Comparison of apoprotein B of low density lipoproteins of human interstitial fluid and plasma

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Virtually all apoprotein B (apoB)-containing lipoproteins of the peripheral interstitial fluid of subjects with primary lymphoedema float in the ultracentrifugal field in the density interval 1.019–1.063 g/ml; in this respect they are similar to plasma low-density lipoproteins (LDL). 2. Virtually all apo-B-containing lipoproteins of interstitial fluid migrate in the electrophoretic field with pre-beta mobility; in this respect they are similar to plasma very-low-density lipoproteins. 3. The apoB of lipoproteins of interstitial fluid does not differ in terms of M_r from apoB-100 of human plasma [Kane, Hardman & Paulus (1980) *Proc. Natl. Acad. Sci. U.S.A.* 77, 2465–2469] as determined by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis. 4. Both apoB of interstitial fluid and plasma are heterogenous in terms of their charge as determined by isoelectric focusing of their complexes with the nonionic detergent Nonidet P40. ApoB of plasma LDL focuses between pH 5.9 and 6.65, and that of interstitial fluid LDL between pH 5.9 and 6.1. Thus the overall charge of apoB of interstitial fluid is more negative than that of its plasma LDL counterpart.

Accumulation of cholesterol within macrophages and proliferating smooth muscle cells of the tunica media of arteries is a salient cellular alteration associated with atherogenesis.

Cultured macrophages take up cholesterol from their environment in the form of lipoproteins. Unlike other cultured cells they take up only little LDL by receptor-mediated endocytosis, but take up by such a pathway LDL in which its apoprotein (apoB) has been modified by acylation (acyl-LDL) (Brown & Goldstein, 1983). All modifications that convert LDL into a ligand of the acyl-LDL

receptor of macrophages that have been described increase the net negative charge of apoB (Brown *et al.*, 1980).

When plasma LDL are incubated in the presence of cultured vascular endothelial cells the lipoprotein increases its net negative charge and becomes a ligand of the acyl-LDL receptor of macrophages (Henriksen *et al.*, 1981). All LDL particles that are present in extravascular extracellular fluids must have been in close contact with vascular endothelial cells while crossing from plasma into the interstitium. We have previously shown that apoB-containing lipoproteins of human peripheral lymph have a higher net negative charge than does plasma LDL (Reichl *et al.*, 1975). Clearly it was important to learn whether this was due to changes in the net charge of apoB. The size of samples of peripheral lymph does not permit such studies. However, samples of interstitial fluid that are large enough for this purpose can be obtained from patients with primary lymphoedema (Johnson & Pflug, 1975). In the present study we compare the LDL and apoB of these lipoproteins obtained from the interstitial fluid of such patients with the LDL and its apoB from their blood plasma.

Abbreviations used: apoB, apoprotein B, the main apoprotein of plasma LDL (in the present study it has been detected immunochemically with antibodies raised against LDL and should be referred to as apoLDL; for convenience it is referred to as apoB); IDL, intermediate-density lipoproteins (ρ 1.006–1.019 g/ml); LDL, low-density lipoproteins (ρ 1.019–1.063 g/ml); VLDL, very-low-density lipoproteins (ρ 1.006 g/ml); NP40, Nonidet P40; SDS, sodium dodecyl sulphate; IEF, isoelectric focusing.

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Experimental

Sample preparation

Interstitial fluid was obtained from four patients with primary lymphoedema of the leg in the course of staged resection of the subcutaneous tissue (Johnson & Pflug, 1975). The clear fluid that accumulates spontaneously in the incision was aspirated with a syringe and transferred into centrifuge tubes that contained 1 mg of disodium EDTA/ml of the fluid. Cell counting revealed that none of the samples contained more than 7000 erythrocytes/ μ l. Gentamycin (1 mg) (Essex Chemie, Lucerne, Switzerland) and soya bean trypsin inhibitor (1 mg) (Sigma) were added to each 1 ml of the fluid and the samples were passed through Millipore filters and kept on ice until used for analysis. Blood was obtained from the cubital vein in the course of the operation; 1 mg of disodium EDTA/ml was added and samples were centrifuged at 5000g and 4°C for 15 min. Plasma was separated and inhibitors added as for interstitial fluid. Specimens of tissue obtained at operation showed no signs of inflammation as judged by light microscopy carried out by Professor N. Huth (Rosenheim, Germany).

