Reduced β -strand content in apoprotein B-100 in smaller and denser low-density lipoprotein subclasses as probed by Fourier-transform infrared spectroscopy

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The secondary structure of apolipoprotein B-100 in low-density lipoprotein (LDL) subfractions was analysed by Fourier-transform IR spectroscopy. LDLs were isolated in three density ranges by gradient centrifugation of human plasma from healthy volunteers. The spectra revealed differences in the lipid content and composition of the three LDL fractions. The secondary structure of apolipoprotein B-100 was the same in the two

fractions corresponding to the large less-dense LDL particles, whereas a lower content of β -strands was found in the third fraction corresponding to the smaller denser LDL particles. Analysis of the spectroscopic data suggests that, in the same set of LDL subfractions, the particle size is probably the cause of the observed differences in apolipoprotein B-100 secondary structure.

INTRODUCTION

Human low-density lipoprotein (LDL), the major carrier of plasma cholesterol, plays an important role in the transport of plasma lipids, regulation of cellular cholesterol metabolism and pathogenesis of atherosclerosis. The LDL particle is organized into two major compartments, a hydrophobic core, comprised primarily of cholesteryl esters and triacylglycerols, surrounded by a surface monolayer of phospholipids with interdigitated free cholesterol [1,2]. One copy of apolipoprotein B-100 (Apo B-100) is present per LDL particle and is believed to wrap around the outer surface, with sites of hydrophobicity immersed in the lipid phase. Although LDLs in the density range 1.019–1.063 g/ml have been most frequently viewed as a homogeneous entity for use in metabolic and structural studies, heterogeneity in particle size and density of LDLs has been demonstrated, and the existence of distinct subclasses of LDL has been identified by ultracentrifugation and gel electrophoretic techniques in normolipidaemic subjects [3–5]. Several lines of evidence suggest that the small more-dense LDL subfractions are more atherogenic than the less-dense LDLs and predispose to coronary artery disease [6,7]. At the molecular level, the proposed higher atherogenicity of small more-dense LDLs has been related to their higher susceptibility to lipid peroxidation than the less-dense LDL subclasses [7] or to abnormal interactions with cell receptors that could allow longer duration in the circulation of small LDLs than less-dense subclasses [8]. As ApoB-100 at the LDL surface plays a key role in recognition of cell receptors, the aforementioned abnormalities could be related to modifications of ApoB-100 conformation and to altered exposure at the surface of the small dense LDLs compared with the less-dense LDLs.

The aim of the present study was to investigate the secondary structure of ApoB-100 in LDL subclasses. We analysed, in duplicate, six samples from two healthy volunteers using Fourier-transform I.R. (FT-IR) spectroscopy, which has been used

previously to characterize the structure of ApoB-100 in total LDLs [9–11].

MATERIALS AND METHODS

Materials

Deuterium oxide (99.9% $^2\text{H}_2\text{O}$), ^3HCl and NaO^2H were purchased from Aldrich. All other chemicals were commercial samples of the purest quality.

Isolation of LDL subfractions

Blood was obtained from two healthy adult volunteers, who were not using vitamin supplements or taking hormones or drugs known to alter plasma lipids or lipoproteins. Blood samples were collected into Vacutainers containing 1 mg/ml EDTA. Cells were removed by low-speed centrifugation (2000 *g* for 10 min) and the plasma was used to isolate LDL subfractions by using density-gradient ultracentrifugation as described by de Graaf et al. [7]. From each subject, 2.75 ml of freshly isolated plasma was pipetted into the bottom of polycarbonate centrifuge tubes. The plasma density was raised to 1.10 g/ml by dissolution of 0.385 g of KBr. The plasma was successively overlaid by solutions of decreasing density (1.85 ml, $\rho = 1.065$ g/ml; 2.73 ml, $\rho = 1.035$ g/ml; 2.73 ml, $\rho = 1.020$ g/ml; 1.375 ml, $\rho = 1.006$ g/ml). The density of each fraction was estimated from the refractive indices of identical fractions isolated from a blank tube run in parallel. Density-gradient centrifugation was performed using a Centrikon T ultracentrifuge and a TST 41.14 rotor (Kontron Instruments) at 200000 *g* at 10 °C for 19.5 h. LDL subfractions from each volunteer were collected in three ranges of density: LDL₁ $\rho = 1.030$ –1.033 g/ml; LDL₂

