Characterization of Triglyceride Rich Lipoproteins with Very Light Density by Ultracentrifugation and Agarose Gel Electrophoresis using Triglyceride- and Cholesterol-Staining

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Abstract. Hypertriglyceridemia is an independent risk factor for atherosclerosis. This risk is most likely due to accumulation of circulating triglyceride rich lipoproteins with heterogeneous particles. The identification and characterization of these triglyceride rich lipoproteins is important to detect abnormality of triglyceride metabolism. In the present study, we developed a new method that combines ultracentrifugation and agarose gel electrophoresis with triglyceride- and cholesterol-staining. We investigated 40 subjects with hypertriglyceridemia. Triglyceride rich lipoproteins with very light density were recovered in the aqueous fraction after ultracentrifugation (17,000 x g, 15 min). The lipoproteins recovered in the aqueous fraction contained chylomicrons, if present, their remnants, and light-VLDL (d < 1.000 g/ml) containing apoB-100, but not normal VLDL (d < 1.006 g/ml) and IDL. Triglyceride rich lipoproteins in the aqueous fraction were characterized by electrophoresis patterns of triglyceride- and cholesterol-staining. Forty patients with hypertriglyceridemia were separated into 8 groups according to their electrophoretic patterns. In lipoproteins recovered in the aqueous fraction from each group, the triglyceride level was correlated with the respective cholesterol level. In summary, a system using ultracentrifugation and agarose gel electrophoresis with triglyceride- and cholesterol-staining is useful for characterization of triglyceride rich lipoproteins and their remnants. (received 23 December 2002; accepted 22 February 2003)

Keywords: hypertriglyceridemia, chylomicron, chylomicron remnant, VLDL remnant, apo-B-48

Introduction

Hypertriglyceridemia is caused by the accumulation of triglyceride rich lipoproteins, chylomicrons, VLDL, and their remnants, and these lipoproteins play an important role in the pathogenesis of atherosclerosis and thrombosis [1-5]. It is important to characterise the triglyceride rich lipoproteins in serum, because they are heterogeneous particles [6] and have different specific roles [7,8].

Standard techniques for isolating and characterizing the lipoproteins, include ultracentrifugation [9,10], high-performance liquid chromatography (HPLC) [11,12], electrophoresis [13,14], and immunoseparation [15,16]. Ultracentrifugation is the “gold standard” method to isolate lipoproteins from other serum proteins [9,10], but it is difficult to achieve subfractionation of heterogeneous particles with similar densities and to characterize their lipid composition. HPLC is an excellent method with mild conditions for fractionation of serum lipoproteins [11,12]. Lipid-staining methods using agarose or polyacrylamide gel electrophoresis are useful to analyze the subfractions in lipoproteins [17], but the electrophoretic pattern of lipid staining with Fat Red 7B or Sudan Black does not reflect the concentration and composition of different triglyceride rich lipoproteins. Remnant-like particles...
(RLP) isolated by immunoseparation with anti apoA-I and anti-apoB-100 antibody include chylomicrons and remnant lipoproteins, containing apoB-48 and apoB-100 [15,16], but the characterization of RLP in serum lipoproteins of patients with hypertriglyceridemia is insufficiently clear.

It is important to measure triglyceride and cholesterol in triglyceride-rich lipoproteins precisely, because of a relationship between the concentration and composition of cholesterol and triglyceride and the development of cardiovascular disease. Agarose gel electrophoresis with triglyceride- and cholesterol-staining is a simple method to measure the concentrations of triglyceride and cholesterol in subfractions of lipoproteins, but use of this technique to characterize serum lipoproteins has not been fully evaluated.

This study shows that using a combination of methods, namely ultracentrifugation and agarose gel electrophoresis with triglyceride- and cholesterol-staining, permits the isolation and characterization of lipoproteins in serum of patients with hypertriglyceridemia. The triglyceride rich lipoproteins (d <1.000 g/ml) are recovered in the aqueous fraction after ultracentrifugation (17,000 x g, 15 min). This characterization provides important information about the metabolism of triglyceride rich lipoproteins in patients with hypertriglyceridemia.