Isolation of LDL

LDL were isolated by sequential ultracentrifugation (Havel *et al.*, 1955). The samples were diluted 10-fold with NaCl/KBr solution of ρ 1.063 g/ml and recentrifuged under the conditions used for their isolation. Alternatively, LDL were isolated from interstitial fluid and plasma from which lipoproteins of $\rho < 1.019$ g/ml had been separated by ultracentrifugation and removed by tube slicing (plasma fraction $\rho > 1.019$ g/ml) according to Warnick & Albers (1978). The resulting precipitates were dissolved in NaCl/KBr solution of ρ 1.063 g/ml and purified by ultracentrifugation.

Electrophoretic procedures

One-dimensional immunoelectrophoresis was carried out according to Grabar & Williams (1953), crossed immunoelectrophoresis in tandem with IEF and quantitative immunoelectrophoresis essentially as described previously (Reichl & Pflug, 1982) and analytical SDS electrophoresis in 3% polyacrylamide gels according to Kane *et al.* (1980).

Preparation of apoB-NP40 complexes

These complexes were prepared by adding to the respective physiological fluid or to isolated LDL 50 mg of NP40 (BDH, Poole, Dorset, U.K.) for each 1 mg of apoB in the sample. Lipid free apoB-NP40 complexes were prepared according to Helenius & Simons (1971). Radio-iodinated LDL

was added as marker before mixing the lipoproteins with NP40. Delipidated apoB-NP40 complexes were extracted with chloroform/methanol (Folch *et al.*, 1957). Cholesterol was determined in the extracts as described previously (Reichl *et al.*, 1980) and phospholipids by the method of Chalvardjian & Rudnicki (1970). Less than 2 μ g of cholesterol and less than 30 ng of lipid phosphorus was found for each 1 mg of protein.

Determination of protein

Protein concentration was determined by the method of Lowry *et al.* (1951) or in the presence of NP40 and Pharmalyte by a modification of the method of Bensadoun & Weinstein (1976). Samples were made to 1 ml with 0.15 M-NaCl, and 1 ml of a 2% (w/v) solution of Dextran 80 (Pharmacia) in water was added, followed by 10 ml of a 15% (w/v) trichloroacetic acid solution in ethanol. After centrifuging at 5000g for 30 min at 4°C the supernatant was removed, 1 ml of ethanol was added to the sediment, followed by 1 ml of a 2% (w/v) aqueous solution of sodium deoxycholate and 10 ml of a 10% (w/v) aqueous solution of trichloroacetic acid. The samples were processed further as in the method of Bensadoun & Weinstein (1976). A subfraction of LDL (ρ 1.025–1.040 g/ml) was used as secondary standard, and crystalline bovine serum albumin as a primary standard. When the subfraction of LDL was radioiodinated and used as internal standard, more than 94% of protein radioactivity was recovered.

Preparative IEF

Preparative flat-bed electrofocusing in Ultrodex (Pharmacia) granulated gels was carried out essentially as recommended by the manufacturer of the LKB 2117 Multiphor apparatus (LKB, Bromma, Sweden), except that the gels contained glycerol at a final concentration of 10% (w/v) and 0.3% NP40. The samples were focused for 16 h at 8 W constant power on a water-cooled plate. The focused gels were cut into zones with the aid of a fractionating grid. Approx. 10% of each zone was taken for pH determination, the rest was extracted by adding to the slurry 0.7 ml of 0.1 M-barbitone buffer, pH 8.6, containing glycerol (10%, w/v), and NP40 (0.2%). The slurry was filtered with suction on sintered glass filters. The recovery of radio-labelled samples was on average 82%, and more than 94% when samples were re-extracted with 1 ml of the buffer.

