Characterization of two lipoproteins containing apolipoproteins B and E from lesion-free human aortic intima

Seppo Ylä-Herttuala,[1] Olli Jaakkola, Christian Ehnholm,[*] Matti J. Tikkanen,[†] Tiina Solakivi, Terttu Särkioja,[**] and Tapio Nikkari

Department of Biomedical Sciences, University of Tampere; National Public Health Institute,[*] Helsinki; III Department of Medicine,[†] University of Helsinki; and Department of Forensic Medicine,[**] University of Oulu, Finland

Abstract Lesion-free areas of aortic intimas from seven men, 30 to 49 years old, were extracted with aqueous buffer within a few hours after an accidental or sudden death. Two lipoprotein fractions could be isolated by density gradient ultracentrifugation from all cases. The mean composition of fraction I (d < 1.012 g/ml) resembled that reported for the cholesteryl ester-rich, beta-migrating very low density lipoprotein (β-VLDL); the composition of fraction II (d 1.021–1.046 g/ml) resembled that of plasma low density lipoprotein (LDL). Mean diameter of the particles was 35 ± 8 nm in fraction I and 25 ± 5 nm in fraction II (22 ± 2 nm in plasma LDL). Both fractions contained apolipoproteins B (apoB) and E (apoE), and had increased electrophoretic mobilities and reduced contents of linoleic acid. The immunoreactivity of apoB to a polyclonal and two monoclonal antibodies in both fractions was not different from that of plasma lipoproteins. The apoE isoform patterns in both fractions were similar to those obtained from the respective postmortem plasmas. When incubated with mouse peritoneal macrophages, fractions I and II enhanced the incorporation of radioactive oleate into cholesteryl esters by 10- to 20-fold and 3- to 4-fold, respectively, in comparison to plasma LDL. In conclusion, our results indicate that lesion-free human aortic intima contains two types of apoB- and apoE-containing lipoprotein particles, both of which might be potentially atherogenic. — **Ylä-Herttuala, S., O. Jaakkola, C. Ehnholm, M. J. Tikkanen, T. Solakivi, T. Särkioja, and T. Nikkari.** Characterization of two lipoproteins containing apolipoproteins B and E from lesion-free human aortic intima. *J. Lipid Res.* 1988. **29:** 563–572.

Supplementary key words apolipoproteins B and E • atherogenesis • chemical composition • electron microscopy • fatty acids • isoelectric focusing • immunoblotting • macrophages • monoclonal antibodies

The role of elevated plasma lipoproteins, particularly LDL, as a source for lipid accumulation in atherosclerotic lesions is undisputed. Several immunohistochemical and immunoelectrophoretic studies have shown that the arterial wall contains apoproteins derived from plasma lipoproteins (1–5), and the content of immunologically detectable apoB in the human aortic intima has a positive relationship to the serum apoB concentration (2, 3). The mechanism whereby intimal lipoproteins cause the accumulation of cholesterol in the cells of the arterial wall with the resultant formation of foam cells is still speculative. Most of the foam cells in atherosclerotic lesions have been identified as monocyte-derived macrophages (6). Although normal plasma LDL does not cause enhanced lipid uptake in macrophages, various forms of modified (7, 8) or abnormal (9, 10) lipoproteins have been shown to accumulate in these cells in vitro, leading to the formation of foam cells.

To understand the early pathogenesis of atherosclerosis, it would be essential to learn what types of lipoproteins are present in the arterial intima before any lesions have developed and whether the lipoproteins are modified in a way enhancing their uptake by the intimal cells. There are two previous reports describing the properties of lipoproteins extracted from minces of normal human aortic intima after mechanical homogenization of the tissue (4, 11). The purpose of the present work was to extend these studies by characterization of lipoproteins removable by gentle extraction with aqueous buffer from lesion-free areas of human aortas within a few hours after death, thus minimizing any deterioration of the lipoproteins due to postmortem autolysis or mechanical trauma. It was found that two types of low density lipoprotein particles were

Abbreviations: apo, apolipoprotein; β-VLDL, β-very low density lipoprotein; DMEM, Dulbecco's modification of Eagle's medium; EC, esterified cholesterol; ELISA, enzyme-linked immunosorbent assay; FC, free cholesterol; FFA, free fatty acids; LPDS, lipoprotein-deficient serum; LP[a], lipoprotein[a]; PBS, phosphate-buffered saline; PL, phospholipid; SDS-PAGE, sodium dodecylsulfate polyacrylamide gel electrophoresis; TG, triglyceride; LDL, low density lipoprotein.

[1]Present address: Department of Medicine, M-013D, Division of Endocrinology and Metabolism, University of California, San Diego, La Jolla, CA 92093.

Downloaded from www.jlr.org by guest, on June 19, 2012

extractable from normal human aortic intima. Both contained apoB and apoE, had reduced contents of linoleic acid, and showed enhanced uptakes into mouse peritoneal macrophages. One of the particles resembled the cholesteryl ester-rich β-VLDL found in type III hyperlipoproteinemia; the other resembled plasma LDL.

MATERIALS AND METHODS

Adult male NMRI (Nordic Medical Research Institute) mice were purchased from Orion (Finland). [^{3}H]Oleic acid (4.3 Ci/mmol) was obtained from the Radiochemical Centre (U.K.). ε-Aminocaproic acid, dipentadecanoyl phosphatidylcholine, glutathione, pentadecanoyl chloride, and oleyl chloride were from Sigma (USA); benzamidine hydrochloride was from Eastman Kodak (USA); pentadecanoate and heptadecanoate were from Supelco (USA); stigmasterol and coprostanol were from Applied Science (USA); and tripentadecanoate was from Nu-Chek-Prep (USA). Stigmasterol pentadecanoate and stigmasterol oleate were synthesized from free stigmasterol and pentadecanoyl chloride or oleyl chloride, respectively (12). Agarose electrophoresis gels were from Corning Medical (USA); polyacrylamide gradient gels (4–30%) and molecular weight markers were from Pharmacia (Sweden); Ampholine (pH 4–6) was from LKB (Sweden); and nitrocellulose membranes and affinity-purified goat anti-rabbit IgG–horseradish peroxidase were from Bio-Rad (USA).

