Differences in carbohydrate content of low density lipoproteins associated with low density lipoprotein subclass patterns

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Abstract The neutral carbohydrate content of both the protein (apoB) and lipid fractions of low density lipoproteins (LDL) from subjects with a predominance of small, dense LDL (subclass pattern B) was found to be lower than in subjects with larger LDL (subclass pattern A): 45 ± 12 versus 64 ± 13 mg/g apoLDL, and 58 ± 8 versus 71 ± 8 mg/g apoLDL ($P < 0.0005$ for both). Sialic acid content of LDL lipids, but not apoB, was also reduced in subclass pattern B. ApoB and glycolipid carbohydrate content of total LDL and LDL density subfractions declined with increasing LDL density and decreasing particle diameter. Moreover, in LDL subfractions from pattern B subjects, carbohydrate content of LDL apoB, but not LDL glycolipid, was significantly lower in comparison with particles of similar size from pattern A subjects. Thus, in LDL subclass pattern B, reductions in LDL carbohydrate content are associated both with reduced concentrations of larger carbohydrate-enriched LDL subclasses, and with reduced glycosylation of apoB in all LDL particles. LDL glycolipids may vary with overall lipid content of LDL particles, but variation in apoB glycosylation may indicate differences in pathways for LDL production, and reduced apoB glycosylation may reflect the altered metabolic state responsible for LDL subclass pattern B. —**La Belle, M., and R. M. Krauss.** Differences in carbohydrate content of low density lipoproteins associated with low density lipoprotein subclass patterns. *J. Lipid Res.* 1990. **31:** 1577–1588.

Supplementary key words apoB • sialic acid • glycosylation

Low density lipoproteins (LDL) in humans function as the major carriers of plasma cholesterol and levels of LDL cholesterol in the plasma have been correlated with the risk of heart disease (1, 2). In addition, a positive correlation has been found between coronary heart disease and high levels of plasma triglyceride, very low density lipoproteins (VLDL), and intermediate density lipoproteins (IDL) (3–5), while levels of high density lipoprotein (HDL) cholesterol show an inverse relationship with heart disease (6, 7).

Earlier work has shown heterogeneity of LDL particle size, density, and composition (8–16) and the existence of distinct subclasses of LDL that can be identified by ultracentrifugal and gel electrophoretic techniques (13–16). Analysis of LDL by gradient gel electrophoresis showed that LDL from most subjects falls into one of two distinct LDL subclass patterns, A or B (16, 17). LDL subclass pattern A is characterized by the presence of a major LDL with a large diameter (usually greater than 255 Å) and a secondary LDL peak of smaller diameter, while LDL subclass pattern B has a major LDL peak of smaller diameter, usually less than 255 Å in diameter, and a secondary LDL peak of larger diameter than the major peak (**Fig. 1**). Complex segregation analysis has shown that these LDL subclass patterns are influenced by a common allele at a single genetic locus (18). The pattern B-associated allele has a population frequency of approximately 25% and is dominant, but with reduced penetrance in young males (<20 yr) and premenopausal women (17). Individuals with LDL subclass pattern B have relatively increased levels of triglyceride, VLDL, and IDL and decreased levels of HDL cholesterol (16–18), a profile that would predict an increased risk of atherosclerosis. A subsequent study of LDL subclass patterns versus risk of myocardial infarction found that individuals with LDL subclass pattern B have up to a threefold increase in risk of myocardial infarction (19).

The smaller LDL species that predominate in LDL subclass pattern B have also been shown to have a higher

Abbreviations: LDL, low density lipoprotein; apoB, apolipoprotein B; VLDL, very low density lipoprotein; IDL, intermediate density lipoprotein; HDL, high density lipoprotein; apoLDL, LDL protein; FH, familial hypercholesterolemia; kD, kilodalton; SDS, sodium dodecyl sulfate; WGA, wheat germ agglutinin; VNTR, variable number of terminal repeats; ER, endoplasmic reticulum; LCP, lipoprotein-complexing proteoglycan; BSA, bovine serum albumin.

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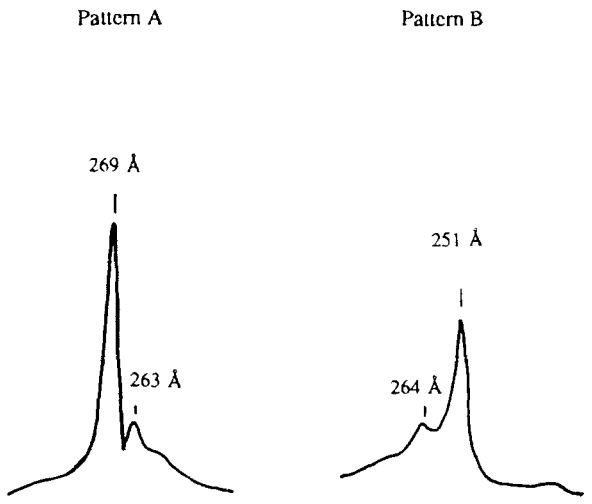

Fig. 1. Densitometric scans of plasma low density lipoprotein particles electrophoresed under nondenaturing conditions on 2% to 16% gradient gel and stained with Oil Red O. Left: scan of LDL subclass pattern A, which is characterized by a relatively large major LDL peak (269 Å) and a smaller diameter (263 Å) secondary peak. Right: scan of LDL subclass pattern B, characterized by a relatively small major LDL peak (251 Å) and a larger diameter (264 Å) secondary LDL peak.

buoyant density and relatively lower lipid:protein ratio than the larger LDL that predominate in subclass pattern A (15). The question arises as to whether there are other structural differences between the LDL found in subclass pattern A and B that may relate to the genetic basis of the LDL subclass patterns and that might contribute to differences in coronary disease risk associated with these patterns. Low density lipoproteins are complex macromolecules consisting of a core of esterified cholesterol (with a small amount of triglyceride) surrounded by a layer of more polar lipids (free cholesterol and phospholipids) and a single large (≈550 kD) glycoprotein, apolipoprotein B (apoB). The amount of carbohydrate present on apoB has been reported to range from 4% to 10% (40 to 100 mg/g apoB) by weight (20–28). At least 50% of the carbohydrate on apoB is present in the form of either of two types of N-linked carbohydrate chains: a high-mannose oligosaccharide containing six mannose and two N-acetylglucosamine residues and a complex-type oligosaccharide containing two sialic acid, two galactose, five mannose, and three N-acetylglucosamine residues (22). In addition, other investigators report the presence of high-mannose N-linked oligosaccharides composed of six to nine mannose residues and one N-acetylglucosamine residue (27). The function of the carbohydrate moiety of apoB in VLDL and LDL is unknown.

