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Postprandial lipoprotein metabolism: VLDL vs chylomicrons

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Since Zilversmit first proposed postprandial lipemia as the most common risk of cardiovascular disease, chylomicrons (CM) and CM remnants have been thought to be the major lipoproteins which are increased in the postprandial hyperlipidemia. However, it has been shown over the last two decades that the major increase in the postprandial lipoproteins after food intake occurs in the very low density lipoprotein (VLDL) remnants (apoB-100 particles), not CM or CM remnants (apoB-48 particles). This finding was obtained using the following three analytical methods; isolation of remnant-like lipoprotein particles (RLP) with specific antibodies, separation and detection of lipoprotein subclasses by gel permeation HPLC and determination of apoB-48 in fractionated lipoproteins by a specific ELISA. The amount of the apoB-48 particles in the postprandial RLP is significantly less than the apoB-100 particles, and the particle sizes of apoB-48 and apoB-100 in RLP are very similar when analyzed by HPLC. Moreover, CM or CM remnants having a large amount of TG were not found in the postprandial RLP. Therefore, the major portion of the TG which is increased in the postprandial state is composed of VLDL remnants, which have been recognized as a significant risk for cardiovascular disease.

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1. Introduction

Plasma triglycerides (TG) are known to be a surrogate for TG-rich lipoproteins (TRL) and are present in chylomicrons (CM), very low density lipoproteins (VLDL) and their remnants. TRL and their remnants are significantly increased in the postprandial plasma and are known to predict the risk of coronary heart disease (CHD) [1,2], independent of the total cholesterol, LDL or HDL cholesterol level. Recently, non-fasting TG levels have come to be known as a significant risk indicator for CHD events [3–5]. Zilversmit [6] first proposed that the postprandial CM is the most common risk factor for atherogenesis in persons who do not have familial hyperlipoproteinemia. The hypothesis of postprandial CM and CM remnants came to be widely accepted as a major cause of common atherogenesis, because it was well established that CM are significantly increased in the intestine after food intake and a large amount of CM flows into the blood stream through the thoracic duct. Therefore, CM and CM remnants have been thought to be the major lipoproteins in the postprandial hyperlipidemia until recently.

Furthermore, Type I and V chylomicronemia, which are associated with severe lipemia, are often confused with common alimentary lipemia. However, severe lipemia is most commonly associated with a deficiency of lipoprotein lipase (LPL) or apoC II, and significantly elevated CM and CM remnants are detected in the fasting plasma of these cases [7]. Type III hyperlipidemia is a kind of postprandial genetic defect which results in significantly elevated apoB48 in beta-VLDL and is associated with the low affinity of apoE2/2 for the receptors which clear remnant lipoproteins [8–11]. Alimentary lipemia is often reflected by the increased turbidity of fat emulsions, not CM, in plasma in terms of lipid concentrations. Therefore, the severity of lipemia in the postprandial plasma often weakly correlates with the plasma triglyceride concentration.

Nevertheless, many of the current textbooks on alimentary lipemia or postprandial hyperlipidemia still indicate CM and/or CM remnants as the major lipoproteins which are increased in plasma after food intake. In addition, approximately 80% of the postprandial increase of triglycerides has been considered to be accounted for by the apoB48 containing lipoproteins until recently [12]. This is based on the fact that the CM and/or CM remnants are the major triglyceride-carrier in the postprandial state, with each particle carrying a very large number of triglyceride molecules. Therefore, large quantities of triglycerides are thought to be transported by a very small number of CM or CM remnant particles. As the CM particle size has not been determined, it has remained unclear whether the major TRL in the postprandial plasma was CM-derived or not.

To investigate the characteristics of postprandial lipoproteins, we developed a new immunoseparation method which enables the direct isolation of remnant lipoproteins as remnant-like lipoprotein particles (RLP) from the postprandial plasma and examination of the concentration and particle size of CM and VLDL remnants [13,14]. This method has the capacity to isolate both CM and VLDL remnants from the plasma simultaneously as RLP, which fulfill most of the biochemical characteristics of CM and VLDL remnant lipoproteins.

In this manuscript, we have demonstrated that the major remnant lipoproteins associated with postprandial hyperlipidemia are in fact not CM remnants, but VLDL. To confirm this finding, three new analytical methods developed in Japan during the last two decades were employed: the isolation of remnant-like lipoprotein particles (RLP) using specific antibodies [13,14], separation and detection of lipoprotein subclasses by a gel permeation high-performance liquid chromatographic (HPLC) system [15] and determination of apoB48 in fractionated lipoproteins by a highly specific ELISA [16].

2. Metabolism of CM, VLDL and their remnants in plasma

Fig. 1 shows the metabolic pathway of CM and VLDL. CM is secreted by the intestine after fat consumption. CM particles contain apoB-48 as a structural protein, which in humans is formed exclusively in the intestine after tissue-specific editing of the apoB-100 mRNA [17,18]. It appears that apoB-48 containing particles are continuously secreted from the enterocyte, and at times of excessive triglyceride availability, lipid droplets fuse with nascent lipoprotein particles, resulting in the secretion of enormous chylomicrons [19,20]. Once the CM particle reaches the plasma compartment, apoA-I dissociates very rapidly [21] and acquires apoCs, in particular apoC-II, to enable efficient unloading of its massive triglyceride content after binding to the lipoprotein lipase (LPL) which is bound to the endothelium [22]. High density lipoproteins (HDL) are a major reservoir for the apoCs and apoE, but in conditions with low HDL concentrations (found most often in hypertriglyceridemic subjects), CM may receive apoCs and apoE from resident VLDL particles. The half-life of CM triglycerides in healthy subjects is very short, approximately 5 min [23]. The half-life of CM particles has been very difficult to estimate due to the difficulty of obtaining adequate labeling of CM. The CM particle half-life is certainly longer than for CM triglycerides and seems to be quite heterogeneous. Certain pools of CM remnants have a very long residence time, at least as long as similarly sized VLDL particles [24,25].