Dulbecco's modification of Eagle's medium (DMEM) was from Flow Laboratories (U.K.); glutamine, penicillin–streptomycin, nonessential amino acids, new-born calf sera were from Gibco (USA); and plastic Petri dishes (35 mm) were from Nunc (Denmark). Phosphate-buffered saline (PBS) used in cell culture experiments contained 0.14 M NaCl–0.01 M sodium phosphate (pH 7.4).

Autopsied cases

Arterial samples were collected from medicolegal autopsies of seven successive cases under the age of 50 years who were brought to postmortem examination immediately after an accidental or sudden death during working hours (**Table 1**). A postmortem plasma sample was taken from the femoral vein for total cholesterol and triglyceride determination (Boehringer Mannheim Kit, FRG). As judged on the basis of autopsy findings and medical records, cases 1, 3, and 7 were obese, case 1 had received treatment for essential hypertension, and cases 1, 2, 3, 5, and 6 were current smokers. None of the cases had any major diseases or overt disorders of lipid metabolism (Table 1).

Dissection of aortas

Thoracic and abdominal aortas were removed at the autopsy, which was performed within 1.5 to 2.5 hr after the death. The vessels were opened, and the extent of the area covered with macroscopic atherosclerotic lesions was evaluated (Table 1). The vessels were washed with cold saline, blotted, moistened with lipoprotein extraction solution containing antioxidants and protease inhibitors (see below), and kept on ice until further processed within 6 hr. Aortas were photographed, after which macroscopically lesion-free areas were dissected free of atherosclerotic lesions. Macroscopically affected areas together with the ostia of the branching arteries were rejected. The surface area of the samples was measured. Intima was isolated under a dissection microscope. The first intact elastic lamina was used as a dissection plane. In histologic controls for the dissection plane, the samples contained the whole intima and less than 10% of the media. In each case approximately ⅔ of the isolated intima was derived from thoracic aorta and ⅓ from abdominal aorta. The

TABLE 1. Autopsied cases[a]

Patient #	Cause of Death	Age	Postmortem Time	Wet Weight of the Isolated Intimal Sample	Aortic[b]		Postmortem Plasma[c]	
					Fatty Streaks	Fibrous Plaques	Total Cholesterol	Triglyceride
		yr	*hr*	*g*	*% of surface area*		*mmol/l*	
1	Ethanol intoxication	39	2.5	2.53	3	4	3.0	2.5
2	Acute myocardial infarction	49	1.5	1.42	10	18	5.5	1.4
3	Ethanol intoxication	30	1.5	2.34	7	8	6.0	3.3
4	Gunshot wounds	32	1.5	5.53	15	5	5.2	2.1
5	Acute myocardial infarction	46	2.0	5.55	5	23	4.8	3.0
6	Carbon monoxide intoxication	44	1.5	4.34	5	7	3.3	2.7
7	Acute myocardial infarction[d]	34	2.0	2.01	4	2	3.8	1.7

[a] All the cases were males.
[b] Estimated from unstained vessels before lesion-free areas were dissected for the extraction of lipoproteins.
[c] Non-fasting samples.
[d] Due to an occlusive coronary thrombus without any significant coronary atherosclerosis.

Downloaded from www.jlr.org by guest, on June 19, 2012

wet weights of the isolated samples varied from 1.42 to 5.55 g per aorta (Table 1).

Extraction of intimal lipoproteins

Intimas were cut into small pieces with a tissue chopper (The Mickle Laboratory Engineering Co., U.K.). Samples were extracted (1 g wet tissue/5 ml extraction solution) overnight at 4°C with gentle agitation in 0.15 M NaCl–0.01 M phosphate buffer, pH 7.4, containing 0.01% sodium azide, 0.05% glutathione, 0.08% benzamidine hydrochloride, 0.1% EDTA, and 0.5% ϵ-aminocaproic acid. The extract was collected by centrifugation (5000 *g*, 4°C, 15 min); the pellet was washed twice with the extraction solution; and the supernatants were combined. The extract was transferred into 12-ml tubes and overlayered with 1.0 ml of fresh extraction solution. The tubes were centrifuged at 100,000 *g* for 30 min at 8°C in a Kontron Centrikon T-2055 ultracentrifuge using a TFT 41.14 swing-out rotor. A pellet and a creamy top layer were discarded. The rest of the solution was used for density gradient ultracentrifugation. Plasma LDL (d 1.019–1.063 g/ml) from healthy human donors was isolated by ultracentrifugation in the presence of 0.05% glutathione, 0.1% EDTA, and 0.13% ϵ-aminocaproic acid.

Density gradient ultracentrifugation

The density of the lipoprotein extract was adjusted to 1.21 g/ml by the addition of solid KBr. A 5.75-ml sample was placed in a tube (12 ml) and overlayered with 5.75 ml of a solution containing 0.154 M NaCl, 0.05% glutathione, 0.08% benzamidine hydrochloride, and 0.1% EDTA (pH 7.4) (d 1.006 g/ml). The tubes were centrifuged at 150,000 *g* for 40 hr at 8°C in a Kontron Centrikon T-2055 ultracentrifuge using a TFT 41.14 swing-out rotor. A tube containing plasma LDL as the sample and a background salt tube were included in each run. Density profile of the gradient was measured by gravimetry. Unless otherwise indicated, lipoproteins were located under indirect light and collected by pipetting. Fraction I and II lipoproteins (see below) were recentrifuged at d 1.020 and d 1.070 g/ml, respectively (150,000 *g*, 8°C, 24 hr). Isolated lipoproteins were dialyzed against 0.15 M NaCl containing 0.05% glutathione, 0.1% EDTA, and 0.13% ϵ-aminocaproic acid (pH 7.4), sterilized by filtration, and stored under nitrogen at 4°C. Usually, the amounts of fraction I and II lipoproteins recovered from one aorta varied between 30 and 100 μg and between 150 and 400 μg of lipoprotein protein, respectively.