In this study we report on differences in the carbohydrate content of the LDL isolated from subjects with LDL subclass pattern A or B. The results demonstrate that LDL from subjects with subclass pattern B have significantly less neutral carbohydrate and sialic acid than LDL from subjects with subclass pattern A and that this difference is due to reduced carbohydrate in both the lipid and protein fractions of the pattern B LDL particles. In addition, there is a linear relationship between LDL carbohydrate content and size of the major LDL peak.

MATERIALS AND METHODS

Materials

BSA, resorcinol, amyl alcohol, copper sulfate, D-mannose, sialic acid, and neuraminidase (Type X) were from Sigma. Precast 2–16% polyacrylamide gradient gels and high molecular weight standards (BSA, lactate dehydrogenase, apoferritin, and thyroglobulin) were from Pharmacia (Piscataway, NJ). Latex beads (0.038 μm diameter) were from Duke Scientific Corp. (Palo Alto, CA).

Subjects

Plasma samples were obtained after overnight fast from volunteer subjects (42 men and 23 women) with plasma cholesterol levels < 300 mg/dl and triglyceride levels < 500 mg/dl (**Table 1**). Two subjects were taking estrogen, one was on tolbutamide and furosemide and one was taking prazosin. None of the other subjects were using drugs known to affect lipid metabolism. Additional studies were carried out in a 31-year-old female with heterozygous familial hypercholesterolemia (total cholesterol 366 mg/dl, HDL cholesterol 70 mg/dl, LDL cholesterol 275 mg/dl, and plasma triglycerides 107 mg/dl).

Methods

Plasma was brought to 0.15% EDTA and LDL (d 1.019–1.063 g/ml) was isolated from the plasma by sequential centrifugation as previously described (29, 30). Subfractions of LDL were prepared by discontinuous density gradient ultracentrifugation in an SW 45 rotor (40,000 rpm for 40 h at 17°C) as previously described (15), except that 11 rather than 7 fractions were collected as follows: the top 0.5 ml, then 1 ml, followed by six 0.5 ml fractions, two 1 ml fractions, and the bottom 0.5 ml. Fraction density was determined based on previous measurements using this centrifugation technique (15). Whole LDL and LDL subfractions were then dialyzed over 24 h against two changes of 20 volumes of 20 mM Tris-HCl (pH 7.5), 1 mM Na_2EDTA, and stored at 4°C. The protein concentration of the dialyzed LDL was determined by a modification of the method of Lowry et al. (31) using BSA as the standard. Measurements of plasma levels of total cholesterol, triglyceride, LDL cholesterol, and HDL cholesterol were performed as previously described (17).

Lipoprotein particle diameter was determined by electrophoresis of 1 μg of LDL protein on nondenaturing 2–16% polyacrylamide gradient slab gels as previously described (32). The gels were stained with Oil Red O to

TABLE 1. Comparison of age, sex, B.M.I. (body mass index), plasma triglyceride levels, cholesterol levels, and diameter of the main LDL of subjects in LDL subclass pattern A versus subjects in LDL subclass pattern B

Pattern	Age	Sex	BMI[a]	Triglycerides	Cholesterol			Particle Diameter
					Total	HDL	LDL	
			kg/cm²	*mg/dl*		*mg/dl*		*Å*
A	21	M	24.8	97	136	46	71	263
n = 31	22	M	23.6	66	155	52	90	258
	26	M	22.0	85	174	69	88	264
	35	M	25.6	120	220	37	159	267
	37	M	20.6	50	139	61	68	263
	37	M	23.0	76	201	63	123	265
	39	M	21.3	95	206	51	136	265
	43	M	22.2	62	188	36	140	267
	45	M	22.0	47	137	47	81	275
	47	M	22.2	51	135	39	86	257
	47	M	24.0	137	194	41	126	261
	51	M	24.4	80	153	70	67	264
	57	M	20.1	132	237	61	150	262
	64	M	27.9	99	176	61	95	267
	72	M	25.4	86	183	48	118	266
	20	F	20.7	93	145	61	65	276
	24	F	20.7	47	186	72	105	275
	26	F	21.8	29	190	72	112	274
	28	F	19.0	50	184	77	97	270
	29	F	25.7	75	166	69	82	272
	29	F	22.5	87	196	77	102	265
	30	F	21.3	37	198	95	96	271
	34	F	20.4	70	248	86	148	272
	35	F	19.8	53	154	66	77	272
	35	F	20.6	92	199	69	112	262
	40	F	25.6	95	174	72	83	272
	42	F	23.3	77	235	93	127	276
	43	F	21.1	78	211	64	131	278
	44	F	21.9	76	150	36	99	262
	55	F	29.1	65	207	60	134	272
	68	F	20.0	145	253	78	146	261
Average	40 ± 14		22.7 ± 2.4	79 ± 28	185 ± 33	62 ± 16	107 ± 27	267 ± 6
B	31	M	28.4	210	217	37	138	247
n = 34	35	M	23.6	151	221	31	160	251
	35	M	23.7	179	193	29	128	248
	36	M	23.7	99	241	39	182	250
	36	M	22.0	88	146	28	100	252
	37	M	25.1	74	180	47	118	259
	39	M	21.6	170	198	44	120	248
	40	M	35.9	223	175	24	106	245
	41	M	21.0	286	222	27	138	245
	42	M	26.3	216	251	27	181	248
	43	M	27	396	200	37	84	246
	44	M	37.4	133	211	34	150	252
	44	M	27.6	275	288	36	197	246
	46	M	29.6	212	205	34	129	245
	46	M	23.7	263	199	35	111	242
	49	M	24.3	237	197	32	118	244
	49	M	22.7	316	294	33	198	248
	50	M	24.3	269	225	32	139	249
	51	M	27.3	104	190	49	120	259
	54	M	26.4	128	219	38	155	246
	54	M	28.3	365	190	39	78	262
	55	M	23.0	222	220	34	142	245
	56	M	23.8	187	243	52	154	249
	57	M	29.3	185	201	31	133	248
	60	M	29.6	251	173	31	92	254
	61	M	28.2	124	222	44	153	260
	68	M	32.2	217	156	22	91	242
	23	F	20.9	129	216	39	151	249
	51	F	27.3	107	261	57	183	249
	65	F	25.2	161	241	37	172	259
	67	F	25.7	180	257	41	180	258
	72	F	25.6	145	258	37	192	250
	82	F	20.7	120	240	63	153	262
	84	F	22.1	436	258	35	136	243
Average	50 ± 14		26.0 ± 3.9	202 ± 88	217 ± 34	37 ± 9	141 ± 33	250 ± 6
Significant at $P <$	0.01		0.0005	0.0005	0.0005	0.0005	0.0005	0.0005

