Distribution of CII and CIII Peptides in Lipoprotein Classes: Methods and Clinical Significance

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We describe a method for measuring apolipoprotein (apo) C distribution between apo B-containing lipoprotein (apo B–LP) and non-apo B–LP. The procedure requires the precipitation of apo B–LP, the redissolution of the pellet, and the quantification of C peptides in the redissolved pellet. The ratio of apo C in non-apo B–LP to apo C in apo B–LP has been calculated for both CII and CIII (R-CII and R-CIII, respectively). R-CII (0.49 ± 0.25) and R-CIII (0.64 ± 0.54) in patients on maintenance dialysis are significantly lower than in the control group (1.14 ± 0.57 and 1.45 ± 0.92, respectively), indicating that hypertriglyceridemia in these patients results from a reduced catabolism of triglyceride-rich LP (TGLRP). Patients with coronary artery disease (CAD) show a distribution of C peptides not different from the control group. Analysis of covariance reveals that the patterns of R-CII and R-CIII are not entirely predictable from the serum concentration of triglycerides. This result seems to support the hypothesis that the underlying metabolic defects involving TGLRP in dialysis patients are not the same as those in patients with CAD.

Indexing Terms: apolipoproteins/triglycerides/coronary artery disease/dialysis/hypertriglyceridemia

The role of the triglyceride-rich lipoproteins (TGLRP) (chylomicrons and very-low-density lipoproteins (VLDL)) in the development of atherosclerosis was recently reported (1).6 The metabolism of these lipoproteins (LP) deeply affects the structure, metabolism, and function of cholesterol-rich LP (low-density lipoproteins (LDL) and high-density lipoproteins (HDL)), and in hypertriglyceridemia these alterations may be atherogenic (2). The atherogenic potential of the TGLRP is mainly supported by the evidence that an individual’s ability to metabolize triglycerides (TG) has a strong influence on HDL cholesterol plasma concentration (3). The catabolic pathway of TGLRP includes the lipolysis of the TG core by lipoprotein lipase (LPL; EC 3.1.1.34) as well as a transfer of surface constituents (phospholipids, free cholesterol, apolipoprotein (apo) C) from TGLRP to HDL3 (Fig. 1). The HDL3 is thus transformed to HDL2, while the remnants of TGLRP are cleared by the parenchymal cells of the liver; the LDL receptor-related protein of these cells binds to the apo E and might be a candidate for the putative chylomicron remnant receptor (4). Apo CII and apo CIII play an important role in this process, modulating the activity of LPL, with CII acting as activator and CIII as inhibitor of the enzyme. The transfer of these peptides from TGLRP to HDL is a marker of an efficient catabolism of TGLRP, because these apolipoproteins (particularly apo CIII) have been shown to retard the apo E-mediated hepatic uptake of TGLRP remnants as long as they are integral components of these LP (5–7).

In case of defective catabolism of TGLRP, an increased plasma concentration of TG, a decreased concentration of HDL cholesterol, and a defective clearance of TGLRP can be observed. Individuals with this condition are at high risk of developing atherosclerosis because a decreased efficiency of reverse cholesterol transport is associated with an increased peripheral uptake of TGLRP remnants by macrophages via the scavenger receptor (1). From the pathophysiology of TGLRP catabolism concisely summarized here, the measurement of total apo CII and CIII and the evaluation of their distribution between apo B-containing LP (apo B–LP) (chylomicrons, VLDL, LDL) and the non-apo B–LP (HDL) could be of great clinical interest (5).

The methods used for measuring these apolipoproteins include immunoaffinity chromatography (8), two-site immunoenzymatic methods (9–11), or separation of LP by ultracentrifugation (12). These procedures are elaborate and time consuming, and require dedicated laboratory equipment. A simpler method, involving the chemical precipitation of apo B–LP, has been proposed (5, 13), but there are some problems with hypertriglyc-

Fig. 1. LPL-induced lipolysis of the TG core by TGLRP and resulting transfer of C peptides, free cholesterol, and phospholipids to HDL3; HDL3 is thus converted to HDL2.
eridemic samples. We describe here a practical method involving the precipitation of apo B–LP and measurement of apolipoproteins by nephelometry. We discuss the application of this method to normal subjects and to patients with different types of hypertriglyceridemia.

Patients and Methods

Patients

The coronary artery disease (CAD) group consisted of 18 patients (17 men, 1 woman), with CAD angiographically documented; mean age ± SD 56.9 ± 9.6 years (range 38–68). The dialysis group consisted of 34 patients (25 men, 9 women) with chronic renal failure, 15 on maintenance hemodialysis and 18 on maintenance peritoneal dialysis; age 63.7 ± 12.9 years (range 31–81). The control group consisted of 18 healthy subjects (11 men, 7 women) from the medical staff; age 50.9 ± 15.2 years (range 23–79).

