phase, which

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was more susceptible to oxidation in the early stages of viral infection.

LDL from the oliguric phase was more sensitive to cupric ion-mediated oxidation, with nearly 2-fold malondialdehyde (MDA) production by 5 and 10 μM treatment (Fig. 2B). This result suggests that LDL in the oliguric phase was more prone to oxidation in the presence of an equal pro-oxidant concentration, thereby indicating that oxidative stress in the oliguric phase might be higher than the normal phase.

**DISCUSSION**

In the current study, changes in the composition, function, and structure of lipoproteins between the oliguric and recovery phases of HFRS were evaluated, particularly with respect to apolipoprotein composition and the enzymatic activity of HDL-C. Although the number of patients was relatively small in this study (due to strict inclusion criteria), we provided more detailed evidence for functional and structural changes in the lipoprotein levels, including severe deprivation of the HDL-associated enzymes, LCAT and PON. Physiologically, the serum bilirubin levels did not change between the oliguric and recovery phases. This phenomenon is comparable to the symptoms of scrub typhus, or tsutsugamushi, in which total bilirubin levels did not change between the oliguric and recovery phases. This result may help to explain why serum TG accumulated in VLDL; the current results demonstrated that LDL from the oliguric phase was smaller than the reference LDL with more sensitivity to cupric ion-mediated oxidation (Fig. 2), supporting the notion that the oliguric-phase LDL was more prone to oxidation. The shift to greater atherogenic lipoprotein predominance could be the result of alterations in the properties of HDL (20), as well as LDL. This result correlates well with the smaller LDL size and faster electromobility in the oliguric phase than the recovery phase.

In conclusion, abnormal lipoprotein parameters in the oliguric phase of HFRS appeared to be quite similar to those of patients with chronic hemodialysis and viral infections, i.e., hypercholesterolemic and hypertriglyceridemic serum profiles with reduced HDL particle size and cholesterol levels, and apoC-III overexpression in the HDL. Serum LCAT and PON activity were significantly reduced in the oliguric phase, with reductions in HDL-C and particle size, while CETP activity was not changed between the phases.

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**Table 2. Sandwich ELISA-based detection of apoC-III in serum and lipoprotein**

<table>
<thead>
<tr>
<th>Lipoprotein</th>
<th>Oliguric (n = 6)</th>
<th>Recovery (n = 6)</th>
<th>Reference (n = 4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum (μg/ml)</td>
<td>344 ± 55†</td>
<td>123 ± 32</td>
<td>95 ± 26</td>
</tr>
<tr>
<td>VLDL (μg/mg of protein)</td>
<td>147 ± 23†</td>
<td>33 ± 7</td>
<td>20 ± 5</td>
</tr>
<tr>
<td>LDL (μg/mg of protein)</td>
<td>9 ± 5</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>HDL1 (μg/mg of protein)</td>
<td>326 ± 48†</td>
<td>90 ± 16</td>
<td>70 ± 21</td>
</tr>
<tr>
<td>HDL2 (μg/mg of protein)</td>
<td>93 ± 23†</td>
<td>30 ± 7</td>
<td>24 ± 6</td>
</tr>
</tbody>
</table>

nd: not detected, *Fifty microliters of individual lipoprotein (2 mg of protein/ml) was reacted with apoC-III antibody (AB821), which was coated on a 96-well plate. † Data are expressed as the mean ± SD from three independent measurements, ‡ P < 0.05 versus recovery phase (paired t-test).
MATERIALS AND METHODS

Blood sampling
Sera were obtained from patients (mean age = 40.8 ± 3.8 years) during the oliguric phase of HFRS (n = 6, body mass index [BMI] = 25.5 ± 0.8 kg/m²) and after recovery 30-40 days after the oliguric phase (n = 6, BMI = 25.8 ± 1.1 kg/m²). None of the patients with HFRS took medications during the oliguric phase, nor did any of the patients drink alcohol excessively before or after the oliguric phase. The patients had no histories of endocrinologic disorders, including hyperlipidemia, diabetes mellitus, or hypertension. Gender and age-matched reference sera were obtained from four healthy volunteers (mean age = 42 ± 4.5 years; BMI = 24.5 ± 1.0). All of the reference individuals were healthy and had unremarkable medical histories. Informed consent was obtained from the patients and the reference subjects, and the protocols of this study were approved by the Institutional Review Board of the Catholic University of Korea (Seoul, Korea).