**Materials and Methods**

**Materials.** Agarose electrophoresis gels (REP LIPO-30 plate), triglyceride- and cholesterol-staining reagents (Choletrimbombo and TG Titan gel S-cholesterol), and total lipid staining reagent (Fat Red 7B) were obtained from Helena Laboratories (Beaumont, TX). Reagents for cholesterol and triglyceride assays were obtained from Kyowa Medex (Tokyo, Japan) and Mizuho Medy (Fukuoka, Japan) respectively. Goat anti-human apo-B polyclonal antiserum was obtained from Daiichi Pure Chemical Co (Tokyo, Japan). Phosphate buffer (pH 7.4) containing 0.15 M NaCl and rabbit IgG anti-goat antibody conjugated with peroxidase was obtained from Medical and Biological Laboratories (Tokyo, Japan).
**Subjects and serum separation.** Fasting blood samples were obtained by venepuncture from 3 healthy members of the hospital staff and from 40 patients with hypertriglyceridemia. The serum samples were separated by centrifugation (1,000 x g, 10 min).

**Preparation of lipoproteins.** Serum lipoprotein subclasses were prepared by ultracentrifugation using the method of Hatch and Lee [10]. Ultracentrifugation was accomplished using a Beckman type TLA 100.3 rotor in an Optima TLX ultracentrifuge system (Beckman-Coulter, Fullerton, CA). Distilled water (0.5 ml) was carefully layered upon 0.5 ml of serum in a centrifuge tube (1.5 ml), and ultracentrifugation was performed at 17,000 x g for 15 min at 5°C. After ultracentrifugation, 0.4 ml samples of the top (aqueous) layer and the bottom layer of the tube were collected respectively (Fig. 1). The lipoproteins recovered in the aqueous fraction (d < 1.000 g/ml) were used for the analysis of triglyceride and cholesterol profiles by triglyceride- and cholesterol-staining using agarose gel electrophoresis. Very low density lipoprotein (VLDL; d < 1.006 g/ml), intermediate density lipoprotein (IDL; d 1.006–1.019 g/ml), low density lipoprotein (LDL;
d 1.020–1.063 g/ml), and high density lipoprotein (HDL; d 1.063–1.21 g/ml) in the bottom part (d > 1.000) were then isolated by ultracentrifugation at 424,000 x g for 2-4 hr at 5°C, respectively [10]. Each lipoprotein was dialyzed overnight against phosphate-buffered saline solution (PBS).

**Agarose gel electrophoresis and lipid staining.** Aagarose electrophoresis and triglyceride- and cholesterol-staining were performed by the following method. After application of 1 µl of serum or 1-2 µl of lipoprotein fractions into two wells of the agarose gel (REP-LIPO), electrophoresis was performed for 25 min at 90 V using the REP-system (Helena Laboratory) according to the manufacturer’s instructions. After electrophoresis, the lipoproteins in the two lanes were incubated with triglyceride- and cholesterol-staining reagents for 10 min at 30°C. The agarose gel was fixed with 5% acetic acid solution and washed with water. Densitometric scans of the gel were performed at 570 nm, and each densitometric pattern was divided in 9 sections, (ie, the maximum number of fractions in this system). The densitometric patterns were analysed using the Cholesterolcom bo system (Helena Laboratories).

**Immunoblotting of Apo-B.** ApoB-100 and B-48 were detected by immunoblotting using SDS-PAGE with minor modifications of a method described previously [18]. After electrophoresis with the SDS-
Results

Ultra-centrifugation, agarose electrophoresis, and lipid profiles. The lipoproteins recovered in the aqueous fraction (d <1.000 g/ml) were prepared by ultracentrifugation at 17,000 x g for 15 min at 5°C. Subsequently, VLDL, IDL, LDL, and HDL from the bottom fraction (d >1.000 g/ml) were isolated by ultracentrifugation at 424,000 x g for 2 to 4 hr at 5°C respectively. The characterization of electrical charge and lipid composition of serum and the 5 different lipoprotein classes was performed by agarose gel electrophoresis with triglyceride- and cholesterol-staining (Fig. 2, panel A and B). The lipoproteins in the aqueous fraction (d <1.000 g/ml) were identified as pre-β to β-fractions by densitometry on agarose electrophoresis (Fig. 2, panel A-2, A-8, B-2); their compositions of triglyceride and cholesterol and their ratios of triglyceride to cholesterol are shown in Fig. 2, panel C-2.