Furthermore, a major proportion of the CM remnants leave the plasma compartment quite rapidly while still quite large, i.e., 75 nm in diameter [24]. There is competition for lipolysis: CM and VLDL mix in the blood and the two TRL species compete for the same lipolytic pathway [25,26]. It has been shown that endogenous TRL accumulate in human plasma after fat intake and the mechanism behind this phenomenon is explained by the delayed lipolysis of the apoB-100 TRL particles due to competition with CM for the sites of LPL action [26]. Similarly, endogenous TRL accumulate in rat plasma due to competition with a CM-like triglyceride emulsion for the common lipolytic pathway [27]. The increase in the number of TRL apoB-100 particles is actually far greater than that of the apoB-48 containing lipoproteins in the postprandial state [28]. Of note, the accumulation of large TRL apoB-100 particles seems to be a particular sign in hypertriglyceridemic patients with CAD compared with healthy hypertriglyceridemic subjects, suggesting a link between the accumulation of large VLDL and the development of atherosclerosis [29].

VLDL particles are secreted continuously from the liver (Fig. 1). In contrast to CM and their remnants, they are characterized by their apoB-100 content. The secretion of VLDL is under complex regulation, as the larger and more triglyceride-rich TRL species are under strict insulin control in a dual sense. First, a number of more or less insulin-sensitive mechanisms regulate the availability of triglycerides for VLDL production. The free fatty acids (FFA) which are generated by sensitive mechanisms regulate the availability of triglycerides for VLDL production. The free fatty acids (FFA) which are generated by competitive mechanisms for the common lipolytic pathway [25,26]. It has been shown that endogenous TRL accumulate in human plasma after fat intake and the mechanism behind this phenomenon is explained by the delayed lipolysis of the apoB-100 TRL particles due to competition with CM for the sites of LPL action [26]. Similarly, endogenous TRL accumulate in rat plasma due to competition with a CM-like triglyceride emulsion for the common lipolytic pathway [27]. The increase in the number of TRL apoB-100 particles is actually far greater than that of the apoB-48 containing lipoproteins in the postprandial state [28]. Of note, the accumulation of large TRL apoB-100 particles seems to be a particular sign in hypertriglyceridemic patients with CAD compared with healthy hypertriglyceridemic subjects, suggesting a link between the accumulation of large VLDL and the development of atherosclerosis [29].

In contrast, the metabolic pathway of VLDL by hepatic triglyceride lipase (HTGL) seems to be still controversial because of the difficulties of measurements. HTGL has been reported to metabolize comparatively small remnant lipoproteins, although to a lesser extent than LPL. However, our recent studies have shown no correlation between HTGL activity and plasma TG, RLP-C, RLP-TG or the small dense LDL-C concentration in humans [33], although we did find a strong inverse correlation between LDL activity and these parameters in both the fasting and postprandial state (Table 1). Previous studies proposed the concept of HTGL role to the remnant metabolism seemed to be mainly based on the animal studies using anti-HTGL antibodies in monkeys and rats and found the accumulation of remnant lipoproteins..
in plasma after the HTGL specific antibody treatment [34,35]. As it is well known that small dense LDL (sdLDL) is positively correlated with TG and remnant lipoproteins in plasma, these data support the concept that remnant lipoproteins are the precursor of sdLDL and are metabolized in the same pathway by LPL [33]. From these data, HTGL does not seem to play a significant role in the metabolic pathway of remnant lipoproteins, in contrast to previous reports [34–38], but instead, plays a definitive role in HDL metabolism in humans.

3. Biochemical characteristics of CM and VLDL remnants

TRL remnants are formed in the circulation when apoB-48 containing CM of intestinal origin or apoB-100 containing VLDL of hepatic origin are converted by lipoprotein lipase (and to a lesser extent by hepatic lipase according to the description commonly given in the literature) into smaller and denser particles [36–38]. Compared with their nascent precursors, TRL remnants are depleted of triglyceride, phospholipid,
apoCs (and apoA-I and apoA-IV in the case of CM) and are enriched in cholesteryl esters and apoE [39,40]. They can thus be identified, separated, or quantified in plasma on the basis of their density, charge, size, specific lipid components, apolipoprotein composition and apolipoprotein immunospecificity [41]. Each of these approaches has provided useful information about the structure and function of remnant lipoproteins, and has helped to establish the role of TRL remnants in the pathogenesis of atherosclerosis. Accurate measurement and characterization of plasma remnant lipoproteins, however, have proven to be difficult for the following reasons: [1] despite their reduced size and triglyceride content, they are difficult to differentiate from their triglyceride-rich precursors; [2] due to their rapid plasma catabolism, they exist in plasma at relatively low concentrations; and [3] since remnants are at different stages of catabolism, they are markedly heterogeneous in size and composition. TRL is known to become progressively smaller, denser and less negatively charged as they are converted into TRL remnants. They gradually lose triglycerides and, in relative terms, become enriched with cholesteryl esters. They also reduce their complement of C apolipoproteins (apoC-I, apoC-II, and apoC-III), which are replaced by apoE. At any given time, there is a continuous spectrum of different-sized remnants in the blood. Some of these particles are of intestinal origin. They contain apoB-48 and are present in greater concentrations after a fat-rich meal. The majority, however in both the fed and fasted states, contains apoB-100 and is derived from the liver. Depending on the extent to which they have been lipolyzed, the different species of TRL contain different proportions of triglyceride and cholesterol and may or may not contain apoCs or apoE. Remnant lipoproteins are thus structurally and compositionally diverse, which has made it necessary to develop a variety of specific biochemical techniques for the detection, quantification and characterization of these lipoproteins. In the light of such difficulties, we endeavored to find a new approach to isolate remnant lipoproteins—an approach which could separate a variety of remnant lipoproteins from the normal apoB100 (nascent VLDL and LDL) carrying lipoproteins. A specific anti-apoB-100 antibody which does not recognize α-helix structure of apoB-51 region was developed and used for the isolation of the apoE-rich VLDL remnants (Fig. 2) [14].