[a]Body mass index was calculated as weight (kg) ÷ (height (cm) ÷ 100)²

identify lipoproteins and with Coomassie Brilliant Blue R-250 to identify the protein standards. Latex beads and Pharmacia high molecular weight standards were used as references to determine particle diameter. LDL particle sizes were determined from stained gels using a Transidyne RFT scanning densitometer (Transidyne Corp., Ann Arbor, MI). Classification of LDL subclass patterns as pattern A or pattern B was determined by two independent observers using previously reported criteria (17–19): pattern B with predominant LDL peak < 256 Å and/or skewing of the LDL peak profile to the left, and pattern A with predominant LDL peak ≥ 256 Å and/or skewing of the LDL peak profile to the right.

Quantitative analysis of the neutral sugar content of both LDL and the lipid extracted from the LDL was by a scaled-down (33) version of the phenol–sulfuric acid assay (34) using mannose as the standard. To determine lipid carbohydrate content, lipid was extracted from 0.1 mg of LDL protein using two extractions with chloroform–methanol 6:5 (v/v), as previously described (35). The two extractions were combined and dried under a stream of N_2. The dried lipids were then partially resuspended in 490 μl of sterile double-distilled H_2O with complete resuspension occurring with the addition of phenol for the carbohydrate assay. Absorbance for both the carbohydrate and sialic acid assays was determined using a Beckman DU-2 spectrophotometer. Each sample (LDL and extracted lipid) was assayed in triplicate (0.1 mg LDL protein/assay) and the results were expressed as the average mg carbohydrate/g LDL protein (apoLDL). In the case of the extracted lipid the results were calculated using the amount of protein in the original LDL sample from which the lipid was extracted. The carbohydrate content of the protein moiety of LDL (apoLDL) was determined by subtracting the lipid carbohydrate from the total LDL carbohydrate values.

Since the phenol–sulfuric acid assay detects only neutral sugars, sialic acid content of the LDL particles (2.5 mg LDL protein per assay) was determined using the resorcinol method (36). Results presented are the average values of duplicate assays. The absorbance in each assay was corrected by subtraction of the average absorbance of duplicate assays on LDL samples in which sialic acid residues had been hydrolyzed by incubating samples for 2.5 h at 100°C in the presence of 2 N HCl. Calculation of the molar ratio of sialic acid to LDL was done assuming a molecular mass of 514,000 daltons for apoB (37) and 309.3 daltons for sialic acid and that the molar ratio of apoB/LDL particle is one. Sialic acid content of LDL apoprotein was determined in subgroups of 12 subjects each with LDL subclass patterns A and B by the method of Warren (38) after extracting LDL lipid (as described above) and releasing sialic acid by mild (1 h at 80°C in 0.05 M H_2SO_4) acid hydrolysis of the lipid extracted LDL (39). This hydrolysis is sufficient to release bound sialic acid without destroying it (37, 38).

Removal of sialic acid residues from LDL carbohydrate was accomplished by dialyzing aliquots (0.1 ml) of LDL overnight at 4°C versus 80 ml of 40 mM sodium acetate buffer (pH 5.0), determining the protein concentration of the dialyzed samples as described above, and then adding 50 mU of neuraminidase (in dialysis buffer) per 0.1 mg of LDL protein. Untreated control tubes had an equal volume of dialysis buffer added. Samples were incubated for 24 h at room temperature before electrophoresis on agarose gels (Paragon, Beckman Instruments, Brea, CA). After electrophoresis LDL was stained with Fat Red 7B according to the Paragon operating manual.

Lectin staining of electrophoretically separated LDL proteins was carried out using a pooled LDL sample from 12 subjects with LDL subclass pattern A and a second LDL pool from 12 subjects with LDL subclass pattern B (40 μg of protein from each subject). The two samples were denatured by incubation at 100°C for 15 min in 1.15 volumes of denaturing buffer (26 mM Tris-HCl (pH 6.8), 3.9 mM Na_2EDTA, 10 M urea, 2.8% (w/v) SDS, 2.8% (v/v) 2-mercaptoethanol, 10% (w/v) sucrose, and 0.04% bromophenol blue). Samples (2.5 μg protein per lane) from the two pools of denatured LDL were electrophoresed on separate lanes of a SDS-glycerol polyacrylamide mini-slab gel (3.5% acrylamide) by the method of Maguire, Lee, and Connelly (40). This technique resolves apolipoproteins with molecular weights ranging from 8,000 to greater than 550,000 (40). One lane of the gel was used for molecular weight standards (phosphorylase (b), mol wt 92.5 kD; BSA, 68 kD; ovalbumin, 46 kD; carbonic anhydrase, 30 kD; trypsin inhibitor, 21.5 kD; lysozyme, 14.3 kD). The proteins were then transferred to nitrocellulose sheets (Genzyme Corporation, Boston, MA; 0.45 μm) in a buffer consisting of 25 mM Tris, 192 mM glycine, pH 8.8. A Bio-Rad Trans-Blot cell was used for the transfer, with a current of 210 mA for 2.5 h at room temperature. To check that the transfer was complete, gels were stained for protein with Coomassie Brilliant Blue R-250 (40). After transfer, the nitrocellulose sheets were cut in half and one half, containing the transferred molecular weight standards and the pooled apolipoproteins, was stained for protein with amido black (41), while the other half was probed for glycoproteins using a biotinylated lectin (wheat germ agglutinin, WGA) from a glycoprotein detection kit (Lectin-Link kit, Genzyme Corporation, Boston, MA). The bound lectins were stained using a complex of avidin and biotinylated alkaline phosphatase according to the manufacturers instructions. WGA specifically binds to N-acetyl-D-glucosamine and sialic acid (42).