Determination of apolipoprotein-B. To characterize the triglyceride rich lipoproteins in the aqueous fraction (d <1.000 g/ml) and the bottom fraction (d >1.000 g/ml) recovered after ultracentrifugation at 17,000 x g for 15 min (Fig. 3, panel A), the molecular weight of apoB in both fractions was analysed by SDS-PAGE using immunoblotting with anti-apoB polyclonal antiserum.

ApoB-48 and apoB-100 were detected in the aqueous fraction (d <1.000 g/ml), but only apoB-
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Table 1. The concentration (mg/dl) and the ratio of triglyceride and cholesterol levels in serum samples from 8 representative patients with hypertriglyceridemia and in corresponding samples of the ultracentrifugal aqueous fraction (d <1.000 g/ml).

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<thead>
<tr>
<th>Patients with hypertriglyceridemia</th>
<th>Ultracentrifugal aqueous fraction (d &lt;1.000 g/ml)</th>
<th>Serum samples</th>
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<td></td>
<td>Cholesterol (mg/dl) Triglyceride (mg/dl) Cholesterol/ triglyceride ratio</td>
<td>Cholesterol (mg/dl) Triglyceride (mg/dl) Cholesterol/ triglyceride ratio</td>
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<tr>
<td>A</td>
<td>34  172  5.06</td>
<td>224  847  3.78</td>
</tr>
<tr>
<td>B</td>
<td>37  106  3.93</td>
<td>255  447  1.75</td>
</tr>
<tr>
<td>C</td>
<td>32  103  3.22</td>
<td>327  558  1.71</td>
</tr>
<tr>
<td>D</td>
<td>26  111  4.27</td>
<td>172  454  2.64</td>
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<tr>
<td>E</td>
<td>13   74   5.69</td>
<td>212  309  1.46</td>
</tr>
<tr>
<td>F</td>
<td>53   241  4.55</td>
<td>297  848  2.86</td>
</tr>
<tr>
<td>G</td>
<td>31   299  9.65</td>
<td>196  600  3.06</td>
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<tr>
<td>H</td>
<td>16   234 14.63</td>
<td>155  420  2.75</td>
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100 was found in the bottom fractions (d >1.000 g/ml) containing VLDL, IDL, and LDL (Fig. 3, panel B). This result shows that the triglyceride rich lipoproteins in the aqueous fraction (d <1.000 g/ml) include chylomicrons or chylomicron remnants containing apoB-48 and light-VLDL (d <1.000 g/ml) containing apoB-100. This is an abnormal VLDL which has lighter density than normal VLDL (d <1.006 g/ml). In serum from subjects with normal triglyceride concentrations, extremely low concentrations of the triglyceride rich fraction were recovered in the aqueous fraction (d <1.000 g/ml) after ultracentrifugation at 17,000 x g for 15 min.

**Agarose electrophoresis of lipoproteins in the aqueous fraction.** To estimate the composition of lipids in lipoproteins recovered in the aqueous fraction (d <1.000 g/ml), we used agarose gel electrophoresis with triglyceride- and cholesterol-staining. After preparation of the lipoproteins recovered in the aqueous fraction (d <1.000 g/ml) by ultracentrifugation at 17,000 x g for 15 min, we measured the triglyceride and cholesterol levels in the aqueous fraction (d <1.000 g/ml). They were then analyzed by densitometry and the lipid ratio determined from triglyceride- and cholesterol-stained gels.