Abbreviations used: FT-IR, Fourier transform IR; LDL, low-density lipoprotein; LDL_a and LDL_b: LDL fractions from volunteers 'a' and 'b' respectively; amide I', amide I band in $^2\text{H}_2\text{O}$ medium; ApoB-100, apolipoprotein B-100.

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$\rho = 1.033\text{--}1.040$ g/ml; LDL₃ $\rho = 1.040\text{--}1.045$ g/ml. After isolation, the six LDL subfractions were dialysed for 24 h against 10 mM phosphate buffer, pH 7.4, containing 150 mM NaCl and 3.5 mM EDTA and stored at 4 °C in the dark.

Analytical methods

Total cholesterol, phospholipids and triacylglycerols in each LDL subfraction were determined using enzymic methods [12]. The apoprotein concentration of the lipoprotein subfractions was determined as described by Lowry et al. [13] using BSA as standard.

Preparation of samples for FT-IR spectroscopy measurements

Each LDL subfraction was transferred to a '300K microsep' micro concentrator (Dasit) and centrifuged at 3000 *g* and 4 °C to a final volume of approx. 50–70 μ l. Then 300 μ l of 10 mM phosphate/150 mM NaCl/3.5 mM EDTA buffer, pH 7.4 ($p^2H = p^1H$ meter reading + 0.4 [14]) was added and the sample concentrated again. This procedure was repeated several times in order to replace 1H_2O completely with 2H_2O . In the final step, the sample was concentrated to a final volume of about 40 μ l. The final apoprotein concentration was approx. 3 % (w/v).

FT-IR spectra

Each LDL subfraction was analysed in duplicate. The concentrated LDL subfractions were placed in a thermostatically controlled Graseby-Specac 20500 cell (Graseby-Specac Ltd, Orpington, Kent, U.K.) fitted with CaF₂ windows, using 6 μ m and 12 μ m tin spacers for samples prepared in 1H_2O and 2H_2O respectively. The cell was maintained at the desired temperature using an external bath circulator (Haake F3); FT-IR spectra were recorded by means of a Perkin-Elmer 1760-x FT-IR spectrometer at 2 cm^{-1} resolution using a normal Beer-Norton apodization function and a deuterated triglycine sulphate detector. Spectral deconvolution was performed using the Perkin-Elmer ENHANCE function which is analogous to the method developed by Kauppinen et al. [15]. Deconvolution parameters were set with the half-bandwidth at 14 cm^{-1} and a resolution enhancement factor of 2.25. Buffer spectra were collected under the same scanning and temperature conditions as sample spectra. An interactive difference routine was used to subtract the spectrum of the buffer from the spectrum of the sample. Correct subtraction of 1H_2O was judged to yield an approximately flat baseline at 1900–1400 cm^{-1} , and subtraction of 2H_2O was adjusted to the removal of the 2H_2O bending absorption near 1220 cm^{-1} [16]. After buffer subtraction, all the absorbance spectra in 2H_2O medium were normalized to the amide I band (amide I' band) area in order to neutralize small differences in protein concentration among the concentrated samples. The band positions, as specified elsewhere or shown in the Figures, are presented rounded to the nearest whole integer.

RESULTS

FT-IR spectra of LDL subfractions and ApoB-100 secondary structure

In this study, we analysed two sets of LDL subfractions (six samples in total) from two healthy volunteers (a and b), which will be referred to throughout the paper as LDL_{1a,2a,3a} and LDL_{1b,2b,3b}.

