Studies of a variant very-low-density lipoprotein with an acquired deficiency of apolipoprotein C-II

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Summary

1. A variant very-low-density lipoprotein was associated with severe hypertriglyceridaemia. Urea-polyacrylamide gel electrophoresis of the tetramethylurea-soluble apolipoproteins of these very-low-density lipoproteins (VLDL) showed that the apolipoprotein C-II content was less than 10% of that in VLDL from hypertriglyceridaemic (3–120 mmol/l) controls.

2. VLDL were incubated with bovine milk lipoprotein lipase (LPL) and a 9,10-3H-labelled triglyceride emulsion. The VLDL deficient in apolipoprotein C-II were a poor activator of LPL, compared with the effect of VLDL with normal content of apolipoprotein C-II obtained from either normal or hypertriglyceridaemic sera.

3. The efficacies of various VLDL as substrates for activated LPL were examined. Apolipoprotein C-II-deficient VLDL were a poor substrate for the activated enzyme compared with normal or hypertriglyceridaemic VLDL, and compared with an artificial triglyceride emulsion.

4. The abnormal VLDL were obtained from a subject with an IgG3 lambda myeloma protein. Intravenous infusion of normal plasma containing apolipoprotein C-II was followed by rapid, complete, but short-lived (5–10 days) clearance of serum triglyceride. The effect was observed on three occasions until treatment of the myeloma was effective.

5. The monoclonal protein behaved as a cryoglobulin, and formed large particle complexes with triglyceride-rich lipoproteins, especially at temperatures below 37°C. The apolipoprotein C-II deficiency, and consequent hypertriglyceridaemia, may be secondary to an autoantibody directed against apolipoprotein C-II. VLDL from relatives with hypertriglyceridaemia, without myeloma, had normal apolipoprotein content, activated LPL, and were efficient substrates for the enzyme.

Key words: apolipoprotein C-II, hypertriglyceridaemia, lipoproteins, lipoprotein lipase, myeloma, very-low-density lipoproteins.

Abbreviations: VLDL, very-low-density lipoproteins; LPL, lipoprotein lipase.

Introduction

Triglycerides carried on chylomicrons and very-low-density lipoproteins are hydrolysed by the enzyme lipoprotein lipase at the peripheral capillary endothelium. Lipoprotein lipase is absent in rare familial type I hyperlipoproteinemia [1]. One peptide component purified from the triglyceride-rich lipoproteins, apolipoprotein C-II, appears to be a potent activator of lipoprotein lipase [2–4], whereas purified apolipoproteins C-III1 and C-III2 may inhibit the activity of the enzyme [2]. In the hypertriglyceridaemia of pregnancy [5] and in type V hyperlipoproteinemia [6] the ratio of apolipoprotein C-II to C-III apolipoproteins is reduced. Breckenridge et al. [7] have reported a 59 year old man with severe hypertriglyceridaemia and undetectable apolipoprotein C-II. Administration of apolipoprotein C-II to the patient by plasma transfusion
rapidly corrected the plasma hypertriglyceridaemia, and family studies suggested an autosomal recessive inheritance [8].

A further rare cause of hyperlipoproteinaemia is autoimmune hyperlipaemia, occurring in myeloma. The monoclonal gammapathy can be associated with failure to catabolize circulating chylomicrons and very-low-density lipoproteins (VLDL) [9-11]. The myeloma protein acts as an autoantibody directed against the lipoprotein [9, 12], possibly against natural heparin-like substances [13] or against the enzyme lipoprotein lipase.

We report the occurrence of variant VLDL with reduced content of apolipoprotein C-II, which was both a poor activator of, and inefficient substrate for, lipoprotein lipase. This abnormal VLDL was associated with severe hypertriglyceridaemia in a patient with multiple myeloma.