We investigated 40 subjects with hypertriglyceridemia. These subjects were separated into 8 groups (group A to H) according to electrophoresis densitometer pattern and lipid composition. Eight subjects with typical electrophoresis patterns from the 8 respective groups are shown in Table 1 and Figs. 4 and 5. The ratio of triglyceride to cholesterol in the aqueous fraction (d <1.000 g/ml) varied from 3 to 15, which was independent of their ratio in serum (Table 1).

The 8 subjects were classified into 2 groups (panel A-D and E-H) as shown in Fig. 4. The first group (A-D) did not show chylomicrons in the aqueous fraction (d <1.000 g/ml) and the second group (E-H) showed chylomicrons, as detected at the origin of the agarose gel. The subjects in the 2 groups were further classified into 4 groups by the electrophoretic mobility and the lipid ratio in the densitogram, based on triglyceride- and cholesterol-staining respectively (Fig. 4). The lipoproteins in the aqueous fraction (d <1.000 g/ml) of 4 subjects (panel A-D), which did not show chylomicrons, predominantly migrated at the middle between pre-β and β (panel A), pre-β (panel B), broad region from pre-β to β (panel C), and β (panel D), respectively. In the other group (panel E-H) the lipoproteins in the aqueous fraction (d <1.000 g/ml) predominantly migrated at pre-β (panel E), pre-β and β region (panel G), and broad region (panels F and H), respectively. In group H, the ratio of triglyceride to cholesterol was higher than in group F (Table 1).
Fig. 5. Lipid profiles of the aqueous fraction (d < 1.000 g/ml) from serum of 8 patients with hypertriglyceridemia (subjects A-H), showing the triglyceride concentrations (open triangles), cholesterol concentrations (open squares), and the ratios of triglyceride to cholesterol (closed circles) for each fraction. No. 1 on the abscissa refers to the α-fraction and no. 9 refers to the chylomicrons that remain at the application point. No. 2-4 and no. 6-8 correspond to the pre-β and β region respectively.
Lipid profile of lipoproteins recovered in the aqueous fraction. To estimate the profile of triglyceride and cholesterol in the aqueous fraction (d <1.000 g/ml) from 8 subjects, the concentrations and ratio of triglyceride and cholesterol in the 9 densitometric fractions are shown in Fig. 5. Panels A-H in Fig. 5 are similar to those in Fig. 4 that show densitometric patterns by triglyceride and cholesterol-staining. Fractions 1 and 9 correspond to alpha-lipoprotein and chylomicron fraction respectively.

In subject A, the lipid compositions of the lipoproteins in the aqueous fraction (d <1.000 g/ml) consisted of a comparatively homogeneous particle (Fig. 5, panel A). In subject B, the lipoproteins in the aqueous fraction (d <1.000 g/ml) migrated predominantly at pre-β region and contained high levels of triglyceride and very little cholesterol, while the cholesterol rich particles migrated at the β region. The ratio of triglyceride and cholesterol varied in each fraction (Fig. 5, panel B). The profile of lipoproteins in subject C included at least two kinds of particles with various electrical charges and lipid compositions: triglyceride rich particles at pre-β region (fraction 3), triglyceride and cholesterol rich particles at β region (fraction 6 and 7) and at the middle region of pre-β and β (fractions 4 and 5). The main peak of subject D was different from that of subject A, but their lipid compositions were similar.

In subjects where chylomicron particles were detected in the aqueous fraction (d <1.000 g/ml), the triglyceride rich lipoproteins that migrated at pre-β region predominantly showed a symmetrical lipid profile, with an almost constant ratio of triglyceride to cholesterol (fraction 3-5; Fig. 5, panel E), and small amounts of cholesterol rich lipoproteins with low ratio of triglyceride to cholesterol in fractions 6-8. Subject F (Fig. 5, panel F) showed three kinds of ratios of triglyceride to cholesterol, in fractions 2-3, 4-6 and 7-9. In subject G the slow-β fraction (fraction 7-8; Fig. 5, panel G) had a higher ratio of triglyceride to cholesterol than fractions 2-6. Subject H showed predominantly triglyceride rich particles with pre-β, mid β, and β mobility. A high level of triglyceride rich chylomicrons was detected at the origin of the gel (Fig. 5, panel H).