IEF in agarose

Horizontal agarose slab gels were prepared essentially according to the instructions of the manufacturers of Agarose IEF (Pharmacia). When

Fig. 1. Immunoelectrophoresis of interstitial fluid and plasma, and LDL from interstitial fluid and plasma
 (a) Interstitial fluid and plasma (5 μ l each) were electrophoresed in 1.5% agarose in 0.1 M-barbitone buffer, pH 8.6, for 3 h and 100 V. At the end of the run troughs were made between the lanes in which samples were electrophoresed. The troughs were filled with anti-apoB serum and the plate was kept in a wet chamber at room temperature for 36 h. Lanes 1 and 3, plasma; lanes 2 and 4, interstitial fluid from the same patient. (b) LDL from intersti-

apoB-NP40 complexes were focused the gels contained additionally 10% (w/v) glycerol and 0.3% NP40. Pharmalyte was added to a final concentration of 2.5% (w/v); 1 ml of the respective mixture was separated and kept at 50°C for the preparation of samples and 29 ml was poured into a horizontal frame (19.0 cm $\times$ 11.0 cm) on a Gelbond film. The samples were heated in a water bath at 42°C and mixed with their own volume of Pharmalyte containing agarose; 40 μ l of this mixture was pipetted into wells 5 mm in diameter punched into the hardened agarose and 3 μ l of a solution of 4.5 mg of isoelectric point markers (wide range kit; BDH) in 200 μ l of redistilled water was applied directly to the surface of the agarose plates centrally between each well that contained the samples. The samples were focused for 3 h at 10 W constant power. Focused samples were either processed and stained as recommended by the manufacturer of Agarose IEF or the gels and supporting film were cut in the cathode-to-anode direction into 1.5 cm-wide lanes and submitted to cross immunoelectrophoresis. Control lanes were focused and cut perpendicularly to the direction of focusing into 1 cm sections that were incubated overnight in 3 ml of redistilled water; the pH of the extracts was measured with a glass electrode.

Immunochemical methods

Commercial goat anti-LDL serum (Miles, Yedda, Israel) was used. A single precipitin line was observed by double diffusion and cross immunoelectrophoresis against isolated LDL or total human serum. Anti-(human serum) serum was also a commercial preparation (Behringwerke, Marburg, Germany). Anti-(apoAI) serum was described previously (Reichl & Pflug, 1982). The concentration of apoB was measured by quantitative immunoelectrophoresis (Reichl *et al.*, 1977).

Radioiodination

Isolated LDL were iodinated by a slight modification (Eisenberg *et al.*, 1972) of the method of McFarlane (1958). Carrier free Na¹²⁵I for protein iodination was obtained from Amersham International. Less than 8% of the radioactivity could be extracted with chloroform/methanol (3:1, v/v). Radioactivity was counted in a LKB-Wallac gamma counter.

tial fluid and plasma (each 5 μ l of 500 μ g/ml) and 5 μ l of plasma were electrophoresed and reacted with antiserum as in (a). Troughs 1 and 2, anti-apoB serum; troughs 3 and 4, anti-(human serum) serum. Lanes 1 and 5, plasma; lanes 2 and 4, plasma LDL; lane 3, interstitial fluid LDL.

Results

Comparison of apoB-containing lipoproteins of interstitial fluid and plasma

On ultracentrifugation of interstitial fluid from the four patients of this study virtually all apoB-containing lipoproteins could be detected in the fraction of ρ 1.019–1.063 g/ml. Thus apoB-containing lipoproteins of interstitial fluid have the ultracentrifugal characteristics of plasma LDL.

In contrast, when interstitial fluid or its LDL was electrophoresed in agarose and apoB-containing lipoproteins detected immunologically it was found that they migrated within a narrow band, corresponding in mobility to plasma VLDL rather than to LDL (Figs. 1a and 1b). ApoB-containing lipoproteins of interstitial fluid also focused in a narrower range of pI (pH 5.7–5.0) than those of the plasma fraction $\rho > 1.019$ g/ml (pH 5.7–6.3) as revealed by crossed immunoelectrophoresis (Fig. 2).

Comparison of apoB of interstitial fluid and plasma

The M_r of apoB of LDL of the two fluids was compared by analytical SDS/polyacrylamide-gel electrophoresis. In the case of interstitial fluid LDL, the only band that could be seen corresponding to apoB-100 (Fig. 3).