None of the enrolled subjects was treated with lipid-lowering drugs or was using oral contraceptives. Venous blood was collected in the morning after a night of fasting and serum was used for the measurements.

Procedures followed were in accordance with the Helsinki Declaration of 1975, as revised in 1983.

Methods

Total cholesterol, TG, HDL cholesterol, apo A-I, and apo B were measured on the same day of collection as part of a lipid profile routinely performed for these patients. Enzymatic methods were used for cholesterol and TG (14, 15); HDL cholesterol was estimated in the supernatant after apo B–LP precipitation (16) with the same enzymatic method used for cholesterol quantification. Apo A-I and apo B were measured with the BNA nephelometer (Behringwerke AG, Marburg, Germany) by using rabbit polyclonal antisera from the same company and a fixed-time method. Specimens for apo CII and apo CIII measurements were stored frozen (−70 °C) until the assays were performed (up to 2 months later). Apo C was measured in serum and in apo B–LP (also with the BNA nephelometer) by using fixed-time assays (6 min for CIII and 24 min for CII) and polyclonal antisera (Eiken Chemical Co., Tokyo, Japan). For measurement of apo C in apo B–LP, apo B–LP was precipitated from serum and then redissolved as previously described (16, 17). Briefly, the apo B–LP was precipitated by mixing 200 μL of serum with 2000 μL of a mixture of polyethylene glycol 6000 (100 g/L), dextran sulfate (Mr, 15 000, 37.4 mg/L), and MgCl₂ (2.6 mmol/L) (16); after centrifugation the clear supernatant was discarded, the tube walls were wiped with filter paper, and the pellet was redissolved in NaCl solution (150 mmol/L) (17). To obtain the same concentration of apo B–LP as in the original serum, we calculated the volume of saline to add to the pellet as the original serum volume minus the pellet volume. The pellet volume was measured by weighing the test tube before precipitation and after the supernatant was discarded. Preliminary studies with dried pellets demonstrated that the error introduced by translating pellet weight into volume was negligible (maximum 1%), if one considers that water in the pellet represented >90% of its weight and that the mean weight of the pellet was 20 mg.

The distribution of CII and CIII peptides between apo B–LP and non-apo B–LP was expressed as the CII ratio (R-CII) and the CIII ratio (R-CIII), respectively (5). The ratios were calculated with the formula: (whole serum apo C – apo C in apo B–LP)/apo C in apo B–LP. The higher the ratios, the higher the content of apo C in non-apo B–LP. The relative amounts of CII and CIII (CII/CIII) in whole serum and in apo B–LP were also calculated.

Statistical Analysis

The comparison among the mean values of the three groups was performed by one-way analysis of variance and Dunnet’s multiple comparison test (18). The influence of sex, age, and TG concentration on CII, R-CII, CIII, and R-CIII variability was investigated by analysis of covariance (18).

Results

The precipitation and the redissolution methods have already been verified for the completeness of the apo B–LP precipitation (even in highly hypertriglyceridemic samples) (16) and of the recovery of the LP in the redissolved pellet (17).

The absence of any coprecipitation of apo A-I-containing LP has also been previously tested, verifying the lack of reactivity for apo A-I in the redissolved pellets by counterimmunoelectrophoresis (detection limit 1 mg/L) (16). As has been verified in previous studies on apo B (17), the precipitation and the redissolution steps do not alter the immunoreactivity of apolipoproteins, thus allowing measurements of comparable accuracy in whole serum and in the redissolved pellet. The precipitation and redissolution steps are quite rapid, and a number of samples can be ready for the nephelometric quantification in <1 h. The between-series CVs of the laboratory internal quality control for total and HDL cholesterol and for TG are always <2.5%; for apo A-I and apo B, the CVs are <5.0%. The immunochromatography methods for apo CII and apo CIII measurements are linear up to 2 and 4.5 mg/L, respectively, thus allowing precise measurements in LP classes with low content of apoproteins. The between-series CVs are 5.3% for CII and 8.1% for CIII.

The groups differ both in regard to age (P = 0.003) and sex distribution (P = 0.6), but the observed differences in the studied variables are unaffected by adjustment for age and sex as tested by covariance analysis.