<table>
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Fig. 6. Correlations of triglyceride and cholesterol level in the aqueous fraction (d <1.000 g/ml) isolated by ultracentrifugation from serum samples of 40 patients with hypertriglyceridemia (panel A, samples without chylomicrons; panel B, samples with chylomicrons).

Lipid profiles of the aqueous fraction. The concentrations and the ratios of triglyceride and cholesterol in the aqueous fraction (d <1.000 g/ml) from 40 subjects with hypertriglyceridemia are shown in Table 2. In groups B and C, the TG/TC ratio was decreased, compared to group A. The TG/TC ratio of the high TG level groups (group G and H) and their content of chylomicrons were significantly higher than group A (group G, p <0.05; group H, p <0.001). Furthermore, the TG/TC ratios of groups E, F, and G were decreased in comparison to group H (Table 2).

Correlation of triglyceride and cholesterol in the aqueous fraction. The correlation of triglyceride and cholesterol concentrations in the aqueous fraction (d <1.000 g/ml) from all subjects shown in Fig. 6.
In the lipoproteins of group A-D and group E-H the levels of triglyceride were significantly correlated with cholesterol ($r^2 = 0.741$, $n = 20$) (Fig. 6, panel A), but only slightly correlated ($r^2 = 0.424$, $n = 20$) in the groups with chylomicrons (Fig. 6, panel B). In groups E, F, and G, the level of triglyceride was significantly correlated with cholesterol ($r^2 = 0.729$, $n = 16$) (Fig. 6, panel B); the coefficient of correlation in group H was $r^2 = 0.936$ ($n = 4$) (Fig. 6, panel B, closed squares). The ratio of triglyceride to cholesterol in the aqueous fraction ($d < 1.000 \text{ g/ml}$) of group H was the highest of all groups (Fig. 6, panel H, closed squares).

**Discussion**

In this study, identification and characterization of triglyceride rich fractions with lighter density lipoproteins (aqueous fraction; $d < 1.000 \text{ g/ml}$) than VLDL ($d < 1.006 \text{ g/ml}$), IDL, and LDL are achieved by a combination of ultracentrifugation and agarose gel electrophoresis with triglyceride- and cholesterol-staining. The lipoproteins recovered in the aqueous fraction isolated by the ultracentrifugation at 17,000 x g for 15 min were separated into 8 groups by the densitometric patterns of triglyceride- and cholesterol-staining.

This characterization highlights the heterogeneity of triglyceride rich lipoproteins, including the very light VLDL particles. It is difficult to characterize the triglyceride rich lipoproteins from serum by a single method using ultracentrifugation, HPLC, or electrophoresis. Other reported methods to separate chylomicrons and their remnants from serum lipoproteins include immunoseparation and ultracentrifugation. The former method uses affinity gel conjugated anti-apoA-I and anti-apoB48; remnant-like particles containing apoB100, apoB48, apoE and apoC-II are recovered in an unbound fraction from the affinity gel [19,20]. The latter is a simple method based on separating by differences in the density of lipoproteins. Measurement of the ratio of RLP-cholesterol to serum total triglyceride has been reported using ultracentrifugation and electrophoresis [21]. The investigators analysed the remnant lipoproteins containing VLDL separated by ultracentrifugation in $d < 1.006$ at 424,000 x g for 4 hr, and cholesterol patterns in lipoproteins by electrophoresis. In our experiments, chylomicrons, their remnants, and lighter density lipoproteins (VLDL, IDL, and LDL) were completely separated in a short time from other lipoproteins by the ultracentrifugation at 17,000 x g for 15 min, and included apo-B100 and apo-B48 similar to the immunoseparation method. Furthermore, chylomicrons were separated from other lipoproteins by agarose gel electrophoresis, because the chylomicrons remained at the origin.