Electron microscopy

Aliquots of the isolated lipoprotein fractions I and II from all cases were dialyzed against 1% ammonium acetate (pH 7.4), negative-stained with 1% sodium phosphotungstate (pH 7.4) (13), and examined with a JEM 100 C electron microscope (JEOL Ltd, Japan). Mean particle size of the lipoproteins was calculated from the measurements of 100 particles in each lipoprotein fraction in each of the analyzed cases.

Chemical analyses

Aliquots of the isolated lipoprotein fractions I and II from all cases were used for lipid analyses. Lipids were extracted according to Folch, Lees, and Sloane Stanley (14) and fractionated with thin-layer chromatography (Silica Gel G, Merck, FRG) to esterified cholesterol (EC), triglyceride (TG), free fatty acids (FFA), free cholesterol (FC), and phospholipid (PL) (15). Before the extraction of the lipids, appropriate amounts of the following recovery standards and carriers were added to the samples: stigmasterol pentadecanoate, stigmasterol, tripentadecanoate, pentadecanoate, and dipentadecanoyl phosphatidylcholine.

The fraction corresponding to EC was saponified with KOH. The liberated FC was recovered by extraction with petroleum ether. After acidification of the mixture with HCl, the liberated cholesteryl ester fatty acids were collected by extraction with petroleum ether (15).

Cholesteryl ester fatty acids and fractions corresponding to TG, PL, and FFA were transesterified with HCl in dry methanol. Methyl esters of the fatty acids were extracted with petroleum ether, and analyzed in a Hewlett-Packard 5880 A gas chromatograph with an OV 351 capillary column (Orion Analytica, Finland) and a flame ionization detector (15). Before the transesterification of the fractions, appropriate amounts of heptadecanoate were added to the samples as a standard. The results were expressed as absolute amounts or as percentages of the total areas of all fatty acid peaks from 14:0 to 22:6 excluding the peaks corresponding to the added standards (15:0 and 17:0). The individual fatty acids were identified using reference mixtures of known methyl esters (Applied Science Laboratories, USA). As judged on the basis of the added standards, the recoveries of the fatty acids in all lipid classes were 60 to 70%.

The concentrations of TG and PL in the isolated lipoproteins were calculated on the basis of the concentrations of total fatty acids in the corresponding lipid fractions. Triolein was taken as an average lipoprotein TG, and linoleate as an average fatty acid of lipoprotein PL. In some cases the concentration of TG was measured with enzymatic methods (Boehringer Mannheim Kit, FRG) and PL as lipid-soluble phosphorus (16).

The fraction corresponding to FC and the extract containing the liberated EC were analyzed in a Hewlett-Packard 5700 A gas chromatograph with an SE-30 capillary column (Orion Analytica, Finland), and a flame ionization detector (15). Coprostanol was used as an external standard. The recoveries of FC and EC were 89

Downloaded from www.jlr.org by guest, on June 19, 2012

ASBMB JOURNAL OF LIPID RESEARCH

to 94%. For the calculation of the cholesteryl ester content of the lipoproteins, all cholesteryl esters were assumed to be in the form of cholesteryl linoleate.

Aliquots of PL extracts of some samples were fractionated with thin-layer chromatography (Silica Gel H, Merck, FRG, containing 0.7% magnesium acetate) to lysolecithin, sphingomyelin, and lecithin (15, 17). The fractions were quantified as lipid-soluble phosphorus (16). All organic solvents in lipid analyses were redistilled and contained 0.005% butylated hydroxytoluene as an antioxidant. Protein content of the lipoproteins was determined by the method of Lowry et al. (18) using bovine serum albumin as a standard. The procedure included chloroform extraction after color development to remove lipid interference. The proportion of isopropanol-insoluble protein of the total lipoprotein protein was analyzed according to Egusa et al. (19).

Immunological analyses

Monoclonal mouse anti-human LDL antibodies were produced as previously described (20). Isolated lipoprotein fractions were made to compete with human plasma LDL for binding to monoclonal antibodies in an enzyme-linked immunosorbent displacement assay (ELISA) as described (21). The results were plotted as competitive displacement curves against apoB content of the added lipoproteins determined by polyclonal anti-human LDL antisera (21, 22). Immunological properties of the isolated lipoproteins were also studied with double immunodiffusion techniques using polyclonal antisera against human LDL (23), Lp[a] (24), apoA-I (23), and apoE (25). ApoB and apoA-I contents of the lipoprotein extracts were quantified by radial immunodiffusion (23).

Isoelectric focusing, immunoblotting, and SDS-polyacrylamide gel electrophoresis

Analytical isoelectric focusing and immunoblotting were performed as previously described (25). The SDS-polyacrylamide gel electrophoresis (SDS-PAGE) (26) and agarose electrophoresis (27) procedures have been described.

Cell culture studies

Resident mouse peritoneal macrophages were prepared according to a modification (28) of the method of Edelson and Cohn (29). Prior to the experiments the cells were incubated for 24 hr in DMEM containing 10% new-born calf lipoprotein-deficient serum (LPDS). Monolayers were washed twice with PBS, and incubated for 10 hr in DMEM containing 10% LPDS, 0.1 mM [^{3}H]oleate-albumin, and 15 μg/ml of the lipoproteins. The incubations were stopped by transferring the vials into an ice bath. The cells were washed three times with ice-cold PBS-0.1% albumin and three times with PBS alone. Stigmasterol and stigmasteryl oleate were added as recovery standards, and lipids were extracted in situ with hexane-isopropanol 3:2 (30). The extract was fractionated by thin-layer chromatography; FC and EC were quantified by gas-liquid chromatography (see above); and cholesteryl ester fatty acids were analyzed for ^{3}H radioactivity in an LKB-Wallac liquid scintillation counter. The cells were dissolved in 5% SDS-0.5 M NaOH. Aliquots of the solution were taken for total protein determination (18, 28). Acetylated LDL was prepared as described (31). All incubation media contained 1% nonessential amino acids, 2 mM glutamine, and 100 U/ml penicillin and streptomycin.