Methods

Lipoprotein preparation

Blood samples from proband, relatives and controls were taken after an overnight (14 h) fast. Serum, or lithium-EDTA-plasma, was separated and kept at 4°C. For lipoprotein quantification samples were also separated by precipitation techniques for chylomicrons and very-low-density lipoproteins [14, 15], and for high-density lipoproteins [15, 16]. Lipoproteins for activation and substrate studies of lipoprotein lipase were prepared and washed by standard ultracentrifugal methods [17]. In view of abnormal flotation of chylomicrons and very-low-density lipoproteins from the proband, these were considered together as triglyceride-rich lipoproteins, at density less than 1.006 g/ml. The concentrations of cholesterol and triglyceride in serum, and in lipoprotein fractions, were determined by semi-automated fluorimetric methods [15, 18].

In some experiments very-low-density lipoproteins from controls were incubated with very-low-density lipoprotein-depleted serum (prepared by ultracentrifugation) [17] from the proband containing monoclonal immunoglobulin, for 1 h at 37°C. The lipoprotein was then re-isolated by ultracentrifugation before analysis for tetramethylurea-soluble apolipoproteins.

Apolipoproteins

The C-apolipoproteins were dissociated from the triglyceride-rich lipoprotein by incubation of the lipoprotein with an equal volume of tetramethylurea for 15 min at 37°C, followed by centrifugation for 10 min at 1000 g [19]. The supernatant (100 µl adjusted to contain 50 µg of protein) was applied to polyacrylamide gel columns (7.5%) containing urea (8 mol/l) at 3 mA per tube until tracking dye reached the bottom of the tube. Gels were stained with Coomassie blue for 30 min and destained for 48 h in methanol/acetic acid/water (17:6:100, by vol.). Stained gels were scanned with a Vitatron densitometer.

Lipoprotein lipase

Adipose tissue samples were obtained from the fasting patient by percutaneous needle biopsy [20], and washed twice in sodium chloride solution (154 mmol/l). Weighed samples (of about 50 mg) were incubated for 1 h at 37°C in isotonic Earle's bicarbonate buffer (pH 7.4; 1 ml) containing glucose (10 mmol/l), fatty acid-free bovine serum albumin (25 mg/ml) and heparin (2 units/ml).

Aliquots of medium were assayed for lipoprotein lipase. A volume (250 µl) of an emulsion containing glyceryl trioleate (2.36 µmol/ml) and glyceryl tri[9,10-3H]oleate (1 µCi/ml) in Tris-HCl buffer (pH 8-1, 1 ml) was used in each assay tube, with 150 µl of medium from adipose tissue incubation, together with 100 µl of fasting normal human serum to activate the enzyme [21]. Enzyme activity against the triglyceride emulsion was shown to be similar to activity against VLDL from normal subjects and hypertriglyceridaemic controls. After incubation for 1 h at 37°C, 200 µl of the mixture was extracted in a Belfrage-Vaughan partition system [22]. Radioactivity in released free fatty acids in 1 ml of upper phase was counted in a liquid scintillation counter (Packard model 2420).

The lipolytic activity in post-heparin plasma was similarly estimated, 50 µl of plasma being incubated with 250 µl of the glyceryl trioleate emulsion.

Blood for post-heparin lipolytic activity was taken before, and 20 and 60 min after, intravenous administration of heparin (5000 units), and serum was frozen at -20°C until assayed.

Activation of lipoprotein lipase

The relative abilities of triglyceride-rich lipoproteins from the proband and from controls to activate lipoprotein lipase was assessed by addition of various quantities of lipoproteins to the assay system containing glyceryl trioleate
Acquired apolipoprotein C-II deficiency

emulsion (2.36 μmol/ml; 1 μCi/ml), and purified bovine milk lipoprotein lipase (28 000 munits/mg of protein; 4 munits/ml) but no addition of normal serum.

The efficiency of triglyceride-rich lipoproteins from the proband and from controls as substrates for lipoprotein lipase were assessed by the reduction in the activity of the enzyme towards the radioactive triglyceride emulsion, as the concentration of added, unlabelled, lipoprotein was increased. In these substrate-efficiency experiments 100 μl of control human serum was added to each assay tube to ensure full activation of the enzyme. Lipoprotein lipase was prepared from fresh cow's milk by batch absorption and affinity chromatography on heparin-Sepharose [23]. The final preparation had a specific activity of 28 000 munits/mg of protein (1 munit = 1 nmol of fatty acid/min). It was diluted with sodium chloride solution (0.5 mol/l) to 200 munits/ml and stored at -20°C.