The net charge of apoB–NP40 complexes of the two fluids was compared by IEF. The method of Socorro & Camejo (1979) that is available requires larger quantities of LDL than can be obtained from interstitial fluid. It was therefore necessary to

adapt the method by focusing apoB–NP40 complexes in agarose in combination with subsequent crossed immunoelectrophoresis. Three experiments were carried out to validate the method.

First, delipidated complexes of LDL apoB–NP40 (Helenius & Simons, 1971) were focused by the adapted method and the results compared with those obtained by IEF in flat-bed granulated gels. Both methods show that apoB–NP40 complexes focus between pH 5.9 and pH 6.65 (Figs. 4 and 5).

Secondly, fractions of apoB–NP40 complexes of differing pI values were obtained by preparative IEF and adjusted to equal apoB protein concentrations were compared by quantitative immunoelectrophoresis under conditions where rocket height was a linear function of protein concentration. No difference in rocket height per mg of protein could be detected among the individual fractions (Fig. 5).

Thirdly, the results of agarose IEF in tandem with crossed immunoelectrophoresis of delipidated apoB–NP40 complexes obtained from plasma LDL were compared with those obtained when the detergent was added to the plasma fraction of $\rho > 1.019$ g/ml (Figs. 4 and 6a). In both cases the range of pI values was 5.9–6.65.

On the basis of these investigations the pattern of focusing of apoB complexes that are formed on adding NP40 to interstitial fluid was compared with those formed by adding the detergent to the plasma fraction of $\rho > 1.019$ g/ml. It was found that interstitial fluid apoB–NP40 complexes focus between pH 5.9 and 6.1, while those of the plasma fraction of $\rho > 1.019$ g/ml focus between 5.9 and

Fig. 2. *Cross immunoelectrophoresis in tandem with IEF in agarose of interstitial fluid and plasma* Interstitial fluid (20 μ l) and plasma (4 μ l) from the same patient were focused in agarose as described in the text. At the end of focusing strips of agarose containing the samples and the underlying Gelbond were submitted to cross immunoelectrophoresis against anti-LDL serum as described in the text. The plates were dried and stained with Coomassie Blue. Strips of agarose containing pI markers that were focused concomitantly with the samples were processed as described in the text. (a) Electrophoretogram of plasma; (b) electrophoretogram of interstitial fluid.

Fig. 3. Analytical SDS/polyacrylamide-gel electrophoresis of apoB from interstitial fluid and plasma LDL of the same patient

Delipidated LDL (20 μ g) from interstitial fluid and plasma were dissolved in 20 μ l of 0.02M-phosphate buffer (pH 7.2) that contained 200 μ g of SDS. The samples were electrophoresed and processed according to Kane *et al.* (1980). Gel 1, apoB of plasma LDL; gel 2, apoB of interstitial fluid LDL.

6.65 (Figs. 6a and 6b). The same difference between the apoB of interstitial fluid and of plasma was obtained when complexes of NP40 with apoB of isolated LDL from the two fluids were compared (Figs. 7a and 7b). It can be concluded that the range of pI values of apoB from interstitial fluid corresponds to the more acidic part of the spectrum of pI values of apoB from plasma.

Discussion

The present results show that apoB-containing lipoproteins of interstitial fluid resemble those of

peripheral lymph of apparently healthy subjects (Reichl *et al.*, 1975) in that they migrate in the electrophoretic field with a mobility similar to that of plasma VLDL (Fig. 1). Studies have shown that peripheral lymph is a representative sample of interstitial fluids of the region it drains (Renkin, 1979). It can therefore be concluded that the present findings are representative of apoB of lipoproteins of peripheral tissues rather than due to the abnormal conditions prevailing in the leg of these patients. Recent studies have shown that this is also true for apoB-containing lipoproteins in extravascular extracellular fluids of other species (Raymond & Reynolds, 1983). This difference between apoB-containing lipoproteins of interstitial fluid and plasma fraction $\rho > 1.019$ g/ml is analogous to that between plasma LDL incubated in the absence and presence of cultured vascular endothelial cells (Henriksen *et al.*, 1981).