RESULTS

Extracts of lesion-free aortic intimas from seven adult males were analyzed. The extracts recovered after centrifugation at 100,000 *g* for 30 min contained apoB, apoA-I, and apoE, as determined by immunodiffusion using polyclonal antisera against human LDL (i.e., apoB), apoA-I, and apoE (data not shown). The mean contents of apoA-I and apoB in the extracts from cases 1 and 2 were quantified using radial immunodiffusion and were (mean ± SD) 0.80 ± 0.23 μg/mg and 3.97 ± 0.70 μg/mg of dry defatted tissue, respectively.

The extracts were fractionated using density gradient ultracentrifugation (**Fig. 1**). In all samples two low density

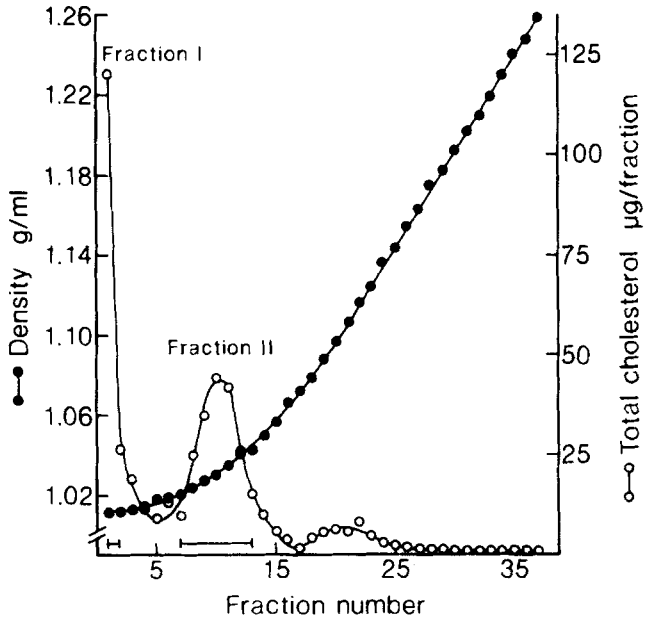

Fig. 1. Density gradient ultracentrifugation of the aortic extract. A 5.75-ml sample of intimal extract (case 4) (d 1.21 g/ml) was overlayered with 5.75 ml of d 1.006 g/ml solution and centrifuged at 150,000 *g* for 40 hr at 8°C. The tube was punctured from the bottom and fractions were collected. Densities were measured by gravimetry. The distribution of total cholesterol in the gradient was determined using gas-liquid chromatography. The positions of the collected lipoproteins are indicated by bars.

Downloaded from www.jlr.org by guest, on June 19, 2012

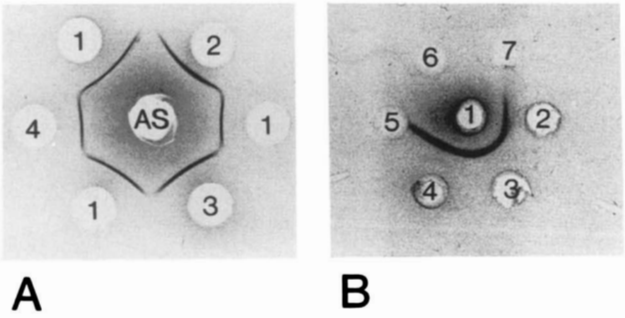

Fig. 2. A: A double immunodiffusion with polyclonal antiserum (AS) against human LDL (i.e., apoB) showing a complete identity between plasma LDL (1), intimal fraction I combined from cases 5 and 6 (2), and intimal fraction II from case 5 (3) and case 6 (4). B: Immunodiffusion of fraction I lipoprotein from case 7 (1) against different dilutions of anti-human apoE (2–4) and anti-human albumin (5–7) antisera. Dilutions of the antisera: (2) 1:8, (3) 1:4, (4) 1:2, (5) 1:44, (6) 1:22, and (7) 1:11; Coomassie brilliant blue staining.

fractions were observed: fraction I, which floated on the top of the tubes as an opaque 2- to 3-mm layer (d < 1.012 g/ml), and fraction II, which had a slightly lower density range (d 1.021–1.046 g/ml) than plasma LDL (d 1.029–1.055 g/ml). Both fractions contained apoB and apoE (**Fig. 2,** see below). After density gradient ultracentrifugation, 7% (range 5 to 15%) and 93% (range 85 to 95%) of the total recovered immunoreactive apoB was detected in fractions I and II, respectively. No albumin (Fig. 2b) or Lp[a] (data not shown) were found in the collected fractions. A fraction corresponding to intimal high density lipoprotein was located near the contaminating proteins and was not studied further.

Electron microscopy of the lipoproteins extracted from lesion-free aortas revealed in each case that particles in the intimal fractions I and II were larger and more heterogenous than plasma LDL (**Fig. 3**). The mean particle size decreased in the order of fraction I, fraction II, and plasma LDL. In agarose gel electrophoresis the mobilities of intimal fractions I and II were increased as compared with plasma VLDL and LDL, respectively (**Fig. 4**).

Chemical composition of the intimal lipoproteins is shown in **Table 2.** Both intimal fractions were rich in cholesteryl esters. In fraction I the percentage of triglyceride was higher and that of protein lower as compared with plasma LDL. No differences in the relative amounts of phospholipid classes were found between fraction II and plasma LDL (**Table 3**). The percentage composition of fatty acids in all lipid classes revealed a higher proportion of stearate (18:0) and a lower proportion of linoleate (18:2) in the intimal lipoprotein fractions than in plasma LDL (**Table 4**).

There was a complete identity between plasma LDL and intimal fractions I and II, when examined with a double immunodiffusion technique using a polyclonal antiserum against human LDL (Fig. 2A). The abilities of intimal lipoproteins to compete with plasma LDL for binding to two monoclonal anti-LDL antibodies were tested (**Fig. 5**). All the curves for the intimal lipoproteins obtained with antibody 2a were shifted to the right. After logit transformation of the data (22) no significant differences were found between the slopes of the intimal lipoproteins and plasma LDL (data not shown). When the displacement obtained with plasma LDL was designated as 100%, the relative displacing capacities of the intimal fractions I and II were 43% and 57% with antibody 2a, respectively, and 57% and 82% with antibody 1a, respectively.