Chylomicrons and their remnants are rarely detected in normal plasma, since they are catabolised and cleared rapidly in the circulation [22]. The accumulations of chylomicrons and their remnants in serum were caused by some impediments of catabolism [23-25], lipolysis with lipoprotein lipase.
(LPL) in the circulation, and/or exchange of lipids and apolipoproteins with other lipoproteins such as HDL. Subsequently, disorder of receptor-mediated uptake, mainly in the liver, results in formation of remnant lipoproteins and heterogeneous particles with various lipids compositions, particles sizes, and electrical charges. Our new method showed that the lipoproteins recovered in the aqueous fraction contain chylomicrons, their remnants, and light-VLDL (d < 1.000 g/ml), as characterized by particle size, electrical charge, and lipid composition.

Agarose gel electrophoresis has two characteristics for analysis of lipoproteins in serum. First, subfractionation of lipoproteins is achieved under mild conditions, with a small volume of sample, and within a short time [17]. Second, one can analyse the concentration and composition of lipids in the subfractions, because the electrophoresis patterns of triglyceride and cholesterol [26,27] in lipoproteins are revealed at parallel positions on the agarose gels and are measured simultaneously by densitometry.

It has been reported that triglyceride rich lipoproteins from hyperlipidemic serum were analyzed by HPLC gel filtration chromatography with mild analytical conditions [28]. Lipoproteins were separated by differences of particle size, and were monitored by triglyceride or cholesterol analysis after agarose electrophoresis. After immunoseparation, RLP was analysed by HPLC with cholesterol and triglyceride detection [29]. However, a long time was required for the analysis; it was difficult to analyse many samples and measure the composition of apolipoproteins. The particle size of fractions from agarose electrophoresis has been measured by native PAGE using 2-dimensional electrophoresis. The compositions of apolipoprotein in triglyceride rich lipoprotein particles on agarose gel were analysed by immunofixation or immunoblotting with anti-apolipoprotein antibody [19]. Thus, agarose gel electrophoresis is very useful for the characterisation of remnant lipoproteins.

The chylomicrons secreted in the lymph undergo hydolysis of triglyceride by LPL in the general circulation, and heterogeneous remnants are formed [30]. The particles of chylomicrons and their remnants include apoB-48. The catabolism of VLDL resembles that of chylomicrons, but particles of VLDL contain only apoB-100 [31,32].
The lipoproteins recovered in the aqueous fraction by ultracentrifugation at 17,000 x g for 15 min consist of chylomicrons, their remnants, and VLDL (d <1.000 g/ml) with light density, based on the detection of apoB-48 and apoB-100 in this fraction. The particles of chylomicrons contain very high levels of triglyceride and small amounts of cholesterol, and the ratio of triglyceride to cholesterol ranges from 4 to 100. Although particles of VLDL are triglyceride rich lipoproteins, the level of triglyceride ranges from 2 to 13 times that of cholesterol. It has been suggested that the characterization of chylomicron remnants and light-VLDL (d <1.000 g/ml) be based on the level of triglyceride, the ratio of triglyceride to cholesterol, and VLDL remnants [33]. Measuring the particle size and composition of apolipoproteins in subfractions is likely to improve the identification and characterization of triglyceride rich lipoproteins.

As shown in Fig. 7, we propose that there are several different triglyceride rich lipoprotein subfractions of chylomicrons, chylomicron remnants, and light VLDL. Based on the present study, we conclude that combined use of ultracentrifugation and triglyceride- and cholesterol-staining after agarose gel electrophoresis enables the identification and characterization of the different triglyceride rich lipoproteins. What remains unknown is whether or not the accumulation of any or all of these lipoprotein particles is associated with increased risk of atherosclerosis. We suggest that the present technique offers an improved way to discriminate between the different triglyceride rich lipoprotein particles that are associated with hypertriglyceridemia. The next step will be to elucidate the potential atherogenicity of each of these triglyceride rich lipoprotein particles.

References


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