The present data show that the increased electrophoretic mobility of LDL from interstitial fluid may, at least to some extent, be due to increased overall net charge of apoB. ApoB-NP40 complexes that are obtained from the plasma fraction $\rho > 1.019$ or from plasma LDL focus within a range of pH 5.9–6.65 (Figs. 4, 6a and 7a). The charge heterogeneity of apoB-NP40 complexes of interstitial fluid LDL is reduced when compared with that of apoB from plasma LDL (Figs. 7a and 7b); they focus between pH 5.9 and 6.1. Thus the overall charge of apoB-NP40 complexes from interstitial fluid LDL is more negative than that of apoB from plasma LDL because of the absence from interstitial fluid of apoB-NP40 complexes that focus between pH 6.1 and pH 6.65. It is possible that this is so because the components of apoB with pI values between pH 5.1 and 6.65 are selectively removed by cells of tissues, e.g. in the course of their crossing the wall of capillaries or by interacting with cells in tissues. It may also be that components of apoB with less acidic values of pI are converted into components that are more acidic.

Recent studies have shown that LDL-apoB is heterogeneous in terms of charge, size of its oligomers (Chen & Aladjem, 1978; Socorro & Camejo, 1979) and in terms of particles that express different epitopes (Curtiss & Edgington, 1982). It is not known whether apoB of interstitial fluid differs from apoB of plasma LDL in terms of the latter two characteristics. However, the present results show that, in the presence of SDS, interstitial fluid apoB does not differ from plasma apoB in terms of particle size.

The biological significance of the present findings remains to be determined. It should be pointed out that apoB-containing lipoproteins of peripheral lymph that exhibit the same electro-

Fig. 4. Cross immunoelectrophoresis of delipidated plasma LDL apoB-NP40 complex in tandem with IEF in agarose. A sample of the complex of apoB with NP40 (200 μ g of protein/ml, 20 μ l) was focused in agarose and subsequently submitted to cross-immunoelectrophoresis against anti-LDL serum as described in the text. The pI marker was also processed as described in the text.

Fig. 5. Preparative flat-bed IEF of delipidated apoB-NP40 complex in a granulated gel

ApoB-NP40 complex (2ml; 1.5mg of protein/ml) prepared according to Helenius & Simons (1971) was applied to a gel of Ultradex and focused as described in the text. At the end of focusing the gel was separated into 30 fractions perpendicular to the pH gradient. Approx. 10% of each fraction was used for determination of the pH with a glass electrode (Δ). The remaining slurry of each fraction was extracted as described in the text. The concentration of protein in the extracts ($\circ$) was determined by a method described in the text, and that of apoB ($\bullet$) immunoelectrophoretically (Reichl *et al.*, 1977).

Fig. 6. Cross-immunoelectrophoresis in tandem with IEF in agarose of apoB-NP40 complexes of interstitial fluid and of the plasma fraction $p > 1.019$ g/ml

Interstitial fluid was mixed with NP40 so that for each 1 mg of apoB 50mg of the detergent was present. The fraction of plasma that was used was first diluted 5-fold with 0.15M-NaCl before NP40 was added as in the case of interstitial fluid. Either fluid (20 μ l) was applied to agarose IEF as described in the text. Focusing, cross-immunoelectrophoresis and processing of the electrophoretograms of the samples as well as of the pI markers was carried out as described in the text. (a) Electrophoretogram of plasma; (b) electrophoretogram of interstitial fluid.

Fig. 7. Cross-immunoelectrophoresis in tandem with IEF in agarose of apoB-NP40 complexes of LDL from interstitial fluid and plasma

Interstitial fluid LDL and plasma LDL were mixed with NP40 as described for interstitial fluid and plasma in the legend to Fig. 4. The samples were processed further also in the same way as described in the text. (a) Plasma LDL; (b) interstitial fluid LDL.

phoretic mobility as those of interstitial fluid are bound, taken up and degraded by cultured fibroblasts in the same way as plasma LDL (Reichl *et al.*, 1978). Studies *in vitro* have shown that the negative charge of apoB must be considerably increased before LDL starts to be recognized by acyl-LDL receptors of macrophages (Haberland *et al.*, 1982). The present data do not give evidence of the presence in interstitial fluid of apoB with a net negative charge higher than the most electro-negative component of plasma apoB.

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