4. Daily rhythm of plasma cholesterol, TG and remnant lipoproteins and the changes in the lipoprotein levels after a fat load

The plasma triglyceride concentration fluctuates throughout the day in response to the ingestion of meals. Even if measured after a 10- to 12-hour overnight fast (as is normal clinical practice), triglyceride levels vary considerably more than LDL and HDL cholesterol levels.

As non-fasting TG levels are now known to be a significant risk factor for CHD events [3–5], the analysis of postprandial lipoproteins, rather than the fasting state, has come to be recognized as more important. We reported that non-fasting TG correlated more strongly with remnant lipoproteins than fasting TG [42]. The correlations between postprandial TG and remnant lipoprotein concentrations were significantly more robust when compared with fasting TG or remnant lipoprotein concentrations. In particular, the increase of postprandial RLP-TG from fasting RLP-TG contributed to approximately 80% of the increase of postprandial total TG from total fasting TG (Table 2). The greater predictive value of non-fasting TG levels associated with cardiovascular events is directly correlated with the increased levels of remnant lipoproteins in the postprandial state.

As shown by Stanhope et al. [43] in Fig. 3 in their fructose treatment study, plasma TG levels are significantly increased during the day associated with food intake. It was only in the early morning that TG levels in all cases returned to the baseline levels. When fructose was administered, a significant increase of TG after food intake compared with the regular meal was observed. Plasma cholesterol levels did not significantly change during the day. Fig. 3 shows that blood samples were collected every hour during the 24 h day. Between blood samplings, each subject consumed a standardized meal (9 am, 1 pm and 6 pm) containing 55% of the energy as carbohydrate, 30% as fat and 15% as protein. The energy content of the meals was based on each subject’s energy requirement as determined by the Mifflin equation [44].

Fig. 3 shows that the TG levels in generally healthy volunteer plasma were the highest at 2 AM in the very early morning, indicating that postprandial conditions continue even past midnight during in the course of a day, except in the early morning. Remnant lipoprotein levels increased significantly for most of the day except the early morning, a similar profile TG. These increases may depend on the kind of foods. The typical carbohydrate-rich Japanese meal did not increase the levels of TG and remnants during a given day compared with a fat-rich meal such as in the typical Western diet [43,45], as shown by Ai et al. [46] and Sekihara et al. [47].

Other postprandial studies have been conducted by oral fat load test. One typical study performed in our laboratory was carried out in 6 male and 6 female (postmenopausal) Japanese volunteers aged 39–60 (mean 52 years) who were generally healthy with no apparent disease [48]. Among these 12 volunteers, there were three cases of mild hyperlipidemia (Type IIb). All participants performed an oral fat tolerance test (OFTT) as previously reported [49]. Briefly, after a 12 h fast, the subjects ingested 17 g fat/m² body surface area (OFTT cream, Jomo foods, Takasaki. Gunma). The test meal (OFTT cream) had a water content of 56.8%, while lipids accounted for 32.9%, protein for 2.5%, carbohydrates for 7.4% and minerals for 0.3%. The fat was 64.3% saturated, 29.3% monounsaturated and 3.5% polyunsaturated. Blood samples were drawn before and 2, 4 and 6 h after an oral fat load. Plasma apoB-48 significantly increased and correlated with the TG levels in postprandial plasma, however, apoB (more than 98% apoB-100) did not (Fig. 4). These results suggest that CM or CM remnants carrying a large amount of TG are the major component of the increase in the postprandial remnant lipoproteins. However, the apoB concentration was far greater than apoB-48 in the plasma, as shown in Fig. 4. The apoB-100 in LDL decreased during a fat load, which resulted in there being no change in apoB despite the increase of apoB100 in the postprandial RLP.

The origin of the cholesterol increase in the postprandial TRL remnants has been investigated by several researchers. Both a single fatty meal and long-term diet have been reported to exhibit a cholesterol accumulation in the TRL fraction less than expected, arguing for a rather limited role of dietary cholesterol in determining the TRL remnant cholesterol level [50,51]. In fact, the calculated proportion of dietary cholesterol present in the CM fraction was very low, i.e. only one out of 99 cholesterol molecules originated directly from the meal [52]. Obviously, the dietary cholesterol is diluted by the cholesterol undergoing enterohepatic recirculation, but the delayed and incomplete absorption of cholesterol (compared to triglycerides) also argues that cholesterol and triglycerides are not incorporated at similar rates into CM particles. It is likely that the majority of the cholesterol is absorbed at a later stage, further down in the gastrointestinal tract, where the abundance of triglycerides is reduced. Therefore, in the postprandial state the accumulation of cholesterol in CM remnant particles is limited in comparison with the cholesterol-enrichment of VLDL remnants. The major source of the cholesterol in the TRL remnants comes from HDL as a result of CETP activity, which will be described later in this manuscript.

5. Isolation of remnant-like lipoprotein particles (RLP) using specific antibodies and the diagnostic characteristics of the RLP-cholesterol (RLP-C) assay

An assay system based on the recognition of TRL remnants according to their apolipoprotein content and immune-specificity has been developed that provides a quantitative and clinically applicable approach to the measurement of plasma remnant lipoproteins [13,14]. This is the first method to separate remnant lipoproteins from TRL using specific antibodies so as to isolate the remnant fraction under moderate...
conditions. In this assay, RLP is separated from plasma in unbound fraction by immunoaffinity chromatography with a gel containing an anti-apoA-I antibody and a specific anti-apoB-100 monoclonal antibody (J-H) (JIMRO II, Otsuka, Tokyo). The former antibody recognizes all HDL and any newly synthesized CM containing apoA-I, whereas the latter antibody recognizes all apoB-100 containing lipoproteins, except for certain particles enriched in apoE. Anti-apoB-100 antibody JI-H recognizes the B51 region of apoB100 and CM has no epitope in this region. Therefore, CM lacking apoA-I, which is defined as the CM remnant [21], is not recognized by the gel and all of the apoB-48 particles in the plasma are isolated in the unbound RLP fraction. The reason the anti-apoB-100 antibody does not recognize the apoE-enriched RLP is not entirely clear, although the amino acid sequence of the epitope region of the apoB-100 antibody is homologous to an amphipathic helical region of apoE, which suggests that apoE should be able to compete for binding of the antibody to its epitope, located between 2270 and 2320 amino acids from N-terminal on apoB-100 [14] (Fig. 2). The amphipathic helical peptide (2293–2301) of the chemically synthesized antibody epitope reacted with the anti apoB-100 antibody (J-H) and exhibited potent reverse cholesterol transport activity as apoA-I, apoE or HDL. Of further note, this peptide showed an inhibitory effect on CETP activity as well (unpublished data).