All statistical procedures including comparisons of means by Student's *t*-test, single and multiple linear regression analysis, and analysis of variance were performed using the Statview II statistical package (Abacus Concepts, Berkeley, CA). All means are presented with standard deviations.

RESULTS

Table 1 presents the clinical characteristics of the study subjects, their plasma lipid levels and the diameters of their predominant LDL peak as determined by nondenaturing gradient gel electrophoresis. As a group, the subjects with LDL subclass pattern B were older and had greater body mass than the subjects with pattern A. Triglyceride, total cholesterol, and LDL cholesterol were all significantly greater ($P<0.0005$) and HDL cholesterol levels were significantly lower ($P<0.0005$) in the LDL subclass pattern B group than in the pattern A group. These differences, as well as differences in particle size of the predominant LDL species, are similar to those reported previously (17, 19).

Determination of the neutral carbohydrate content of isolated LDL samples (**Table 2**) revealed significantly lower levels of total LDL carbohydrate in pattern B than in pattern A ($P<0.0005$). This difference was due to significantly lower levels of carbohydrate in both the glycolipid ($P<0.0005$) and apoprotein ($P<0.0005$) components of LDL in pattern B subjects.

Table 2 also shows that sialic acid content of total LDL was significantly lower in subjects with LDL subclass pattern B than in subjects with pattern A, by approximately one mole per mole of LDL. However, sialic acid content of apoLDL, determined in subgroups of 12 subjects each with LDL subclass patterns A and B, was similar in the two groups: 3.2 ± 0.7 versus 3.3 ± 0.8 mol/mol apoLDL. Thus, the difference in total LDL sialic content between LDL subclass patterns A and B is due to the lower glycolipid content of LDL in pattern B subjects. Comparison of LDL electrophoretic mobility on agarose before and after exposure to neuraminidase in 10 pattern A and 9 pattern B subjects showed a significantly greater reduction in pattern A versus pattern B mobility (54 ± 4% vs 45 ± 4%, $P<0.01$), consistent with the presence of less sialic acid in glycolipids from pattern B LDL.

TABLE 2. Comparison between subjects with LDL subclass pattern A and those with LDL subclass pattern B, of the average values for total carbohydrate content of the LDL, carbohydrate content of the LDL glycolipid, the carbohydrate content of the apoLDL fraction, and the molar ratio of sialic acid to LDL

Parameter	Pattern A	Pattern B
Total LDL carbohydrate[a] (mg/g apoLDL)	135 ± 16	103 ± 16
Glycolipid carbohydrate[a] (mg/g apoLDL)	71 ± 8	58 ± 8
Apoprotein carbohydrate[a] (mg/g apoLDL)	64 ± 13	45 ± 12
Sialic acid[a] (mol/mol LDL)	52 ± 10	39 ± 8

[a]Differences significant at $P < 0.0005$.

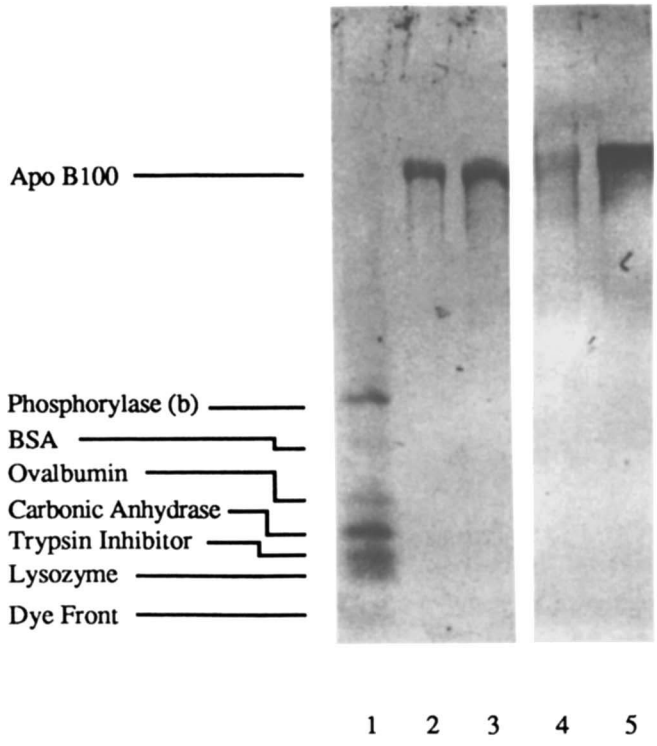

Fig. 2. Western blot of denatured pooled LDL from LDL in subclass pattern A or subclass pattern B. Lanes 1, 2, and 3 of the Western transfer were stained for protein with amido black while lanes 4 and 5 were probed for the presence of glycoproteins by incubation with the biotinylated lectin wheat germ agglutinin. Lane 1: molecular weight standards; lanes 2 and 4 have samples from the pooled subclass pattern B LDL while lanes 3 and 5 show the results for samples from pooled subclass pattern A LDL.

To determine whether the increased carbohydrate content of apoLDL in subclass pattern A was due to the presence of glycolipoproteins other than apoB-100, such as apoE (43), apoD (44), or apoC-III (45), nitrocellulose blots of electrophoretically separated apoLDL samples pooled from 12 subjects each with LDL subclass pattern A and B were stained for protein or probed for glycoprotein with the biotinylated lectin WGA (**Fig. 2**). In both samples, only a single apoB-100 band was visualized by each procedure. Since WGA binds specifically to N-acetyl-D-glucosamine and sialic acid (42), and N-acetyl-D-glucosamine is part of the core structure of both high mannose and complex N-linked carbohydrate chains, this result argues against the presence of significant amounts of glycoproteins other than apoB-100 in the LDL preparations.