Of the total protein in the fractions I and II, 56 ± 7.5% and 90 ± 5.1% (mean ± SD), respectively, were insoluble in isopropanol, which gives an estimate of the content of apoB in the isolated lipoproteins (19). In SDS-PAGE, apoB-100 was the major protein of the intimal fractions (**Fig. 6**). Slight to moderate degradation of apoB was

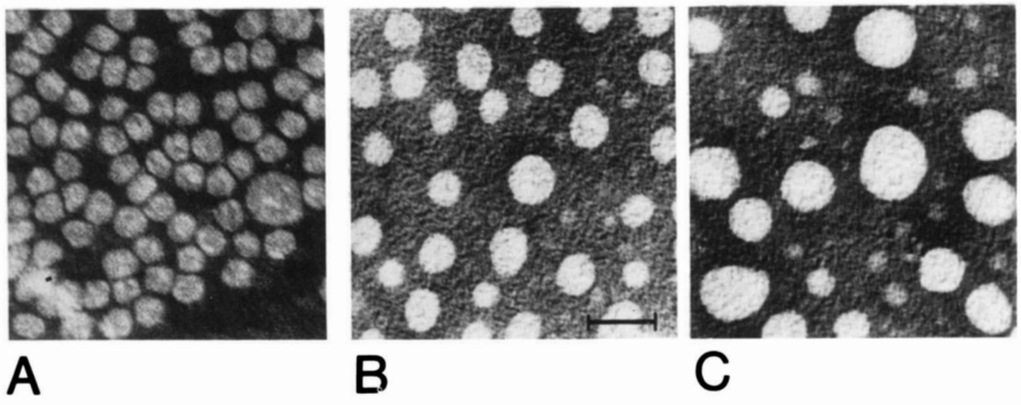

Fig. 3. Representative electron micrographs of negatively stained lipoproteins. A: Plasma LDL, d 1.029–1.055 g/ml, mean diameter 21.5 ± 1.6 (SD) nm; B: intimal fraction II (case 6), d 1.021–1.046 g/ml, mean diameter 24.8 ± 4.5 nm; C: intimal fraction I (case 6), d < 1.012 g/ml, mean diameter 35.1 ± 8.2 nm. Original magnification 100 000×; bar = 50 nm.

ASBMB JOURNAL OF LIPID RESEARCH JLR

Downloaded from www.jlr.org by guest, on June 19, 2012

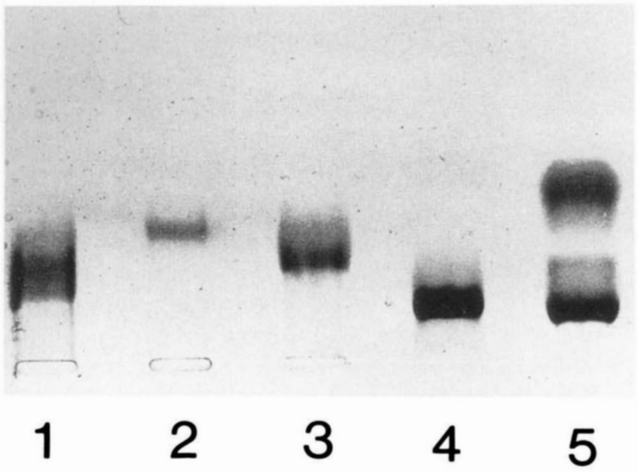

Fig. 4. Agarose electrophoresis showing increased electrophoretic mobility of the intimal lipoproteins. Plasma VLDL from a nonfasting donor (1), fraction I (2) and fraction II (3) from case 7, plasma LDL (4), and fasting human plasma (5). Fat Red 7B staining. The anode (+) is at the top of the figure.

observed. Fraction I also showed apoE and apoC bands (Fig. 6). The presence of apoE in both intimal fractions was demonstrated using immunoblotting (**Fig. 7, A and B**). ApoE in the fractions I and II was further characterized using isoelectric focusing. The same isoforms of apoE were present in the intimal lipoproteins as in the corresponding postmortem plasma samples (Fig. 7C). Intimal fraction II and postmortem plasma (Fig. 7, lines 5 and 8) also contained some sialylated forms of apoE.

When lipoprotein fractions I and II were incubated with mouse peritoneal macrophages in the presence of [^{3}H]oleate, intimal fraction I enhanced the formation of cholesteryl esters over that observed with plasma LDL 10- to 20-fold, while fraction II caused a 3- to 4-fold increase (**Fig. 8**).

DISCUSSION

In order to find out whether lesion-free aortic intima contains lipoproteins capable of causing lipid accumulation in macrophages, which would help to explain early stages of atherogenesis, we studied the chemical, immunological, and biological properties of lipoproteins extractable from lesion-free aortic intimas obtained from relatively young men after accidental or sudden death. The results obtained from autopsy material may always be distorted by postmortem changes. The short postmortem times (<2.5 hr) employed in the present study reduce the risk of such changes. In addition, lipoproteins were isolated using a gentle extraction procedure, and antioxidants and protease inhibitors were added to the extraction buffer to prevent possible formation of artifacts. Nevertheless, because of the limited number of the cases, the present material cannot be regarded as representative of the whole population. On the other hand, no major diseases or overt disorders of lipid metabolism (such as type III hyperlipoproteinemia) were detected in the present series.

Two types of low density lipoproteins were detected by density gradient ultracentrifugation in all seven samples from lesion-free intimas. The composition of the smaller particles (fraction II) resembled plasma LDL and they apparently represent plasma LDL that has penetrated into the arterial wall. This finding is in line with earlier data (4, 11). Fraction I contained cholesteryl ester-rich particles, whose chemical composition, density distribution, and mean diameter resembled those reported for d 1.006–1.019 g/ml particles isolated from the plasma of human type III hyperlipoproteinemic patients (32, 33) and small β-VLDL particles of cholesterol-fed dogs (34). The electrophoretic mobility of fraction I was somewhat higher than that reported by Fainaru et al. (34) for small β-VLDL particles of cholesterol-fed dogs. As compared with plasma LDL, fraction II had an increased electrophoretic mobility, which is in line with previous observations from intimal LDL-like particles (2, 4, 11).