The results of the classical lipid and apolipoprotein measurements show that the dialysis group is significantly different from the control group for TG, HDL cholesterol, and apo A-I; the CAD group differs for HDL cholesterol and apo A-I (Table 1). Cholesterol, apo B, and TG in the CAD group are higher than in the control group but do not reach statistical significance (Table 1). The mean values of CII and CIII and the related ratios (R-CII, R-CIII, CII/CIII) of the patients on maintenance dialysis are statistically different from those of the con-
control group; only serum CII of the CAD group shows statistical difference when compared with the control group (Table 2). The CIII/CII ratios both in whole serum and in apo B–LP are significantly higher in the dialysis group than in the controls. The analysis of covariance to test the influence of TG on the variability of CII, R-CII, CIII, and R-CIII shows that the differences among the three groups persist even after adjustment for TG. The statistical significance of the test is $P = 0.0001$ both for R-CII and R-CIII; it is slightly less for serum CIII ($P = 0.002$). If the hemodialysis group is separated from the peritoneal dialysis group (Table 3), we can verify that the above-cited differences from the control group are more pronounced in patients on peritoneal dialysis, the two ratios showing statistical differences between the two groups of dialysis patients.

Discussion

Given the increasing evidence of the pathogenetic influence of TGRLP remnants on the development of atherosclerosis, it has become important for the clinical chemistry laboratory to measure the efficiency of TGRLP catabolism. The shift of CII and CIII peptides from apo B–LP to non-apo B–LP during the hydrolysis of TG is postulated to be an index of the body's ability to efficiently metabolize TGRLP (1, 5).

The proposed methods include separation of LP by ultracentrifugation and measurement of apolipoproteins in the separated fractions (12), two-site immunoenzymatic methods (9-11), and immunoaffinity chromatography (8). Ultracentrifugation fractionates LP on the basis of their hydrated density, but LP are functionally heterogeneous in this physicochemical characteristic (19). Furthermore, the technique is time-consuming and cannot be used to handle large series of samples. The immunoenzymatic methods involve the use of two or more different antisera and require a number of successive measurements. The fractionating of LP by immunoaffinity chromatography is probably the most accurate procedure and can be regarded as the method of choice for measuring the apolipoprotein composition of different LP classes. Because it is elaborate and requires dedicated equipment, it could be considered more a Reference Method than a routine assay (8, 20).

A more practical method involving the quantification of apo C in heparin-Mn supernatants (non-B–LP) and in precipitates (apo B–LP) has been proposed (5, 13). However, this precipitation method may result in incomplete separation of TGRLP in hypertriglyceridemic samples, to the extent that the Centers for Disease Control standardization of the heparin-Mn method for the quantification of HDL cholesterol requires the prior removal of VLDL by ultracentrifugation (19). The precipitation mixture used in this study allows complete separation of apo B–LP even at high concentrations of TG (16). This feature could be useful in view of the fact that hypertriglyceridemia is likely to be present in conditions requiring CII and CIII quantification. Furthermore, a complete recovery and a lack of any alteration in the immunoreactivity of precipitated LP in the redissolved pellet have been demonstrated (17).

A number of studies suggest that the underlying metabolic defect in patients on maintenance dialysis results from reduced catabolism of TGRLP (21-25). Some findings in our study support this hypothesis. The dialysis group was characterized by an increase in TG concentrations accompanied by increased concentrations of serum CII and CIII; the increase was more prominent in the apo B–LP, as demonstrated by low R-CII and R-CIII, indicating an impaired clearance of TGRLP remnants (5). These data agree with previous reports of increased CIII in serum and in apo B–LP in subjects on maintenance dialysis (9, 23, 25, 26). As for serum apo CII, there were some discrepancies because increase, decrease, and no change have been reported (9, 26); to the best of our knowledge, there are no reports on R-CII. The increase of apo CIII in whole serum and in apo B–LP has been claimed to contribute to hypertriglyceridemia in dialysis patients because the peptide acts as an inhibitor of LPL (27). The finding of a similar increase in apo CII (which acts as an activator) does not oppose such a hypothesis because the dialysis patients showed significantly increased CIII/CII ratios both in serum and in separated LP (Table 2). The lipolysis reaction may re-

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Table 1. Lipid and apolipoprotein concentrations (mean ± SD) in patients and controls.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Total chol, mmol/L</th>
<th>TG, mmol/L</th>
<th>HDL chol, mmol/L</th>
<th>Apo A-I, g/L</th>
<th>Apo B, g/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>5.41 ± 1.07</td>
<td>1.17 ± 0.52</td>
<td>1.39 ± 0.37</td>
<td>1.59 ± 0.26</td>
<td>1.18 ± 0.33</td>
</tr>
<tr>
<td>CAD</td>
<td>5.54 ± 1.06</td>
<td>2.03 ± 0.74</td>
<td>1.10 ± 0.26*</td>
<td>1.27 ± 0.22a</td>
<td>1.33 ± 0.32</td>
</tr>
<tr>
<td>Dialysis</td>
<td>5.50 ± 1.64</td>
<td>2.73 ± 1.99*</td>
<td>1.03 ± 0.29*</td>
<td>1.27 ± 0.17a</td>
<td>1.39 ± 0.46</td>
</tr>
</tbody>
</table>

* Significantly different from controls at $P < 0.01$.