Myeloma

Staining of marrow smears with Jenner–Giemsa stain showed large numbers of plasma cells. Unstained smears showed specific immunofluorescent reactions between plasma cells and both anti-IgG3 and anti-λ antisera. The para-protein was characterized as an IgG3λ immunoglobulin, and behaved as a cryoglobulin.

Subjects

Variant VLDL were obtained from an obese (115% of desirable weight) 53 year old postman who complained of 1 week of numbness, pain and pallor of fingers and toes, and of a diffuse rash on trunk and limbs on cold exposure. Severe Raynaud's phenomenon and a white reticular rash were visible. Lipaemia retinalis was present, but discrete lipid droplets in the retinal vessels suggested lipoprotein particle aggregation.

![Graph](image-url)

**Fig. 1.** Changes in serum triglyceride (●—●), serum cholesterol (●⋯●) and whole blood viscosity, in relation to treatment by prednisone and melphalan, and by plasma exchange. Blood removed is shown in units, and was replaced with units of packed cells (c) and/or units of fresh frozen plasma (p) at the points indicated (■). Anticoagulant in fresh frozen plasma (and during plasma exchange) was citrate/phosphate/glucose.
Full blood cell count and coagulation screen were normal, as were urea, electrolytes, liver function and glucose tolerance. Serum was grossly lipaemic, and cholesterol and triglycerides were 7.4 and 28.5 mmol/l. Whole blood viscosity at a shear rate of 23 s⁻¹ was 18 cps (normal less than 7). Immunoglobulins G, A and M were 5.7 g/l (normal 5–18), 1.13 g/l (0.8–4.5) and 1.16 g/l (0.2–2.0) respectively. However, a discrete immunoglobulin band was characterized as an IgGκ monoclonal protein, and behaved as a cryoglobulin.

The subject's father died at age of 73 years of carcinoma of the stomach, and mother at age 70 years of unknown cause. Four brothers had died, two in infancy, one of tuberculosis at age 21 years and the fourth at age 63 years of carcinoma of the bladder. Two surviving brothers had serum cholesterol concentrations of 6.3 and 4.0 mmol/l, and serum triglyceride concentrations of 13.0 and 9.0 mmol/l respectively. Two sisters had values for serum cholesterol of 5.5 and 4.6 mmol/l respectively. The cholesterol and triglyceride values for the only son of the proband were 3.7 and 1.65 mmol/l. Their serum samples separated normally on centrifugation.

Plasma exchange was carried out shortly after admission in view of the hyperviscosity, high serum fibrinogen and hypertriglyceridaemia (Fig. 1), and was followed by return of lipid levels to normal. Further symptoms, and hypertriglyceridaemia, were improved by subsequent plasma exchange, but not by removal of two units of blood without plasma replacement. Units of blood removed were replaced with units of packed cells (c in Fig. 1) and/or with fresh frozen plasma (p).

After confirmation of multiple myeloma treatment with melphalan and prednisone intermittent therapy was instituted, and was well tolerated. No monoclonal immunoglobulin was detected after day 60, the patient being clinically in remission, with concentrations of cholesterol and triglycerides of 2.3 and 0.4 mmol/l respectively. VLDL were also obtained from control subjects and from hypertriglyceridaemic control subjects (serum triglycerides 3–120 mmol/l).

Results

Lipoprotein analyses

Initial cholesterol and triglyceride concentrations were elevated at 7.4 and 28.5 mmol/l respectively. Centrifugation of blood showed a heavy layer of chylomicrons, a moderately turbid serum and, unusually, a heavy creamy layer above the erythrocytes. Blood centrifuged at 37°C showed much less aggregation of lipoprotein particles. On agarose gel electrophoresis the majority of the triglyceride-rich lipoproteins remained at the origin. Concentrations of high-density lipoprotein cholesterol in initial samples were low at 0.4–1.0 mmol/l.