The IR absorbance spectra of LDL subfractions in 1H_2O (not shown) and 2H_2O (Figure 1) are similar to those of total LDL

Figure 1 Original absorbance spectra (1800–1500 cm^{-1} region) of LDL subfractions at 20 °C in 2H_2O

The spectra were normalized at the amide I' band area. Top and bottom, LDL subfraction spectra of volunteer a and b respectively. Spectra were obtained by subtraction of buffer spectra from sample spectra as described in the Materials and methods section.

reported by other authors [9–11]. In 2H_2O (Figure 1) the area-normalized amide I' bands (1700–1600 cm^{-1}) of LDL_{1a} and LDL_{1b} superimpose completely on the corresponding amide I' band of LDL_{2a} and LDL_{2b} samples respectively. Conversely, the amide I' bands of LDL_{3a} and LDL_{3b} are different from the amide I' bands of the corresponding LDL₁ and LDL₂ subfractions. These findings indicate that in LDL_{3a} and LDL_{3b} the secondary structure of ApoB-100 is different from that in the corresponding LDL₁ and LDL₂ subfractions. Differences between the FT-IR spectra of the different LDL subfractions can also be observed in the 1750–1700 cm^{-1} region where the lipoprotein lipids absorb [9–11].

The amide I' band as well as the lipoprotein lipid band can be resolved into their components by deconvolution techniques [15], allowing a detailed analysis of the ApoB-100 secondary structure. The deconvoluted FT-IR spectra of LDL subfractions in 1H_2O and 2H_2O display the same amide I' components as the corresponding spectra of total LDL shown in previous studies [9–11]. In 1H_2O , six amide I' band components at 1617, 1631, 1653, 1671, 1682 and 1695 cm^{-1} (spectra not shown) were found. In 2H_2O , eight amide I' components at 1604, 1617, 1630, 1643, 1653, 1668, 1680 and 1694 cm^{-1} are present, as shown in Figure 2 for LDL_{1a,2a,3a} and LDL_{1b,2b,3b} subfractions. The deconvoluted spectra shown in Figure 2 were calculated after normalization of the original absorbance spectra at the amide I' band area. The 1630, 1643 and 1653 cm^{-1} bands were assigned to β -sheet, unordered structures and α -helix respectively [9,10]. The 1670 and 1680 cm^{-1} bands were assigned to turns, and the 1617 cm^{-1} band was assigned to 'low-frequency β -sheet' [11] or a β -structure not accessible to the solvent [9]. Recently, the 1617 and 1694 cm^{-1} bands were assigned to β -strands, i.e. extended structures not forming β -sheet [10]. We agree with this interpretation and with the arguments given by the authors for the assignment of the bands [10]. The 1604, 1583, 1565 and 1515 cm^{-1} bands are attributable to amino acid side-chain absorption [17], and the

Figure 2 Deconvoluted FT-IR spectra of LDL subfractions at 20 °C

The three LDL subfractions from each volunteer were named LDL_{1a,2a,3a} (top) and LDL_{1b,2b,3b} (bottom). The deconvoluted spectra were calculated after normalization of the original absorbance spectra to the amide I' band area. The band positions are rounded to the nearest whole integer.

1736 and the 1747 cm⁻¹ bands are related to the C=O stretching vibration of the lipoprotein lipids [9–11]. The higher intensity of the 1747 cm⁻¹ band in LDL_{3a} than in LDL_{1a,2a} probably reflects a different lipid composition in this subfraction.

Comparison of FT-IR spectra of LDL subfractions

The deconvoluted spectra of LDL_{1a} and LDL_{2a} are indistinguishable, indicating no differences in the secondary structure of ApoB-100 in these samples. The same was observed in the LDL_{1b} and LDL_{2b} spectra.