HDL, LDL, large CM and the majority of VLDL are thus retained by the gel. The unbound RLP are made up of remnant VLDL containing apoB-48 particles in the plasma isolated in the unbound RLP fraction. The reason the anti-apoB-100 antibody does not recognize the apoE-enriched RLP is not entirely clear, although the amino acid sequence of the epitope region of the apoB-100 antibody is homologous to an amphipathic helical region of apoE, which suggests that apoE should be able to compete for binding of the antibody to its epitope, located between 2270 and 2320 amino acids from N-terminal on apoB-100 [14] (Fig. 2). The amphipathic helical peptide (2293–2301) of the chemically synthesized antibody epitope reacted with the anti apoB-100 antibody (J-H) and exhibited potent reverse cholesterol transport activity as apoA-I, apoE or HDL. Of further note, this peptide showed an inhibitory effect on CETP activity as well (unpublished data).

Table 2
Lipids, lipoproteins, RLP-TG/RLP-C ratio and RLP-TG/total TG ratio after oral fat in 18 male volunteers.

<table>
<thead>
<tr>
<th></th>
<th>0 h</th>
<th>2 h</th>
<th>4 h</th>
<th>6 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>TC (mg/dL)</td>
<td>200</td>
<td>217</td>
<td>219</td>
<td>210</td>
</tr>
<tr>
<td>TG (mg/dL)</td>
<td>85</td>
<td>104</td>
<td>169</td>
<td>206</td>
</tr>
<tr>
<td>HDL-C (mg/dL)</td>
<td>62</td>
<td>74</td>
<td>73</td>
<td>71</td>
</tr>
<tr>
<td>RLP-C (mg/dL)</td>
<td>4.8</td>
<td>6.1</td>
<td>7.7</td>
<td>9.4</td>
</tr>
<tr>
<td>RLP-TG (mg/dL)</td>
<td>12</td>
<td>16</td>
<td>77</td>
<td>101</td>
</tr>
<tr>
<td>RLP-TG/RLP-C</td>
<td>2.5</td>
<td>2.7</td>
<td>10.1</td>
<td>13</td>
</tr>
<tr>
<td>RLP-TG/TG</td>
<td>0.13</td>
<td>0.15</td>
<td>0.47</td>
<td>0.56</td>
</tr>
<tr>
<td>ΔTG (mg/dL)</td>
<td>–</td>
<td>–</td>
<td>0.47</td>
<td>0.56</td>
</tr>
<tr>
<td>ΔRLP-TG (mg/dL)</td>
<td>–</td>
<td>–</td>
<td>0.47</td>
<td>0.56</td>
</tr>
<tr>
<td>ΔRLP-TG/ΔTG (ratio)</td>
<td>–</td>
<td>–</td>
<td>0.47</td>
<td>0.56</td>
</tr>
</tbody>
</table>

ΔTG; postprandial minus fasting (0 h) TG, ΔRLP-TG; postprandial minus fasting (0 h) RLP-TG.

apoB-100 and CM containing apoB-48, which are routinely measured in terms of cholesterol, although they can also be quantified in terms of triglycerides or specific apolipoproteins (i.e., apoB, apoC-III, or apoE) [53,54]. The plasma concentration of RLP-C has been shown to be significantly correlated with the plasma concentration of total TG, VLDL-TG and VLDL-C. It has not been significantly correlated with LDL cholesterol or LDL apoB [55,56].

The physical and chemical properties of lipoproteins which are not recognized by the apoB-100 monoclonal antibody JI-H, subsequently isolated by ultracentrifugation at a density 1.006 g/mL, have been described [57]. These lipoproteins contained more molecules of apoE and cholesteryl esters than those that were bound, consistent with them being remnant-like lipoproteins. They had slow pre-b electrophoretic mobility compared with the bound VLDL fraction and ranged in size from 25 to 80 nm. Other lipoproteins, however, may be present when the JI-H monoclonal antibody (together with an anti-apoA-I antibody) is used to isolate RLP by immunoaffinity chromatography from total plasma in the absence of ultracentrifugation [13,14]. HPLC analysis of RLP fractions isolated in this way from normolipidemic and diabetic subjects [14], and fast protein liquid chromatographic analysis of RLP from type III and type IV patients [54], have revealed considerable size heterogeneity in RLP, with particles ranging in size from VLDL to LDL. The relative amount of lipid and apolipoprotein in RLP can also vary considerably from one individual to another. Hypertriglyceridemic patients have more triglyceride and apoC-III, and less apoE, relative to the apoB in RLP, than do normolipidemic subjects [53,54]. Hypertriglyceridemic patients invariably have elevated levels of RLP-C, and the clinical usefulness of this assay depends on the studies which show that RLP-C concentration predicts the presence of coronary or carotid atherosclerosis independently of the plasma triglyceride level [58,59].