After 2 months' treatment the concentrations of cholesterol in serum very-low-, low- and high-density lipoproteins were 2.3, 0.3, 1.3 and 0.7 mmol/l respectively, and for triglycerides were 1.6, 0.85, 0.6 and 0.15 mmol/l respectively. The concentrations of cholesterol in low- and high-density lipoproteins were below the fifth percentiles for a normal, randomized, stratified male population from the same city (2.2 and 0.8 mmol/l respectively) (unpublished data). By 80
Acquired apolipoprotein C-II deficiency

exchange transfusion led to a rapid and large fall in triglyceride and cholesterol concentrations, which was not explainable on the basis of the plasma volume exchanged (Fig. 1). A nadir in lipid levels was reached 4 days after exchange (day 6), but hypertriglyceridaemia recurred until the next plasma exchange on day 18, after which triglycerides fell from 14.4 to 2.5 mmol/l. A series of plasma exchanges from day 47 was followed by a fall in triglycerides from more than 9.8 to 1.6 mmol/l. Removal of two units of blood, without plasma exchange, on day 38, did not influence serum lipid levels.

Polyacrylamide gels of the tetramethylurea-soluble apolipoproteins are shown in Fig. 2 for the variant VLDL and for VLDL from a normal subject. The major apolipoprotein bands from normal VLDL are, from below upwards in Fig. 2, C-III, C-II and C-II. In the gel from variant VLDL distinct C-II and C-II bands were present, but the band in the C-II region was greatly reduced. The proportions of each lipoprotein, determined by scanning densitometry, are shown in Table 1 for triglyceride-rich lipoproteins from the proband, relatives (four siblings and son) and from hypertriglyceridaemic controls. Hypertriglyceridaemic relatives had normal patterns of tetramethylurea-soluble apolipoproteins, and in particular a normal apolipoprotein C-II content. The variant VLDL showed a considerable reduction in apolipoprotein C-II levels, and in the ratio of C-II to C-III apolipoproteins. After treatment of the myeloma, and regression of the hyperlipidaemia, serum cholesterol and triglycerides fell to subnormal levels. It was not then possible to obtain very-low-density lipoproteins in sufficient amount to allow estimation of apolipoprotein C-II.

**Table 1**. Apolipoprotein composition of triglyceride-rich lipoproteins from proband, relatives and hypertriglyceridaemic controls

Concentrations of apolipoproteins were estimated by scanning densitometry of stained polyacrylamide gels.

<table>
<thead>
<tr>
<th>Apolipoprotein</th>
<th>Apolipoprotein concentration (% of total apolipoproteins C-II, C-III, C-III, E) Proband Relatives Controls (n = 5) (n = 27)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C-II</td>
<td>1.3                                                                  15.7 ± 3.0</td>
</tr>
<tr>
<td>C-III</td>
<td>13.3                                                                 39.5 ± 3.3</td>
</tr>
<tr>
<td>C-III</td>
<td>11.3                                                                 21.3 ± 4.7</td>
</tr>
<tr>
<td>E</td>
<td>74.0                                                                 23.7 ± 4.6</td>
</tr>
<tr>
<td>Ratio C-II/C-III</td>
<td>0.054                                                        0.26</td>
</tr>
</tbody>
</table>

**Lipoprotein lipase**

Injection of heparin caused the appearance in serum from the proband of triglyceride lipase activity.

![Graph showing the reduction of activity of fully activated, purified, bovine milk lipoprotein lipase towards a 9,10-3H-labelled triglyceride emulsion when incubated in the presence of triglyceride-rich lipoproteins from the proband (○—○; mean ± SEM; n = 3) or from hypertriglyceridaemic (type V) control subjects (○—○; mean ± SEM; n = 4). The 'expected' pool dilution curve is that expected if labelled triglyceride emulsion and lipoprotein-triglyceride are equivalent substrates for the enzyme.](image-url)
activity, which was increased by the addition of serum from fasting controls.