In SDS-PAGE both lipoprotein fractions contained apoB and apoE. ApoB-100 was the major band in the intimal fractions but, in contrast to previous studies of intimal LDL-like particles (11, 35), only slight to moderate degradation of apoB was observed. A previously unreported finding is that apoE was present in both isolated fractions. In SDS-PAGE intimal apoE showed one band and had a molecular weight similar to plasma apoE. Isoelectric focusing showed the presence of the same isoforms of apoE in the intimal lipoproteins as in the postmortem plasma samples. Thus, apoE in the intimal lipoproteins

TABLE 2. Composition of lipoprotein fractions I and II extracted from normal aortic intima and plasma LDL

Composition	Intimal Fraction I (n = 7)	Intimal Fraction II (n = 7)	Plasma LDL (n = 4)
	% of total weight ± SD		
Phospholipid[a]	16.4 ± 2.9	20.4 ± 2.2	20.3 ± 2.5
Triglyceride[b]	12.7 ± 2.6[c]	7.4 ± 1.3	6.2 ± 0.8
Free cholesterol	11.0 ± 1.5	10.8 ± 2.3	9.8 ± 0.9
Cholesteryl esters[a]	45.6 ± 5.1	39.3 ± 4.4	40.0 ± 4.9
Protein	14.3 ± 2.2[c]	22.0 ± 4.0	23.8 ± 3.6

[a] For the calculation of phospholipid and cholesteryl ester contents, linoleic acid was taken as the average fatty acid esterified to the lipid fractions.

[b] For the calculation of triglyceride content, oleic acid was taken as the average fatty acid esterified to glycerol.

[c] $P < 0.05$, compared with plasma LDL (*t*-test).

Downloaded from www.jlr.org by guest, on June 19, 2012

TABLE 3. Phospholipid classes in lipoprotein fraction II extracted from normal aortic intima and plasma LDL

Class	Intimal[a] Fraction II (n = 2)	Plasma LDL (n = 4)
	% of total phospholipid ± SD	
Lysolecithin	7.3 ± 1.6	7.0 ± 1.8
Sphingomyelin	18.6 ± 2.0	24.3 ± 2.7
Lecithin	69.2 ± 4.8	66.4 ± 3.9
Others	4.9 ± 0.7	2.3 ± 0.4

[a]Two pooled samples from cases 2, 3, 4, and 5.

was similar to plasma apoE, although the proportion of sialylated forms may be increased in fraction II. Whether some apoE in the intimal lipoproteins is derived from tissue macrophages (36) remains to be determined.

Our results indicate that, in addition to LDL-like particles (fraction II), normal aortic intima contains "remnant"-like particles (fraction I), the presence of which has not been reported earlier in arterial wall. The origin of fraction I particles remains speculative, but the most likely explanation is that they are derived from a direct transendothelial transport of plasma VLDL or intermediate density lipoproteins (37–40), perhaps followed by lipolysis

TABLE 4. Percentage composition of the major fatty acids in different lipid classes in lipoprotein fractions I and II extracted from normal aortic intima and plasma LDL

Composition	Intimal Fraction I (n = 7)	Intimal Fraction II (n = 7)	Plasma LDL (n = 4)
		Mean ± SD[a]	
Phospholipid			
16:0	32.8 ± 6.7	29.2 ± 2.5	28.6 ± 2.7
18:0	23.0 ± 5.6	20.9 ± 3.1	17.8 ± 2.1
18:1 (n-9)	9.7 ± 2.7	15.6 ± 3.7	11.9 ± 1.8
18:2 (n-6)	13.1 ± 2.1[b]	15.9 ± 3.2[c]	23.0 ± 1.3
20:4 (n-6)	4.0 ± 2.5	7.4 ± 1.7	7.7 ± 0.8
Triglyceride			
16:0	32.7 ± 5.7	29.0 ± 4.6	24.0 ± 2.3
18:0	22.2 ± 7.8[b]	12.6 ± 4.5	4.8 ± 2.2
18:1 (n-9)	17.6 ± 11.2[c]	32.3 ± 8.8	38.2 ± 3.3
18:2 (n-6)	4.9 ± 3.6[b]	9.0 ± 5.2[c]	18.5 ± 3.1
20:4 (n-6)	1.2 ± 0.5	1.5 ± 0.7	1.7 ± 0.5
Cholesteryl esters			
16:0	19.6 ± 6.5	13.8 ± 2.8	11.5 ± 0.3
18:0	10.2 ± 6.8[c]	3.3 ± 1.7	1.7 ± 0.8
18:1 (n-9)	20.8 ± 2.7	22.9 ± 3.4	19.3 ± 1.2
18:2 (n-6)	35.0 ± 10.4[b]	46.2 ± 5.6[c]	54.2 ± 2.3
20:4 (n-6)	3.0 ± 2.9	4.3 ± 1.6	5.5 ± 0.6
Free fatty acids			
16:0	30.3 ± 4.7	30.7 ± 5.2	28.8 ± 5.8
18:0	27.4 ± 4.1	26.8 ± 4.6	25.6 ± 2.3
18:1 (n-9)	6.2 ± 1.7	7.3 ± 4.4	6.1 ± 2.9
18:2 (n-6)	2.1 ± 0.8	3.1 ± 1.3	4.8 ± 2.0
20:4 (n-6)	1.5 ± 0.4	1.5 ± 0.7	2.4 ± 0.5

[a]Percentage of all fatty acid peaks from 14:0 to 22:6 except 15:0 and 17:0, which were used as standards (see Methods).
[b]$P < 0.01$, [c]$P < 0.05$, compared with plasma LDL (*t*-test).