Individual values for LDL carbohydrate measurements are shown in **Fig. 3** in relation to the particle diameter of the predominant LDL subspecies on gradient gel electrophoresis. Significant positive correlations with peak LDL diameter were observed for total LDL carbohydrate, LDL glycolipid carbohydrate, and apoLDL carbohydrate (Fig. 3A–C), consistent with the mean differences be-

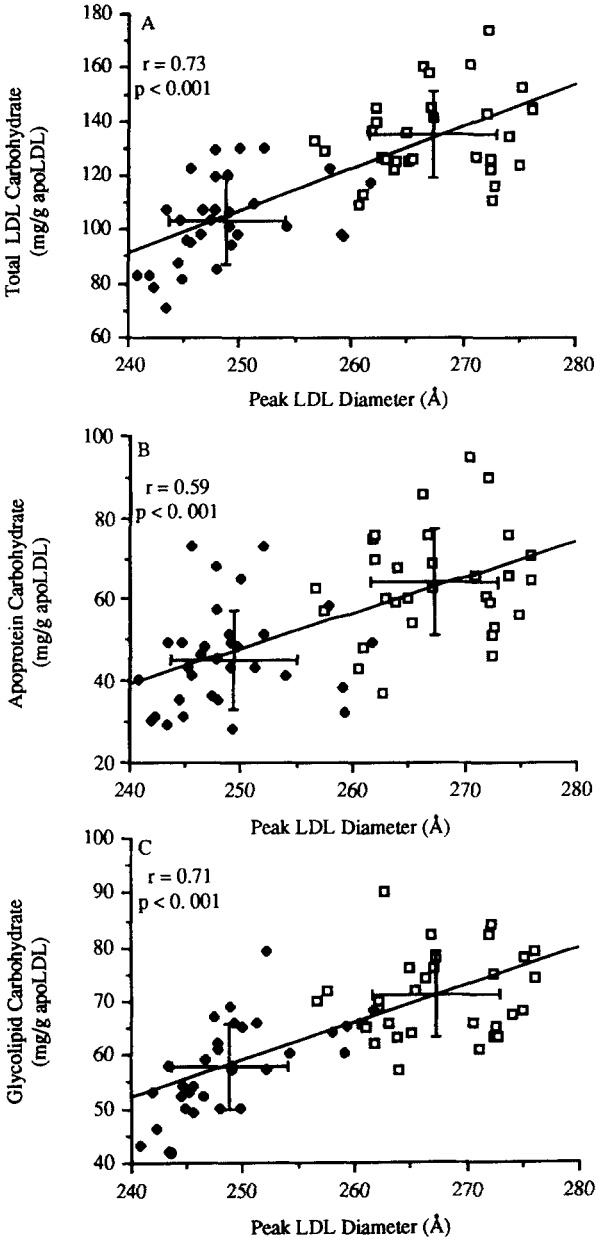

Fig. 3. Plot of the diameter of the major LDL, as determined by gradient gel electrophoresis, versus the amount of carbohydrate present in (A) LDL, (B) LDL protein, and (C) the LDL lipid fraction. In each graph both the coefficient of correlation (r), as calculated by linear regression analysis, and the P value, the significance level of the correlation coefficient, are given for each line. Carbohydrate content for LDL, LDL protein, and LDL lipid was determined using the phenol-sulfuric acid method as described in Methods. LDL that is in LDL subclass pattern A is identified in the graphs with a (□) symbol while LDL in LDL subclass pattern B is identified with a (♦) symbol. The center and length of each of the two double error bars in each graph show the average and standard deviation, respectively, for the values for the two LDL subclass patterns.

tween subjects with predominantly smaller and larger LDL. Correlations of carbohydrate content with peak LDL particle diameter within the phenotypes were not statistically significant.

In a subject with familial hypercholesterolemia, the peak LDL particle diameter was 274 Å (LDL subclass pattern A) and LDL carbohydrate content was 170 mg/g apoLDL (105 mg/g apoLDL in protein and 65 mg/g apoLDL in lipids). In addition, sialic acid content was 4.9 ± 0.3 mol/mol LDL. These findings were all consistent with those of normolipidemic subjects with LDL subclass pattern A.

In view of the differences in plasma lipid levels as well as mean age and body mass index between subjects with differing LDL subclass patterns, relationships were sought between these variables and carbohydrate and sialic acid content of isolated LDL. There were significant inverse correlations (r) of carbohydrate measurements with levels of plasma triglyceride: −0.71 for glycolipid carbohydrate, −0.57 for apoprotein carbohydrate, and −0.58 for total sialic acid (all $P<0.001$). Stepwise multiple logistic regression analysis showed that LDL particle diameter was a significant predictor of LDL total carbohydrate, apoprotein carbohydrate, and glycolipid carbohydrate content. Plasma triglyceride level contributed independently but less strongly to LDL total carbohydrate and glycolipid carbohydrate content, as well as to total sialic acid content. Inclusion of HDL cholesterol, LDL cholesterol, body mass index and age in the regression models did not change these results. **Fig. 4** shows the relationships of plasma triglyceride concentration to LDL content of total carbohydrate (Fig. 4A), apoprotein carbohydrate (Fig. 4B), and glycolipid carbohydrate (Fig. 4C).

HDL cholesterol concentration was positively correlated with glycolipid carbohydrate (r = 0.39) and with glycoprotein carbohydrate (r = 0.49, $P<0.01$ and $P<0.001$, respectively) and less strongly with sialic acid (r = 0.31, $P<0.05$). LDL cholesterol level showed a weak inverse correlation with LDL sialic acid content (r = −0.32, $P<0.05$), but not significantly with other carbohydrate parameters. Body mass index was inversely correlated with LDL apoprotein carbohydrate (r = −0.39, $P<0.01$), and age was weakly correlated with glycolipid carbohydrate (r = −0.28, $P<0.05$).