Lipoprotein lipase activity was also released from adipose tissue biopsies, the activity being 115 ± 28 nmol of fatty acids released h⁻¹ g⁻¹ (mean ± 1 SD; n = 4) compared with control activities of 193 ± 136 nmol h⁻¹ g⁻¹ (n = 11 subjects).

In view of the low concentration of apolipoprotein C-II in the variant triglyceride-rich lipoproteins, the activation by variant VLDL of lipoprotein lipase, purified from bovine milk, was studied (Fig. 3). At lipoprotein concentrations of 15 μg of protein/ml, variant triglyceride-rich lipoproteins gave only 10% of the enzyme activation seen with control lipoproteins.

As triglyceride-rich lipoproteins are the natural substrate for lipoprotein lipase, the ability of variant VLDL to act as a substrate for the enzyme was also assessed, by a substrate dilution experiment in which purified bovine milk lipoprotein lipase, fully activated by fasting normal human serum, was incubated with a standard amount of emulsified glyceryl tri[9,10-

3H]oleate, together with increasing amounts of either variant or normal unlabelled triglyceride-rich lipoproteins (Fig. 4). At an added triglyceride concentration of 0-6 mmol/l the formation of labelled free fatty acids from the emulsion was reduced by 35% (n = 4) with control lipoproteins compared with only 16% with variant triglyceride-rich lipoproteins.

Discussion

The variant triglyceride-rich lipoproteins were found to have abnormal concentrations of tetramethylurea-soluble apolipoproteins, with a greatly reduced content of apolipoprotein C-II to less than 10% of that in control hypertriglyceridaemic VLDL. This reduced concentration of apolipoprotein C-II, and reduced ratio of C-II to C-III₁,₂ apolipoproteins, are likely to be related to the hypertriglyceridaemia and increased serum VLDL concentration. This is in contrast to the concentrations of apolipoprotein C-II in other hypertriglyceridaemic VLDL, which are similar to concentrations in normotriglyceridaemic VLDL.

However, apolipoprotein C-II is a specific activator of the enzyme lipoprotein lipase, which is necessary for the catabolism and clearance of VLDL. Further, Breckenridge et al. [7] have described congenital deficiency of apolipoprotein C-II associated with hypertriglyceridaemia and later described similar defects in hypertriglyceridaemic relatives [8]. Our C-II deficient variant VLDL failed to activate lipoprotein lipase normally, a defect which in vivo would allow accumulation of VLDL, and hypertriglyceridaemia. Lipoprotein lipase activated by normal VLDL was shown to use normal VLDL and labelled triglyceride emulsion similarly as substrates. With a deficit of apolipoprotein C-II the variant VLDL were poorly recognized as a substrate by lipoprotein lipase. The poor efficiency of the variant VLDL as substrate for the activated enzyme suggests that normal apoprotein composition of VLDL may be necessary for normal enzyme–substrate binding. Lipoprotein lipase was not deficient, being normally extracted from adipose tissue, and being released into plasma by heparin. These features suggest that the acquired C-II deficiency is responsible for the failure to catabolize and remove circulating VLDL, in a manner similar to familial apolipoprotein C-II deficiency. The apolipoprotein E content of the tetramethylurea-soluble apolipoproteins of the variant VLDL was elevated compared with hypertriglyceridaemic control VLDL. No obvious explanation is apparent, but a similar increase in apolipoprotein E has been reported in primary apolipoprotein C-II deficiency [7]. The relative reduction in apolipoprotein C-III as a percentage of tetramethylurea-soluble apolipoproteins is a consequence of the increased apolipoprotein E in the variant lipoproteins.