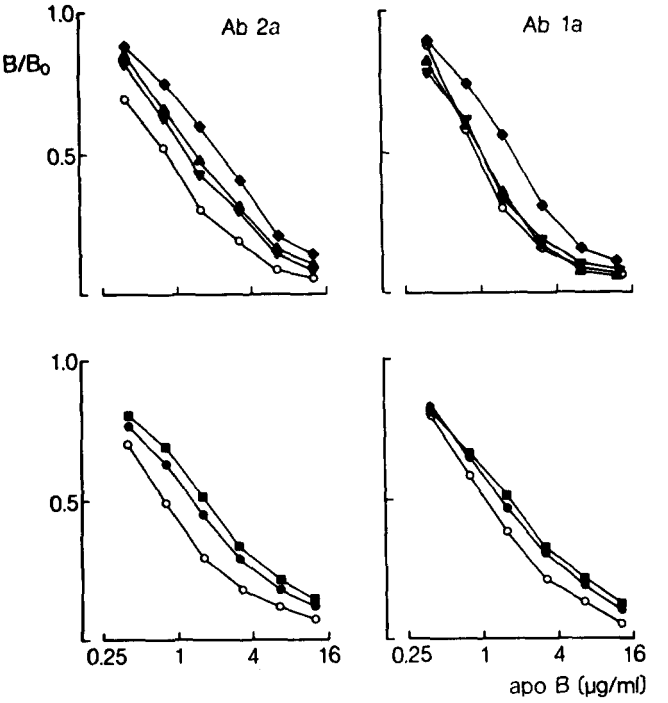

Fig. 5. Competitive displacement curves produced by intimal lipoprotein fractions I and II and human plasma LDL with monoclonal antibodies 2a and 1a. Plasma LDL (○), intimal fraction I combined from cases 5 and 6 (◆), and intimal fraction II from cases 1 (■), 2 (●), 5 (▼), and 6 (▲). ApoB content of the added lipoproteins was determined using polyclonal antisera against human LDL. The results of the competition ELISA analysis are expressed as B/B_0, where B denotes an absorbance in the presence and B_0 in the absence of a competing lipoprotein. Each point represents the mean of duplicate determinations.

in the intima. Another possibility is that these particles represent products of the local action of lipoprotein lipase on chylomicrons and/or VLDL at the surface of the endothelium followed by transport through the endothelium, as suggested by Zilversmit (41). To rule out possible effects of lipases during the extraction of lipoproteins, plasma VLDL was added to aortic minces in a separate experiment; no significant hydrolysis of VLDL triglyceride was observed.

A conspicuous chemical alteration in both isolated intimal fractions in comparison to plasma lipoproteins was the reduction of linoleic acid in all major lipid classes. This finding agrees with that reported by Hoff and Gaubatz (11), who found even a greater reduction of linoleic acid in intimal LDL-like particles isolated after mincing of the intima. The reason for the reduction of linoleic acid is unknown, but it may reflect peroxidation of the lipids, since corresponding changes have been reported from lipoproteins oxidized in vitro (42). Because the changes were similar in all lipid classes and no change was found in the composition of phospholipid classes, the action of lecithin:cholesterol acyltransferase in the intima seems less likely.

Downloaded from www.jlr.org by guest, on June 19, 2012

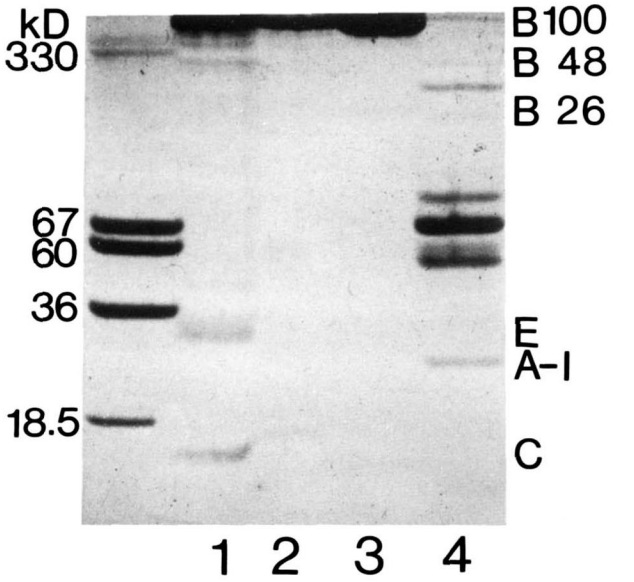

Fig. 6. SDS-PAGE on a gradient gel (4–30%) of the intimal lipoproteins. Fraction I (1), and fraction II (2) from case 6, plasma LDL (3), and chylomicrons from a hypertriglyceridemic patient (4). Molecular weight markers from the top: thyroglobulin, bovine serum albumin, catalase, lactate dehydrogenase, and ferritin. The locations of the apolipoproteins B, E, A-I, and C are indicated; Coomassie brilliant blue staining.

To test the presence of subtle immunological alterations in apoB which may not be detectable in double immunodiffusion analysis with polyclonal antibodies, competitive displacement assays were performed using two monoclonal anti-human LDL antibodies: antibody 2a, which was directed against an epitope located near the receptor recognition domain of apoB, and antibody 1a, which was directed against an epitope other than the receptor recognition domain of apoB (20). All the displacement curves for the intimal lipoproteins tested with antibody 2a (but not with 1a) were shifted to the right, which might imply a slight decrease in the immunoreactivity. The results obtained using fraction I particles agree with previous data showing reduced immunoreactivity with increasing flotation rate of lipoprotein fractions (22). The curves for the fraction II particles lie within the variation of the immunoreactivity of plasma LDL obtained from different human donors (43). The results indicate the presence of immunologically intact apoB in the fractions I and II.