Table 3 presents apoprotein and glycolipid carbohydrate content of 11 LDL density subfractions isolated from six subjects each with LDL patterns A and B. Also shown are the buoyant densities and protein content of each fraction, as well as mean particle diameters determined by gradient gel electrophoresis. The distribution of protein mass across the density fractions differed as expected between the two groups of subjects, with a relative increased mass of more buoyant and larger LDL in fractions 3 and 4 in subjects with pattern A ($P<0.01$ and $P<0.05$, respectively, by analysis of variance), and increased mass of more dense and smaller LDL in fraction 7 in subjects with pattern B ($P<0.05$). Both LDL particle diameter and LDL glycolipid carbohydrate declined significantly with increasing density of the LDL fractions,

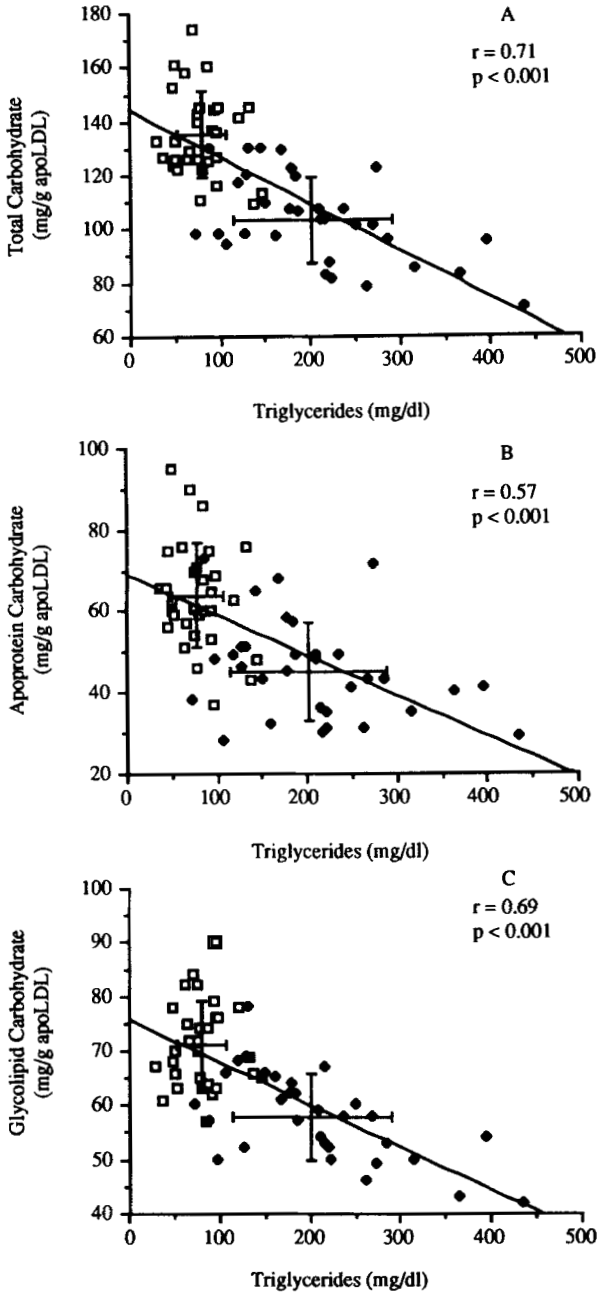

Fig. 4. Plot of the level of plasma triglycerides in subjects with LDL subclass pattern A (□) and LDL subclass pattern B (♦) versus the carbohydrate content of (A) LDL, (B) LDL protein, and (C) the lipid fraction of the LDL. The values for the average and standard deviation for the two LDL subclass patterns are shown by the center and length of each of the two double error bars in each graph. The coefficient of correlation (r), as calculated by linear regression analysis, and the P value, the significance level of the correlation coefficient, are given for each line in the graphs.

but analysis of variance showed no significant differences in these variables between pattern A and pattern B subjects. In contrast, analysis of variance revealed significant differences of apoLDL carbohydrate content both among the LDL subfractions ($P<0.0001$) and between subjects with LDL subclass patterns A and B ($P<0.0001$), with no significant interaction between these parameters.

The results in Table 3 may also be compared with those shown in Table 2 and Fig. 2 for apoprotein and glycolipid carbohydrate content of unfractionated LDL from subjects with LDL subclass patterns A and B. For subjects with pattern A, the peak particle diameter (267 ± 6 Å, Table 1) and glycolipid and apoprotein carbohydrate content (Table 2) of unfractionated LDL are consistent with the size and carbohydrate content of LDL particles isolated in density gradient fractions 3–6, which contain the bulk of LDL mass in pattern A subjects (Table 3). On the other hand, for subjects with pattern B, peak LDL diameter (250 ± 6, Table 1) and LDL protein and lipid carbohydrate content (Table 2) of unfractionated LDL are comparable to the values for LDL particles in density fractions 7–9 (Table 3).

DISCUSSION

Previous studies using density gradient ultracentrifugation and nondenaturing gradient gel electrophoresis (13–16) have established the existence of multiple distinct subclasses of plasma LDL. Differences in lipid content (15, 46–48), apolipoprotein content (49, 50), and apoB immunoreactivity (32) have been reported across the LDL particle spectrum. The present study provides evidence that LDL carbohydrate content also differs as a function of LDL diameter, since variation in total LDL carbohydrate content among individuals is proportional to LDL particle size as determined by nondenaturing gradient gel electrophoresis. Approximately one-half of the total carbohydrate is present as glycolipids, and the variation in glycolipid carbohydrate appears to be directly proportional to the total lipid content of LDL. Thus, it is possible that LDL glycolipid content may depend on metabolic events, such as lipolytic and transfer activities, that affect other LDL lipids. Consistent with this is the observation that plasma triglyceride level, which is related to relative LDL lipid content (49), was significantly associated with LDL glycolipid content.

On the other hand, carbohydrate content of apoB also varies over a wide range (approximately 30–100 mg/g apoLDL), and the relationship of apoLDL (apoB-100) carbohydrate to peak LDL particle diameter in both unfractionated LDL and LDL density subfractions suggests that the previously reported differences in apoB carbohydrate in the range of 40–100 mg/g apoLDL (20–28) may be related in part to factors responsible for predominance of LDL subclasses of varying size.