Chol, cholesterol.

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Table 2. Measurements of CII and CIII (mean ± SD) in patients and controls.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Serum CII, mg/L</th>
<th>Serum CIII, mg/L</th>
<th>R-CII</th>
<th>R-CIII</th>
<th>Serum CII/CIII</th>
<th>Apo B–LP CII/CIII</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>30.3 ± 8.1</td>
<td>75.7 ± 30.5</td>
<td>1.14 ± 0.57</td>
<td>1.45 ± 0.92</td>
<td>2.20 ± 0.42</td>
<td>1.92 ± 0.38</td>
</tr>
<tr>
<td>CAD</td>
<td>51.6 ± 17.8a</td>
<td>98.6 ± 34.1</td>
<td>1.52 ± 0.96</td>
<td>2.05 ± 2.18</td>
<td>2.23 ± 0.85</td>
<td>1.78 ± 0.41</td>
</tr>
<tr>
<td>Dialysis</td>
<td>59.8 ± 33.6b</td>
<td>168.0 ± 99.4</td>
<td>0.48 ± 0.25b</td>
<td>0.84 ± 0.54*</td>
<td>3.00 ± 0.71*</td>
<td>2.41 ± 0.50a</td>
</tr>
</tbody>
</table>

* Significantly different from controls at $P < 0.05$.

* Significantly different from controls at $P < 0.01$.
quire an optimum apoprotein composition for normal 
TG hydrolysis; a more pronounced increase of the inhibitor (such as that observed in patients on maintenance 
dialysis) can probably decrease the activity of the 
enzyme, contributing to defective removal of TGRLP (12, 
27-29). The higher concentration of TG and the lower 
R-CII and R-CIII of patients on peritoneal dialysis 
compared with patients on hemodialysis (Table 3) suggest 
that disturbances of TGRLP metabolism are more severe 
in patients undergoing peritoneal dialysis. We postu-
late that transperitoneal transfer of dialysate glucose 
plays an important role, since the enzymatic system involved in TG hydrolysis is insulin-dependent (22, 24).

In patients with CAD we observed a different pattern: 
Moderate increases of TG and serum CII and CIII were 
not accompanied by a decrease of R-CII and R-CIII; 
moreover, the relations between the two apoprotein 
modulators of LPL were preserved because the CIII/CII ratios were similar to those found in the control group 
(Table 2). Because biochemical alterations indicating an 
impaired clearance of TGRLP (i.e., presence of apo B-LP 
enriched in apo C, and prevalence of the inhibitor over 
the activator of LPL both in serum and in apo B-LP) 
were not observed in the CAD group, one can postulate 
that the increase of TG in these patients cannot be 
attributed to a defective removal of TGRLP. The hypo-
thesis that the underlying metabolic defects involving 
TGRLP in patients on maintenance dialysis are not the 
same as those in patients with CAD seems supported by 
the results of analysis of covariance, which showed that 
the variability of R-CII, R-CIII, and serum CIII are not 
totally predictable from the serum content of TG. In 
other words, the shift of apo C from non-apo B-LP to apo 
B-LP seems not to be a mere function of the serum 
content of TG but an effect dependent on the pathogene-
sis of hypertriglyceridemia.

In conclusion, the quantification of apo CII and apo 
CIII in apo B-LP may be of help in highlighting the 
metabolic defects underlying the different types of hy-
pertriglyceridemia. These apolipoproteins can be mea-
sured easily with a sensitive nephelometric method af-
ter chemical separation of apo B-LP at relatively low 
cost. The accuracy of the measurements requires both 
an effective separation of LP and an accurate immuno-
chemical quantification. Whereas a functional identifi-
cation of LP requires separation on the basis of apolipo-
protein content (immunoaffinity chromatography), the 
accuracy of the immunochemical assay requires the use 
of appropriate calibration materials and antisera. Stan-
dardization of the apolipoprotein measurements is not 
entirely accomplished for apo A-I and apo B and is in its 
initial stages for "minor" apolipoproteins. Nevertheless, 
the values of the present control group are in good 
agreement with those reported by others (13, 24, 25), 
indicating a satisfactory performance of the method il-
lustrated here. The clinical importance of the suggested 
variables needs to be further confirmed by studying 
patients with various types of hypertriglyceridemia.

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