However, small differences in the secondary structure of ApoB-100 are present in each set in LDL subfractions, as previously shown by the original absorbance spectra (Figure 1). In particular, the 1617 cm⁻¹ band intensity is lower in spectra of LDL_{3a} and LDL_{3b} than in spectra of LDL_{1a,2a} and LDL_{1b,2b} subfractions respectively. This finding indicates a reduced β -strand content in ApoB-100 in the smaller denser LDL₃ fraction. Although small, the reduced intensity of the 1617 cm⁻¹ band in the LDL_{3a} and LDL_{3b} samples was reproducible, as revealed by the repetition of the IR measurements.

More evident differences are present in the C=O stretching vibration region: the 1736 cm⁻¹ band intensity is almost the same in LDL_{1b,2b}, but decreases from LDL₁ to LDL₃ in both sets of subfractions, and the 1747 cm⁻¹ band shows a higher intensity in the LDL_{3a} fraction than in LDL_{1a,2a}.

The small differences in the amide I' region of LDL subfraction spectra are better displayed in Figure 3, showing the difference spectra obtained by calculating the difference between the absorption spectra of the different subfractions for each volunteer. These spectra may appear quite complex: the characteristics of the bands appearing in these spectra (e.g. negative and positive peaks, band position, etc.) depend on differences in band position, band shape and band intensity between the original peaks, and they depend also on the characteristics of nearby bands [18]. More simply, the positive 1617.1 and 1617.4 cm (right panel) and 1618 and 1617.8 cm⁻¹ (left panel) bands indicate a higher content of this amide I' component (β -strands) in LDL₁ and LDL₂ than in LDL₃ in both volunteers. The positive bands at 1734.8 and 1735.7 cm⁻¹ (right panel) indicate a higher content

Figure 3 Difference spectra of LDL subfractions at 20 °C

The original absorption spectra were normalized at the amide I' band area, then difference spectra were generated by calculating the difference between the absorption spectra of the indicated LDL subfractions. The difference spectra are displayed in an expanded view in order to show small spectral differences.

Table 1 Spectral parameters and composition of LDL subfractions

All absorbance spectra of LDL subfractions at 20 °C were normalized at the same amide I' band area (100). The 1736 cm⁻¹ band area was calculated by curve fitting [19]. C/P, PL/P, TG/P and TL/P mean cholesterol, phospholipid, triacylglycerol and total lipid to protein weight ratio respectively.

	LDL _{1a}	LDL _{2a}	LDL _{3a}	LDL _{1b}	LDL _{2b}	LDL _{3b}
Amide I' band area	100	100	100	100	100	100
1736 cm ⁻¹ band area	30.27	28.08	27.16	28.55	28.52	27.12
C/P	1.58	1.24	0.74	1.32	1.26	0.54
PL/P	1.48	1.31	1.08	1.1	1.2	1.2
TG/P	0.16	0.12	0.13	0.20	0.18	0.18
TL/P	3.22	2.67	1.95	2.62	2.64	1.92

of lipids in LDL_{1b} and LDL_{2b} than in LDL_{3b}. No marked differences in the lipid or amide I' region can be observed in the LDL_{1b}–LDL_{2b} difference spectrum (right panel), indicating minor or no differences in lipid content and in secondary structure of the LDL_{1b} and LDL_{2b} subfractions. The left panel shows positive peaks at 1734 cm⁻¹, indicating differences in lipid content among the three LDL subfractions. The negative peaks at 1748.2 cm⁻¹ are a consequence of the higher 1747 cm⁻¹ band intensity in the LDL_{3a} spectrum than in LDL_{1a} and LDL_{2a} spectra.

The content of phospholipids, triacylglycerols and total cholesterol (non-esterified and esterified) was determined in each LDL subfraction by analytical methods [12]. In the deconvoluted spectra (Figure 2) the 1736 cm⁻¹ band area, which reflects the amount of total lipids containing the C=O moiety in their molecule, was determined by curve fitting [19]. The estimation of total lipids obtained by the two methods gives similar results. In particular, Table 1 shows the decrease in the lipid content in each set of LDL subfractions, which, in turn, reflects a decrease in LDL particle size.