The median concentration of RLP-C is 5.9 mg/dL in 35- to 54-year-old American men and 4.6 mg/dL in similarly-aged women [56]. RLP-C is higher in older vs younger subjects [13,55], men vs women [55,56], postmenopausal vs premenopausal women [55], the fed vs the fasted state [49,60], individuals with diabetes [61], patients with familial dysbetalipoproteinemia [13,54,62,63], hemodialysis patients [47,64] and patients with coronary artery restenosis after angioplasty [65]. It has been demonstrated that the RLP-C concentration is significantly higher in patients with CAD than in control subjects [13,55,56,66–68]. The potential atherogenicity of RLP-C is supported by the observation that RLP can promote lipid accumulation in mouse peritoneal macrophages [69], stimulate whole-blood platelet aggregation [70,71] and impair endothelium-dependent vasorelaxation [72]. The physiological and pathophysiological aspects of RLP have been investigated...
extensively by many researchers using isolated RLP fraction in various diseases [73], but relatively little is known about the biochemical composition of RLP or the extent to which this composition varies from one individual to another.

6. Fractionation and analysis of RLP with a gel permeation HPLC system

Gel permeation HPLC is a method for quantifying lipoproteins by particle size [15,74]. Online post column detection of lipid components in lipoproteins particles after separation by size provides quantitative lipoprotein size distribution from whole serum or the RLP fraction. The following combined techniques were used: 1) TSKgel LipopropakXL columns which can separate a wide range of lipoprotein particle sizes from CM to HDL [75], 2) an on-line enzymatic reaction for TC and TG in the separated effluents from the column [76] and 3) 20 component peak analysis using a Gaussian curve fitting technique [77]. These techniques can provide CM-cholesterol (CM-C), VLDL-cholesterol (VLDL-C), LDL-C, and total cholesterol (TC) and their triglycerides (CM-TG, VLDL-TG, LDL-TG, HDL-TG and total TG) together with their VLDL, LDL and HDL subclasses. This HPLC method has satisfactory performance on sensitivity, reproducibility and accuracy compared to the reference methods [75]. The Liposearch HPLC analytical service for the TC and TG concentration in the major subclasses of lipoproteins was conducted at Skylight Biotech, Inc (Akita, Japan: http://www.skylight-biotech.com/eng/service.html).

This HPLC system is appropriate for the profiling of TG rich lipoproteins and postprandial samples because of its capacity to separate CM and VLDL. The high sensitivity of this method enables an examination of the particle size distribution in the RLP fractions separated by immunoaffinity gels, which are very low in concentration compared with LDL and HDL in plasma. Moreover, the dual detection system of TC and TG provides useful information for determining the heterogeneity of the RLP particles separated by immunoaffinity gels. Using 100 µL of supernatant in immunoaffinity gel suspension solution after 2 h of incubation (5 µL serum or plasma with 300 µL immunoaffinity gel solution), the RLP-C and RLP-TG profiles can be directly observed with an on-line dual detection method using gel permeation HPLC [76], Fig. 5 shows a typical profile of the postprandial RLP monitored by TC and TG reagents in normolipidemic and hyperlipidemic cases after a fat load [48]. The particle sizes are shown in a range of VLDL to LDL before (0 h) and after (2, 4, 6 h) an oral fat load. A small peak at the void retention time was detected at 2 h and 4 h, in each case as large TG-rich lipoproteins. The clearance of the VLDL fraction was significantly delayed in a hyperlipidemic subject compared to a normolipidemic subject.

Another approach is to isolate a large amount of RLP from the plasma for further HPLC fractionation. For example, 0.2 mL aliquots of plasma were applied to columns containing 2 mL of immunoaffinity mixed gel. The plasma samples were incubated with the immunoaffinity mixed gel at room temperature for 30 min. Lipoproteins unbound from the gel (containing primarily CM and VLDL remnants) were eluted with 3.5 mL of 10 mM phosphate buffered saline (pH 7.2). The unbound fraction was concentrated with an Amicon Ultra filter (Millipore, USA) for HPLC fractionation and analysis. The concentrated RLP fraction was fractionated by HPLC and 0.35 mL of aliquot in each tube was collected for the determination of TC, TG, apoB-48 and apoB-100 [48].

7. Determination of serum apoB-48 with ELISA

The characteristics and development of the apoB-48 ELISA assay using monoclonal antibodies have been reported by the two groups in Japan [16,78,79]. The ELISA for apoB-48 for this study was obtained from Shibayagi (Shibukawa, Gunma) [16]. The assay uses a monoclonal antibody raised against a C terminal decapetide of the apoB-48 protein and was calibrated using recombinant apoB-48 antigen [80]. The monoclonal antibody has no cross-reactivity with apoB-100, as verified by ELISA and Western blotting, with more than 90% recovery of apoB-48, and the assay has within- and between run coefficients of variation of 4.8% and 5.4%, respectively. The assay was tested in healthy fasting Japanese volunteers with mean reported values of 0.460±0.15 mg/dL (range 0.27–0.81 mg/dL). In healthy volunteers tested at 0, 2, 4 and 6 h after a 40 g fat load, serum apoB-48 and TG increased approximately 2-fold, with a similar peak time of 3 to 4 h [16,79]. The apoB48 ELISA determined the apoB48 protein in the large TG-rich lipoprotein particles in severe lipemic plasma by pre-treatment with detergent (0.1% Triton X–100). The assay had a sensitivity of 0.25 ng/mL and a linear dynamic range at 2.5 to 40 ng/mL. Interference with the assay values was noted in the hemoglobin values of at least 106 mg/dL and bilirubin values of at least 10 mg/dL. To further validate this assay, whole plasma and the TRL fraction (after subjecting plasma to ultra centrifugation at its own density of 1.006 g/mL for 18 h) was studied [81]. TRL samples were subjected to gel electrophoresis for the separation of apoB-100 and apoB-48, followed by gel scanning, and showed that the mean TRL apoB-48 value was 1.01 mg/dL (SD, 0.43 mg/dL) using ELISA and 0.50 mg/dL (SD, 0.20 mg/dL) using gel scanning. The correlation coefficient or r value between the 2 methods was 0.82 [81], highly correlated. Therefore, although the 2 methods correlate well, and the gel method yielded TRL apoB-48 concentrations that were approximately 50% lower than the ELISA values, indicating that a correction factor of 2.02 would need to be applied to prior kinetic studies [12] in which the TRL apoB-48 levels had been assessed by gel scanning. Furthermore, Nakano et al. [82] determined the concentration of apoB-48 and apoB-100 carrying lipoprotein particles extracted from human aortic atherosclerotic plaques in sudden death cases. The plasma apoB-48 level was shown to be significantly elevated in type III cases, as previously reported [54,79]. Therefore, this method was applicable to the determination of the apoB48 concentration in the fractionated RLP using an HPLC system.