Isolation of lipoproteins
VLDL (1.006 < d < 1.019 g/ml), LDL (1.019 < d < 1.063), HDL2 (1.063 < d < 1.125), and HDL3 (1.125 < d < 1.210) were isolated from the sera via sequential ultracentrifugation, in accordance with the method described by Havel et al. (21), at 100,000 x g for 22 hours at 10°C using a Himac CP-90 α (Hitachi, Tokyo, Japan).

Determination of serum lipids and proteins
Serum parameters, lipids, and glucose concentrations were determined using an automatic blood analyzer (Fuji DRI-CHEM, FDC-3000; Tokyo, Japan). Protein concentrations in lipoprotein species were determined via the Lowry protein assay, as previously reported (24-26). The migration of each lipoprotein is dependent on its intact charge and size of particle, and was compared via electrophoresis in agarose gels in native conditions (27). The gel was dried and stained with 0.125% Coomassie brilliant blue in order to visualize the lipoprotein bands.

Calculation of GFR
The GFR was calculated using the Cockcroft-Gault formula (23) as follows: GFR = [(140 – age x body weight) / (72 x serum creatinine)].

HDL-associated enzyme assay
LCAT, CETP, and PON assays were carried out as described in the supplemental materials, as previously reported (24-26).

Enzyme linked immunosorbent assay (ELISA)
For the sandwich ELISA, diluted primary antibody (1 : 2,000 or 1 : 4,000) was coated onto a 96-well plate (Nunc Maxisorp #439454) overnight at 4°C. As primary antibodies, apoA-I (#ab7613; Abcam), anti-apoA-I (#ab7613; Abcam), anti-LCAT (#ab786; Abcam), anti-PON-1 (#ab24261; Abcam), and anti-CETP antibodies (#ab 19012; Abcam). The relative band intensities were compared via band scanning using a Gel Doc XR (Bio-Rad, Hercules, CA, USA) with Quantity One software, version 4.3.2.

Electrophoresis and Western blot analysis
The apolipoprotein/lipoprotein compositions were compared via SDS-PAGE with the same amount of protein loading, and the level of expression of apolipoprotein was analyzed by immunodetection using anti-apoC-III (#AB821; Chemicon), anti-apoA-I (#ab7613; Abcam), anti-LCAT (#ab786; Abcam), anti-PON 1 (#ab24261; Abcam), and anti-CETP antibodies (#ab 19012; Abcam). The relative band intensities were compared via band scanning using a Gel Doc XR (Bio-Rad, Hercules, CA, USA) with Quantity One software, version 4.3.2.

Electromobility of lipoproteins
The migration of each lipoprotein is dependent on its intact charge and size of particle, and was compared via electrophoresis in agarose gels in native conditions (27). The gels were dried and stained with 0.125% Coomassie brilliant blue in order to visualize the lipoprotein bands.

Susceptibility of LDL oxidation
In order to evaluate susceptibility via copper-mediated LDL-oxidation, each diluted LDL (108 μg/ml) in phosphate buffered saline (PBS; pH 7.4) was incubated for 4 hours at 37°C with different concentrations of CuSO4. After incubation, the degree of LDL-oxidation was determined via measurements of the quantities of thiobarbituric acid reactive substances (TBARS) generated at 517 nm (28).

Electron microscopy
Transmission electron microscopy (TEM) was performed with a Hitachi electron microscope (Ibaraki, Japan), model H-7600, operating at 80 kV. Each LDL was negatively stained with 1% sodium phosphotungustate (PTA; pH 7.4) at a final protein concentration of 0.3 mg/ml in PBS. Five μl of the LDL suspension was blotted with filter paper and replaced immediately with a 5 μl droplet of 1% PTA. After a few seconds, the stained LDL fraction was blotted onto a Formvar carbon-coated 300 mesh copper grid and air-dried. The shape and size of the LDL were determined via TEM photography at a magnification of 30,000x.

Statistical analysis
All values were expressed as the mean ± standard deviation (SD), and changes between the oliguric and recovery phases were evaluated by a paired t-test using SPSS (version 14.0;
SPSS, Inc., Chicago, IL, USA). Statistical significance was defined as a P < 0.05.

Acknowledgements

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REFERENCES