The rapidity and extent of the fall in serum triglyceride after partial plasma exchange and the subsequent return to hypertriglyceridaemia, repeated on three occasions, suggests an activation of endogenous lipoprotein lipase by an infused factor present in normal serum but not in the serum of the proband, and which could be apolipoprotein C-II. This would be similar to the response in familial apolipoprotein C-II deficiency in which intravenous infusion of 250 ml of normal plasma increased the apolipoprotein C-II content of abnormal VLDL to about 5–10% of normal, and was associated with a fall in serum triglyceride for approximately 1 week [7]. The fall in triglycerides was several fold greater than can be attributed to dilution by infused plasma, or to antibody removal.

It is thought that apolipoprotein C peptides normally transfer from high-density lipoproteins to newly synthesized triglyceride-rich lipoproteins of hepatic and intestinal origin, and are subsequently lost from these lipoproteins during their catabolism and triglyceride hydrolysis. Clearance of variant VLDL after infusion of normal apolipoprotein C-II-containing VLDL may result from activation of lipoprotein lipase alone, but
apoprotein C-II might also pass to variant VLDL, rendering them a more efficient substrate for activated lipoprotein lipase.

The hypertriglyceridaemia in relatives might suggest that our variant VLDL was familial, but these triglyceride-rich lipoproteins were shown to have normal C-apoproteins, to activate lipoprotein lipase normally and to be effective substrates for this enzyme, and in these respects their triglyceride-rich lipoproteins behaved similarly to those from hypertriglyceridaemic or normal controls. The mechanism of hypertriglyceridaemia in these relatives is therefore different, and not due to the specific clearance defect of apolipoprotein C-II-deficient variant VLDL, while the proband’s hypertriglyceridaemia resolved and was not familial.

The resolution of the hypertriglyceridaemia after adequate treatment of the myeloma provides clinical evidence of the relationship between acquired variant VLDL and the monoclonal gammopathy. It would have been helpful to have examined VLDL composition after effective treatment of the myeloma but the low–normal serum triglyceride concentrations at this time prevented recovery of sufficient VLDL to examine the tetramethylurea-soluble apolipoproteins. This might in itself suggest that structure and function of VLDL had returned to normal.

The lipoprotein patterns occurring in myeloma have been examined [24, 25], showing a reduced serum cholesterol (the reduction occurring in both low- and high-density lipoproteins) and normal concentrations of the triglyceride-rich lipoproteins. Severe hypertriglyceridaemia, however, may occasionally occur in patients with multiple myeloma [9, 11, 26], usually in association with κ chains, and IgA monoclonal protein. The hyperlipaemia appears to have an autoimmune basis [27] with autoantibodies against lipoprotein, natural heparin-like substances or lipoprotein lipase. Autoimmune hyperlipaemia has been seen without myeloma, with or without other evidence for an immune disturbance [9, 13, 28, 29].

The immunoglobulin of monoclonal origin was characterized as an IgGκλ protein, and was demonstrated on the surface of marrow plasma cells. These cells did not demonstrate a specific anti-lipoprotein activity. Normal lipoproteins did not alter their content of C-apolipoproteins during incubation with the proband’s serum containing abnormal immunoglobulin but depleted of variant VLDL. This provides no direct support for, but does not exclude, a reactivity of abnormal immunoglobulin against apolipoprotein C-II in vivo. Similarly autoantibodies did not prevent release of lipoprotein lipase into blood after intravenous heparin, and the enzyme was extractable from adipose tissue.

Variant VLDL deficient in apolipoprotein C-II were associated with hypertriglyceridaemia and an IgGκλ myeloma. The variant VLDL were a poor activator of, and inefficient substrate for, lipoprotein lipase, which was present in normal amounts. The relationship between the deficiency of apolipoprotein C-II and hypertriglyceridaemia was further demonstrated by the response to plasma exchange. The resolution of hypertriglyceridaemia with successful treatment of the myeloma supports a causative role of an abnormal myeloma immunoglobulin acting as an antibody, possibly specifically against apolipoprotein C-II.

Severe hypertriglyceridaemia may therefore occur secondary to acquired and reversible deficiency of this apolipoprotein. This study provides further evidence for the importance of the lipoprotein peptides in modulating the fate and rate of metabolism of triglyceride-rich lipoproteins.

References


100