8. Postprandial remnant metabolism in CETP deficiency

Plasma cholesteryl ester transfer protein (CETP) mediates the CE/TG exchange from HDL to TG-rich lipoproteins which forms remnant lipoproteins (Fig. 1). Fielding et al. [83] reported that CETP activity was significantly increased in the postprandial state almost in parallel with the increase of plasma TG. The RLP-C and RLP-TG levels were increased along with elevated CETP activity in patients with nephrotic range proteinuria [84]. Therefore, CETP activity and remnant formation are evidently closely associated.

CETP deficiency results in a low LDL/high HDL phenotype including apoE-rich large HDL. Large HDL may provide cholesteryl ester and apoE to CM/VLDL during lipolysis in the postprandial state, accelerating remnant lipoprotein formation and uptake in the liver. To investigate the role of CETP in postprandial lipoprotein metabolism, the lipid levels of plasma RLP were determined in one homozygous and three heterozygous CETP deficiency cases and controls with an apoE3/3 phenotype by Inazu et al. [85]. After oral fat-load, the area under the curve (AUC) of the TG, RLP-TG and apoB48 levels was remarkably decreased in heterozygous CETP deficiency as compared to the controls (Fig. 6). Similarly, the homozygote had a significantly low AUC for the TG, RLP-TG and apo-B-48 levels.

HPLC analysis in the homozygote showed that the increased RLP-C was not due to the conventional VLDL size RLP, but to those of large HDL size. In heterozygotes, a bimodal distribution of the RLP-C level had been assessed by gel electrophoresis. Furthermore, Nakano et al. [81] determined the concentration of apoB-48 and apoB-100 carrying lipoprotein particles extracted from human aortic atherosclerotic plaques in sudden death cases. The plasma apoB-48 level was shown to be significantly elevated in type III cases, as previously reported [54,79]. Therefore, this method was applicable to the determination of the apoB48 concentration in the fractionated RLP using an HPLC system.
The former mutation is common, but the latter had not been previously reported in the Japanese population. This is the reason the CETP level was less than the detectable limit. Because of this CETP deficiency, HDL is unable to exchange its cholesteryl ester with the TG of other lipoprotein particles, including RLP. In this case, it is not clear whether the CETP deficiency was the cause of the profile of the postprandial RLP-C and RLP-TG. A similar CETP-deficient case with abnormally elevated TG was previously reported by Ritsch et al. and precise genetic analysis of CETP was performed to find the cause of the dissociation between the cholesteryl ester and TG transport in plasma. However, they could not find any specific cause in that case, and she was completely CETP deficient as well as being an apo-E2 carrier. The serum levels of RLP-C and RLP-TG as well as total TG usually increase and decrease in parallel after an oral fat load in normal individuals. Also, both RLP-C and RLP-TG in CETP deficient cases are usually reduced, as reported by Inazu et al. [85]. However, in this subject, the serum RLP-C level was highly elevated in the fasting state and did not increase after a fat load, but rather decreased, while the RLP-TG and total TG levels significantly increased after a fat load, with a delayed peak time compared with normal control subjects. This phenomenon indicated that CETP and HDL played an important role in the formation of RLP-C (but not RLP-TG), as has been reported for postprandial lipid metabolism in homo- and heterozygous CETP deficiency cases [85] and in the vitro formation of RLP with CETP deficiency by Okamoto et al. [87]. However, interestingly, the RLP-TG and total TG levels in this case subject increased significantly at 240 min after a fat load, like those in common hyperlipidemic cases. The trend of the case subject was similar to that of individuals treated with estrogen, whose serum RLP-C level is reduced, but RLP-TG level increases, after the treatment [89,90]. This means that the major metabolic pathways of RLP-C and RLP-TG in the postprandial state are controlled independently, although the RLP particle itself is of the same structure as other lipoproteins i.e. composed of TC, TG, phospholipids and apolipoproteins, and isolated by the same immunoseparation method. In the case subject, found an extremely elevated plasma ANGPTL3 level, which was discovered as an inhibitory modulator of LPL and HTGL in mice [91]. However, it was recently reported that ANGPTL3 associates more strongly with EL or HTGL, which controls HDL-C metabolism, but not with TG or remnants in humans [33,92]. As the case subjectAi et al. reported [86] showed nearly normal LPL and HTGL activities [33]. However, the lack of CETP together with enhanced EL or HTGL inhibition by elevated ANGPTL3 may have affected a significantly increase of the HDL-C level, especially apo-E-rich HDL in this case.

Another interesting dissociation between RLP-C and RLP-TG in the postprandial plasma was observed in one of the heterozygous CETP-deficient cases in a normolipidemic subject. This subject was treated with estrogen, which reduced the RLP-C level but increased the RLP-TG level. However, in this case subject, the serum RLP-C level was highly elevated in the fasting state and did not increase after a fat load, but rather decreased, while the RLP-TG and total TG levels significantly increased after a fat load, with a delayed peak time compared with normal control subjects. This phenomenon indicated that CETP and HDL played an important role in the formation of RLP-C (but not RLP-TG), as has been reported for postprandial lipid metabolism in homo- and heterozygous CETP deficiency cases [85].
deficient subjects (CC-2) after a fat load [86]. The serum RLP-C and RLP-TG levels reportedly increase and decrease in parallel after an oral fat load in most of the study cases [45,93,94]. However, one heterozygous CETP-deficient subject with increased RLP-C and RLP-TG (VLDL size remnants) after a fat load exhibited RLP-TG which started to separately decrease at 240 min and RLP-C in 360 min. This dissociation may be associated with the magnitude of CETP deficiency, in which case CC-2 still enhances the formation of VLDL size RLP-C and RLP-TG, but is apparently insufficient to complete the normal pathway between CETP and LPL [95]. These cases may be associated with some genetic disorder of either CETP or its activity, resulting in the dissociated clearance of RLP-C and RLP-TG.