DISCUSSION

In this FT-IR spectroscopic study we have demonstrated that, among the six samples analysed, small but significant modifications in the secondary structure of ApoB-100 are present only in the small dense LDL_{3a} and LDL_{3b} fractions. The data have also shown that the lipid content as well as the lipid composition may be different within the same set of LDL subfractions or between the LDL subfractions from different subjects. These data reflect the heterogeneity of LDL subfractions that has been observed by others [8] and suggest that many factors could influence the secondary structure of ApoB-100 in LDL subfractions. However, in the same set of LDL subfractions, the analysis of FT-IR data suggests that the secondary structure of ApoB-100 is most likely affected by a particular lipid/protein weight ratio (≈ 1.90 , see Table 1), and thus by the LDL particle size, rather than by the LDL lipid composition. In fact, (i) the content of the component related to the 1617 cm⁻¹ band is almost the same in LDL_{1a,2a} and LDL_{1b,2b}, which show different and the same total lipid contents respectively (Figures 2 and 3, Table 1). Moreover, (ii) the 1617 cm⁻¹ band intensity is lower in LDL_{3a}, which has a different lipid composition and a lower lipid content than LDL_{1a} and LDL_{2a} (Figures 1–3). Also, (iii) the 1617 cm⁻¹ band intensity is lower in LDL_{3b}, which has only a lower total lipid content than LDL_{1b} or LDL_{2b}. Finally, (iv) the 1617 cm⁻¹ band is lower in intensity in both LDL_{3a} and LDL_{3b} than in the corresponding LDL_{1,2} subfractions. From these data,

it appears that when the total lipid content is reduced (i.e. the LDL particle size), as in the LDL_{3a} and LDL_{3b} subfractions, the 1617 cm⁻¹ band appears reduced in intensity. This finding is indeed in accordance with the hypothesis of Teng et al. [20] that a conformational change in ApoB would occur on decreasing the LDL particle size.

The 1617 cm⁻¹ band has been related to β -strands not forming β -sheets, and the amino acids involved in this structure form a filament that has been hypothesized to penetrate the phospholipid layer on the surface of the LDL particle [10]. In LDL₃ subclasses, which are smaller than LDL₁ or LDL₂, the reduced surface area of the particles might not be sufficient for the interaction of phospholipids with the β -strand (belonging to the 1617 cm⁻¹ band) unlike in LDL₁ or LDL₂ subfractions. The lower β -strand content in LDL₃ subclasses could be a consequence of local rearrangement in the β -strand filament, because of the small particle size, that could result in altered exposure of some of the amino acid sequences on the LDL surface. Even though the difference in the content of the 1617 cm⁻¹ band in LDL₃ subclasses with respect to LDL₁ or LDL₂ is not large, it is probably responsible for the modified receptor recognition [8,21] in small LDL and for the different antigenicity observed by Teng et al. [20] and Chen et al. [22] in these subfractions with respect to the less-dense LDLs.

As the particle size seems to be an important factor in the modification of the ApoB-100 secondary structure in the same set of LDL subfractions, it cannot be excluded that other factors, for example lipid-peroxidation products, may influence the ApoB-100 conformation. These products could react with specific amino acids and induce conformational changes and/or different interactions of the polypeptide amino acid side chains with the microenvironment surrounding them. Several lines of evidence suggest that the small more-dense LDL subfractions are more atherogenic than the less-dense LDLs [6,7], and at a molecular level this higher atherogenicity has been related to the high susceptibility to lipid peroxidation of small LDLs. However, in the light of the present data, it is possible that the modification of the ApoB-100 secondary structure induced by the small LDL particle size could also significantly contribute to the high atherogenicity of small more-dense LDL particles.

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