The relationship of apoB carbohydrate content to LDL particle size contributes to differences in apoB carbohydrate between individuals with a predominant peak of larger LDL subclasses (pattern A) and a major peak of

TABLE 3. Comparison, between six subjects with LDL subclass pattern A and six with LDL subclass pattern B, of the LDL particle size and the carbohydrate content of the glycolipid and protein components of the LDL in each of the LDL subfractions

			Total Protein		Mean LDL Diameter		Carbohydrate: Protrein		Carbohydrate: Lipid	
Fraction	Volume	Density	A	B	A	B	A	B	A	B
	ml	*g/ml*	%		*Å*			*mg/g apoLDL*		
1	0.5	1.024	1.8 ± 1.4	3.6 ± 1.5	288.4 ± 4.4	288.7 ± 9.1	58 ± 26	62 ± 19	115 ± 21	90 ± 13
2	1.0	1.027	3.8 ± 2.1	4.1 ± 1.3	277.3 ± 3.5	276.1 ± 6.8	79 ± 8	67 ± 16	91 ± 16	87 ± 14
3	0.5	1.030	12.3 ± 2.9	6.6 ± 2.3	271.8 ± 2.4	270.5 ± 5.6	83 ± 13	64 ± 18	77 ± 9	82 ± 15
4	0.5	1.032	18.3 ± 5.0	9.6 ± 5.1	268.1 ± 2.8	266.2 ± 3.9	79 ± 14	53 ± 17	74 ± 7	73 ± 12
5	0.5	1.034	18.1 ± 5.6	14.0 ± 7.9	264.2 ± 2.9	262.7 ± 2.8	74 ± 14	52 ± 17	67 ± 9	71 ± 15
6	0.5	1.037	14.9 ± 3.5	15.0 ± 4.5	262.4 ± 3.5	260.1 ± 2.1	65 ± 13	47 ± 13	62 ± 10	66 ± 7
7	0.5	1.040	10.4 ± 2.8	14.0 ± 2.5	256.9 ± 1.8	253.7 ± 3.8	57 ± 8	43 ± 12	58 ± 7	59 ± 12
8	0.5	1.043	7.8 ± 3.8	12.9 ± 5.2	252.2 ± 3.5	250.9 ± 4.8	47 ± 9	40 ± 9	56 ± 10	57 ± 11
9	1.0	1.049	4.2 ± 1.9	6.5 ± 4.1	248.6 ± 2.9	248.0 ± 5.4	38 ± 12	28 ± 15	51 ± 9	56 ± 11
10	1.0	1.060	1.6 ± 0.8	2.4 ± 1.6	243.3 ± 3.5	241.5 ± 1.6	20 ± 17	19 ± 8	51 ± 20	48 ± 6
11	0.5	1.069	1.2 ± 0.5	0.7 ± 0.6	239.8 ± 0.4	239.3 ± 3.9	12 ± 9	2 ± 2	40 ± 20	30 ± 3

smaller LDL (pattern B). Previous studies have shown that these two subclass patterns appear to be under the influence of a dominant gene with population frequency of approximately 0.25 and full penetrance in men above age 20 and in women after menopause (17, 18). The present findings suggest that differences in glycosylation of apoB, and possibly in glycosylation of LDL lipids, may be an important feature distinguishing the LDL of individuals with these two lipoprotein subclass phenotypes, and conceivably could relate to the genetic determinant of these phenotypes.

Reduced carbohydrate content of LDL apoB in subjects with LDL subclass pattern B is not due simply to the predominance of smaller, denser, carbohydrate-depleted LDL subspecies. LDL particles isolated across the entire size and density range from pattern B subjects showed reduced apoB carbohydrate content (but no differences in glycolipid carbohydrate content) in comparison with particles of comparable size and density from pattern A subjects. Thus, in subjects with LDL subclass pattern B, reductions in LDL apoB and glycolipid carbohydrate content are associated both with reduced concentrations of more buoyant, carbohydrate-enriched LDL subspecies, and with reduced glycosylation of apoB in all LDL particles.

Differences in apoB carbohydrate content between LDL particles of differing size and density and between the two LDL subclass patterns could originate with intrahepatic glycosylation of LDL precursors, or could result from intravascular processing of lipoprotein particles. Intravascular modification of LDL glycoprotein or glycolipid could involve differential action of plasma glycosidases. Plasma is known to contain a number of lysosomally derived glycosidases (51–58) including α- and β-mannosidases and a neuraminidase activity. In subjects with LDL subclass pattern B, there could be high activity of one or more of these enzymes, or possibly greater exposure to enzyme activity due to prolonged LDL plasma residence time. However, with the exception of neuraminidase (pH optimum of 5.5), these acid glycosidases have pH optima below pH 5.0 and, based on their pH activity curves (54, 56–58), should not be active at plasma pH of 7.4. In addition, kinetic studies in humans with primary hypertriglyceridemia (59) have found that relatively dense LDL with reduced cholesterol content typical of the smaller LDL species which predominate in LDL subclass pattern B (15) had a higher turnover rate, and thus a shorter plasma residence time than did less dense LDL. To further test for a possible role of plasma residence time, we isolated LDL from a subject heterozygous for familial hypercholesterolemia, a condition resulting from defective or absent LDL receptors and associated with reduced LDL clearance rate (60). If prolonged plasma residence time were responsible for reduced LDL carbohydrate con-

tent, LDL carbohydrate would be expected to be reduced in such subjects. However, the hypercholesterolemic subject was found to have a predominance of large LDL particles and the high levels of carbohydrate characteristic of pattern A LDL. Although this observation will require confirmation in larger numbers of subjects, it is consistent with the findings described above which suggest that plasma glycoside activities are not responsible for observed differences in LDL carbohydrate content between pattern A and B LDL.