As LPL metabolizes TRL and CETP enhances the formation of both CM and VLDL remnants, the balance of LPL and CETP activity may determine the major components of the postprandial remnant lipoproteins. Further, CETP deficiency itself may not be atherogenic, whereas together with elevated RLP it may be atherogenic and pose a risk for CHD. These cases may help to clarify the controversy whether CETP deficiency is atherogenic or not.

9. RLP-TG as a marker for the analysis of postprandial remnant lipoproteins

RLP-C has been considered as a risk factor for cardiovascular disease for the last two decades. Most of the studies in the literature have reported the RLP-C concentration in the fasting state [73]. The lack of sensitivity of the TG measurement in the RLP fraction made us delay an intended investigation of RLP-TG. Recently, we have established a satisfactory assay of RLP-TG which enabled us to determine the concentration of RLP-TG reference range in the Japanese population [96].

The simultaneous measurement of TG and cholesterol in RLP resulted in a RLP-TG/RLP-C ratio which reflects the RLP particle size, as shown by the HPLC profile reported by Okazaki et al. [97]. The RLP-TG/RLP-C ratios showed a variety of the RLP particle sizes in various lipid disorders and under different physiological conditions; for example, Type III cases exhibited a significantly lower RLP-TG/RLP-C ratio which indicated the presence of a higher cholesterol content in RLP (mainly IDL), while the RLP-TG/RLP-C ratio was increased significantly in the postprandial state, indicating a significantly increased TG content in RLP (mainly large VLDL), as shown in Fig. 5. Therefore, the RLP-TG/RLP-C ratio predicted the particle size of RLP in a manner comparable with the HPLC profile.

Although RLP-C also increased after an oral fat load, the changes in the RLP-C/total TG ratio in the postprandial state were not significantly different from the ratio in the fasting state. This is because the percentage of RLP-C in the total TG (RLP-C/total TG ratio) was approximately only 5% in the fasting and 4% in the postprandial state. However, a RLP-C/total TG ratio above 10% in the fasting state is now commonly used for the detection of Type III hyperlipidemia [62,63,98]. In contrast, the RLP-TG/total TG ratio was approximately 10–15% in the fasting state, and the ratio increased significantly to more than 40% in the postprandial state. Therefore, we investigated the postprandial changes in the RLP-TG/total TG ratio after an oral fat load [48,96]. The postprandial RLP-TG/total TG ratio increased more than 3 fold in 2 h and RLP-TG made up almost half of the total TG (Table 2). In terms of total TG, we have also studied the TRL-TG ratio with RLP-TG, which is the major fraction of the total TG as VLDL (d < 1.006). The RLP-TG/TRL-TG ratio changed significantly more than the RLP-TG/total TG ratio.

The particle size predicted by the RLP-TG/RLP-C ratio reflects a significant time-dependent increase of TG in RLP particles in the postprandial state. RLP-TG increased 5.3 fold in 4 h, while RLP-C increased 1.5 fold, as shown in Table 2. Therefore, we have found that RLP-TG is a better maker than RLP-C for a direct comparison with total TG in the postprandial state. The RLP-TG/total TG ratio may reflect LPL activity, because LPL activity was shown to be inversely correlated with the concentration of RLP and TG [33]. If a subject has a fasting RLP-TG/total TG ratio above 0.13 (95% percentile in TG less than 150 mg/dL) [96] or an RLP-TG above 20 mg/dL in the fasting state, it may be a subject who is still in the postprandial state and this reflects the delayed remnant lipoprotein metabolism because of disturbed LPL activities. A higher RLP-TG/total TG ratio may be associated with an increased risk for CHD [29,58,99].

10. Characteristics of the RLP isolated from postprandial plasma

As reported by Karpe et al. [29], CM is more susceptible to lipoprotein lipase (LPL) than is VLDL. Therefore, the greater susceptibility to LPL of CM rather than VLDL may more readily result in the formation and accumulation of VLDL remnants than CM remnants in the postprandial state. Therefore, the accumulation of large RLP particles in 4 h after an oral fat load may be due to the delayed metabolism of VLDL by LPL.
Schneeman et al. [100] also reported the postprandial responses (after
fat load) of apoB-48 and apoB-100 were highly correlated with those of
TRL triglycerides. Although the increase in apoB-48 represented a 3.5-
fold difference in concentration as compared with a 1.6-fold increase in
apoB-100, apoB-100 accounted for approximately 80% of the increase in
lipoprotein particles in TRL. The increase in the number of apoB-100
particles in RLP (VLDL remnants) is actually far greater than that of the
apoB-48 particles (CM remnants) in the postprandial state [100,101].

The RLP isolated from plasma in healthy subjects after an oral fat load
using an immunoaffinity mixed gel was significantly increased [48].
Fig. 5 shows the typical profile of RLP particle size in the range of VLDL to
LDL at 0, 2, 4 and 6 h after an oral fat load, when monitored by TC and TG
with HPLC in a normolipidemic and hyperlipidemic subject, respective-
lv. CM or CM remnants (apoB48) could be detected at void volume (a
retention time of 15 min). Fig. 7 shows a typical RLP profile isolated from
the plasma of a Type Iib hyperlipidemic subject 4 h after an oral fat load.
RLP was fractionated by HPLC equipped with gel permeation columns
(TSK Lipopropak XL, TosO, Tokyo) [76] and 0.35 mL of aliquot each was
collected for the determination of TC, TG, apoB-48 and apoB-100 (Fig. 7).
The peak of the TC and TG retention time in RLP fractionated by HPLC
was observed almost at the same particle size as the apoB-100 peak, but
was not the same as the apoB48 peak, as shown in Fig. 7. Furthermore,
the apoB-48 particle size was similar or smaller than that of apoB-100.
These results clearly demonstrate that postprandial RLP do not have
any apoB-48 particles carrying a large amount of TG in the void fraction,
which may thus be categorized as nascent CM [75,76]. The scale
(perpendicular axis) of the apoB-100 (left) and apoB-48 (right)
concentration in Fig. 7 is 4 fold different, but there are similar areas
under the curve. Therefore, the significantly higher concentrations of
apoB-100 than apoB-48 in postprandial RLP of similar particle size were
found in both normolipidemic and hyperlipidemic cases [48]. These
results show that approximately 80% of the RLP in the postprandial
state is composed of large VLDL with apoB100. The increase of small
VLDL such as intermediate density lipoproteins (IDL, SF 12–20) in the
postprandial state has been reported to not be increased in the
postprandial state [12,102], even though IDL has often been defined as
a typical remnant lipoprotein.

11. Postprandial remnant hyperlipoproteinemia in sudden cardiac
death (SCD)

Clinical studies have shown that elevated plasma TG levels greatly
increase the risk of sudden cardiac death. Results from the Paris
Prospective Study [103] and the Apolipoprotein Related Mortality Risk
Study (AMORIS) in Sweden [104] as well as the coronary heart disease
mortality in a 24-year follow-up study in China [105] demonstrated that
increased TG was a strong risk factor for fatal myocardial infarction.
However, plasma TG levels vary often over even a short period of time.
Therefore, it has been difficult to identify the relationship between
clinical events and elevated TG in the long term prospective studies until
recently [3–5].

If the lipid and lipoprotein levels in postmortem plasma correctly
reflect the antemortem levels, these data would provide the same
values with the results obtained from the prospective studies, which
are difficult to obtain in that they require long-term observation to
evaluate. The plasma levels of lipids and lipoproteins in sudden death
cases may reflect the condition of the subject at the moment of fatal
clinical events followed by certain inevitable postmortem alterations,
but nevertheless still usefully reflect the physiological conditions
when the fatal events had occurred. Therefore, we analyzed
postmortem plasma under well-controlled conditions to clarify the
cause or risk of sudden cardiac death. Plasma RLP-C and RLP-TG levels
vary greatly within a short time do the TG levels, unlike other stable
lipoprotein markers such as HDL-C and LDL-C. Hence, the cross-
sectional study of RLPs at the moment of sudden death may be superior to a prospective study of RLP in determining the potential risk for CHD [106]. During the investigations of sudden death cases, we found that the postmortem alterations of lipoproteins in plasma were unexpectedly slight [107] compared with the proteins or other bio-markers. Moreover, these plasma lipoprotein levels were very similar to those determined in living patients, based on the clinical studies in our laboratory.

More than two thirds of the SCD cases observed in our studies, including Pokkuri death syndrome (PDS; sudden cardiac death cases without coronary atherosclerosis), showed stomach full, indicating a strong association with postprandial remnant hyperlipoproteinemia. Significant remnant hyperlipoproteinemia was observed in the plasma of SCD cases compared with the control death cases [68,101,108–112]. These data suggest that the increased RLP in SCD cases may be mainly composed of CM remnants. However, unexpectedly, we found no significant differences in the apoB-48 levels in plasma or in terms of RLP apoB48, but found a significant increase of RLP apoB100 levels in SCD compared to the control cases [101]. The RLP apoB100 levels were significantly increased in the SCD cases in the postprandial state (when RLP-C and RLP-TG were significantly increased), however, neither the plasma apoB48 nor RLP apoB48 level was significantly increased. These results strongly suggested that the major subset of RLP associated with fatal clinical events with the stomach full was the apoB-100 carrying particles, not the apoB-48 particles.

The absolute amount of apoB-100 in RLP is much greater (approximately 7 fold) than that of apoB-48 in RLP. Also the particle size of apoB-48 and apoB-100 was very similar, as shown in Fig. 7 [112]. Furthermore, we often found SCD cases who had consumed alcohol on a full stomach. It is known that alcohol increases fatty acids in the liver and enhances VLDL production while inhibiting LPL activity [113]. Alcohol intake with a fatty meal is well known to increase TG significantly compared with the proteins or other lipoproteins. We found that the postmortem alterations of lipoproteins in plasma; (Postprandial TG concentration of the increased remnant lipoproteins in the postprandial state) exceeded by a factor of two more than fasting state. Furthermore, we often found SCD cases who had consumed alcohol on a full stomach. It is known that alcohol increases fatty acids in the liver and enhances VLDL production while inhibiting LPL activity [113]. Alcohol intake with a fatty meal is well known to increase TG significantly compared with the proteins or other lipoproteins.

12. Conclusions

Three new analytical methods were used to investigate the characteristics of postprandial lipoprotein metabolism. These were a method of isolating the remnant lipoproteins as RLP using specific antibodies, gel permeation HPLC and apoB48 ELISA. The major portion of the TG which increased in the postprandial state was shown to be TRL remnant lipoproteins. Although Zilversmit proposed CM or CM remnants as the major lipoproteins which increased in the postprandial lipemia, we have shown that the major portion of the postprandial lipoproteins which increased after food intake was composed of VLDL remnants. The amount of apoB48 particles was shown to be much lower than apoB100 particles in the postprandial RLP, and the particle sizes of apoB48 in RLP were similar with those of apoB100 or smaller when analyzed by gel permeation HPLC system. We did not find CM or CM remnants having a large amount of TG in postprandial RLP. Therefore, the major part of postprandial TRL remnants is composed of VLDL remnants (approximately 80% or more), not CM remnants. It was also found that TG vs RLP-TG concentrations in the postprandial state correlated significantly higher than in the fasting state. Furthermore, it was demonstrated that approximately 80% of the increased TG in the postprandial state consisted of the TG in remnant lipoproteins. Taken together, we propose the following equation for the estimated concentration of the increased remnant lipoproteins in the postprandial plasma; (Postprandial TG – Fasting TG) × 0.8 = RLP-TG, to reflect the increase in the postprandial plasma as being mainly composed of VLDL remnants.

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