There are several possible mechanisms by which carbohydrate content of LDL precursors could be affected prior to secretion. Conceivably, the glycolipid and glycoprotein differences between pattern A and B LDL could be due to a variation in the apoB gene. Restriction fragment length polymorphisms (RFLPs) in the apoB gene have been associated with altered triglyceride, cholesterol, and apoB levels (61–65) and several of these RFLPs have also been found to be associated with increased risk of coronary heart disease (65, 66). Examination of the nucleic acid sequence of apoB (37) shows that there are 19 potential sites for N-glycosidic linkage (67) and at least 16 of these sites are reported to be glycosylated (68). Genetic differences in apoB structure might affect the number of sites available for glycosylation or the transport of apoB through the Golgi apparatus with resulting differences in the degree of glycosylation of both protein and lipids. Recently we have used analysis of the 3' hypervariable region of the apoB gene (69) in six families to exclude linkage between the apoB gene and LDL subclass patterns (70). Thus variation in the apoB gene does not appear to be the basis for differences in LDL between subjects with LDL subclass patterns A and B.

There are several steps in the post-translation processing of the apoB where differences in carbohydrate could be introduced. Although glycosylation begins in the rough ER during protein synthesis, the majority of carbohydrate processing occurs after transfer to the Golgi apparatus where further processing of the oligosaccharide chains continues as the nascent glycoprotein is transported from the *cis* Golgi through the medial and *trans* Golgi. Since subclass pattern B LDL have less neutral carbohydrate than pattern A LDL, there may be a difference in transport to the *trans* Golgi or in oligosaccharide processing of complex chains in the *trans* Golgi. As a result there may be fewer and/or less complete carbohydrate chains present in LDL from subjects with the LDL subclass pattern B phenotype. Possibly such differences in Golgi processing could be associated with other lipid abnormalities found in subjects with LDL subclass pattern B (17, 19). Investigation of these possibilities will require isolation and sequencing of carbohydrate chains from both the pattern A and pattern B LDL subclasses, and further information regarding mechanisms involved in hepatic apoB glycosylation.

Another mechanism that could contribute to the differences in LDL carbohydrate between subjects with the two LDL subclass patterns is differential plasma clearance of carbohydrate-rich LDL. In this regard, it is interesting to note that clearance of many plasma glycoproteins involves a system of receptors that recognize desialylated (i.e., mannose, N-acetylglucosamine, or L-fucose terminated) glycoproteins (71–74). However, it is unlikely that such a system accounts for the differences we find since it has been reported that there are no differences in catabolism of native and desialylated LDL in the pig (25) or binding to skin fibroblasts (75). Further, treatment of LDL with a mixture of glycosidases, which removed at least 80% of the carbohydrate on apoB, did not affect LDL binding and uptake by human fibroblasts (26). However, at present we cannot exclude the possiblity that there is selective removal of LDL or LDL precursors based on differences in carbohydrates other than sialic acid, such as the absolute number or relative content of high mannose and complex chains. Either possibility could result in differential clearance rates such as those reported for rat liver glycoproteins (76); those with high mannose chains were cleared much more rapidly than unglycosylated glycoproteins which, in turn, were cleared faster than glycoproteins with the complex type carbohydrate chains.

Differences in clearance or catabolism of LDL with differing carbohydrate content could not, however, account for the presence of differing amounts of apoB glycosylation among LDL particles in individual subjects. Given the evidence reviewed above that these differences are likely to arise from variation in intrahepatic processing of LDL precursors prior to secretion, the present observation suggests that some or all of the multiple discrete subclasses of LDL described in previous reports arise in parallel from different hepatic precursors, rather than sequentially from intravascular metabolism of a common precursor. Such a conclusion is compatible with previous evidence for the presence of multiple discrete VLDL and IDL subclasses in human plasma (77), which in turn give rise to different LDL products with in vitro lipolysis (78) and intravascular metabolism in rats (79). Studies are currently in progress to evaluate the possiblity that differential glycosylation of apoB among these precursors corresponds to differences in apoB glycosylation among their LDL products.

An important question raised by this study is what role, if any, differences in LDL carbohydrate content may play in the increased risk of heart disease present in individuals with LDL subclass pattern B (19) and a predominance of small, dense LDL (26, 50). Direct interaction of LDL with components of artery walls is thought to play an important role in the development of atherosclerosis. Immunochemical methods have shown that apoB is present in atherosclerotic lesions (80) and LDL particles, in the form of insoluble proteoglycan–lipoprotein complexes,

have also been found to be present preferentially in these lesions (80–82). A negatively charged lipoprotein-complexing proteoglycan (LCP) has been isolated from human artery wall and was found to form soluble and insoluble complexes with LDL (83). Initially, it was found that LDL from different individuals formed different amounts of insoluble complexes with LCP, with the largest amount of insoluble complex formed from LDL with higher affinity for LCP (84, 85). This led to the hypothesis that LDL with the higher affinity for LCP could be preferentially involved in the development of atherosclerosis. Later work found that the LDL population with greater affinity for LCP, and the greater tendency to form insoluble LDL-LCP complexes, possessed significantly less sialic acid than the LDL subclass with relatively low affinity for LCP (84). In addition, desialation of LDL with neuraminidase was found to increase affinity of LDL for LCP (84) and, in vivo, to increase uptake of LDL by the arterial intima-media (85). Based on these reports, LDL subclass pattern B LDL, with its lower sialic acid content, would be predicted to show stronger binding than LDL from subclass pattern A to at least one human arterial proteoglycan with potential for increasing deposits of an insoluble LDL-LCP complex in the arterial wall. Since we have shown here that the reduction in LDL sialic content in pattern B subjects is related to differences in LDL glycolipids and not to apoB glycosylation, it may be that altered LDL glycolipid composition could contribute to the increased risk of coronary heart disease found in individuals with LDL subclass pattern B.

The authors wish to thank Adelle Cavanaugh for obtaining plasma samples, Laura Glines and Charlotte Brown for technical assistance, Dr. Melissa Austin, Dr. Hudson Freeze, and Professor Clinton Ballou for helpful discussions during this study, and Marybeth Gonzales for preparation of the manuscript. This work was supported by NIH Program Project Grant HL 18574 from the National Heart, Lung, and Blood Institute of the National Institutes of Health, and was conducted at the Lawrence Berkeley Laboratory (Department of Energy contract DE-AC03-76SF00098 to the University of California).

Manuscript received 16 January 1990 and in revised form 30 April 1990.

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