As compared with plasma LDL, intimal fractions I and II stimulated the synthesis of cholesteryl esters in macrophages 10- to 20-fold and 3- to 4-fold, respectively. The magnitude of the stimulation of cholesteryl ester synthesis by fraction I lipoproteins was of the same order as that reported by Fainaru et al. (34) for small β-VLDL particles and by Goldstein et al. (9) for β-VLDL isolated from the plasma of cholesterol-fed dogs. Our results thus imply that grossly normal aortic intima contains β-VLDL-like lipoproteins capable of initiating atherosclerotic changes. The stimulation of cholesterol esterification by the LDL-like fraction II was approximately half of that reported from experiments using LDL-like particles isolated by anti-

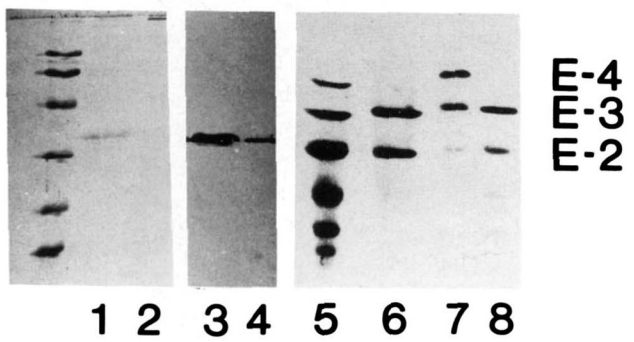

Fig. 7. A: SDS-PAGE (10% gel) of the fraction I (1) and fraction II (2) from case 7. Molecular weight markers from the top: phosphorylase B (92.5 kD), bovine serum albumin (67 kD), ovalbumin (45 kD), carbonic anhydrase (31 kD), soybean trypsin inhibitor (21.5 kD), and lysozyme (14.4 kD); Coomassie brilliant blue staining. B: Immunoblot of the SDS-gel shown in (A) using anti-human apoE antiserum; fraction I (3) and fraction II (4). C: Immunoblot of apoE after isoelectric focusing of the intimal lipoproteins and a postmortem plasma sample (case 7); intimal fraction II (5), intimal fraction I (6), postmortem plasma treated with cysteamine (7), and untreated postmortem plasma (8). The locations of the main apoE components (E-4, E-3, and E-2) are indicated. Bands below the level of E-2 are different sialylated forms of apoE.

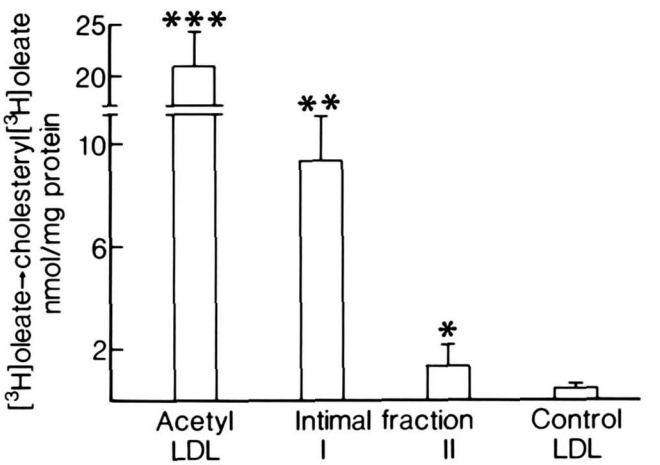

Fig. 8. Cholesteryl ester synthesis in mouse peritoneal macrophages incubated for 10 hr with various lipoproteins. Cells were preincubated for 24 hr in DMEM containing 10% LPDS. The cultures were washed twice with PBS, and incubated in DMEM containing 10% LPDS, 0.1 mM [^{3}H]oleate–albumin and 15 μg of lipoprotein protein/ml culture medium. After the incubation the cells were washed, and the content of [^{3}H]oleate incorporated into cellular cholesteryl esters was determined. Intimal lipoprotein fractions I and II were combined from cases 3 and 4. Each value represents the mean of three determinations (± SD). The contents of cellular esterified cholesterol were determined simultaneously and were as follows: acetyl LDL 22.1 ± 4.6, intimal fraction I 6.0 ± 1.9, intimal fraction II 4.1 ± 1.3, and plasma LDL 2.1 ± 0.3 ng/μg of cell protein (mean ± SD). The experiment was repeated twice with essentially similar results using lipoproteins isolated from the cases 2 and 6 (data not shown); *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ by Student's t-test in comparison to control LDL.

Downloaded from www.jlr.org by guest, on June 19, 2012

apoB affinity chromatography after mechanical homogenization of atherosclerotic aortas (35, 44). The reason for the enhanced uptake of fraction II lipoproteins is not known, but it is possible that plasma LDL has been altered in the intima by various processes, such as peroxidation (45, 46) or retroendocytosis (47), although we cannot exclude the possibility that a subclass of plasma LDL is selectively retained in the intima. The presence of apoE in these particles may also contribute to the higher uptake observed. Further studies are needed to clarify the exact origin and the pathobiological significance of the potentially atherogenic fraction I and II particles in normal aortic intima.

The authors thank Dr. Markku Pelto-Huikko, M.D., for his collaboration in electron microscopy, Mrs. Marja Jousimies, Mrs. Tarja Arvela, Miss Seija Koivuniemi, and Mrs. Merja Taatila for technical assistance, and Miss Eija Kyrölä for typing the manuscript. This work was supported by grants from the Finnish Foundation for Cardiovascular Research, the Foundation of the University of Tampere, and the Sigrid Juselius Foundation. During the study, Dr. Ylä-Herttuala was a Research Assistant of The Research Council for Medicine, The Academy of Finland.

Manuscript received 18 May 1987, in revised form 9 September 1987, and in re-revised form 17 November 1987.

REFERENCES

1. Kao, V. C. Y., and R. W. Wissler. 1965. A study of the immunohistochemical localization of serum lipoproteins and other plasma proteins in human atherosclerotic lesions. *Exp. Mol. Pathol.* **4:** 465–479.
2. Smith, E. B. 1974. The relationship between plasma and tissue lipids in human atherosclerosis. *Adv. Lipid Res.* **12:** 1–49.
3. Hoff, H. F., C. L. Heideman, J. W. Gaubatz, A. M. Gotto, Jr., E. E. Erickson, and R. L. Jackson. 1977. Quantification of apolipoprotein B in grossly normal human aorta. *Circ. Res.* **40:** 56–64.
4. Hollander, W., J. Paddock, and M. Colombo. 1979. Lipoproteins in human atherosclerotic vessels. *Exp. Mol. Pathol.* **30:** 144–171, 172–189.
5. Ylä-Herttuala, S., T. Solakivi, T. Nikkari, J. Hirvonen, H. Laaksonen, M. Möttönen, E. Pesonen, J. Raekallio, and H. K. Åkerblom. 1987. Glycosaminoglycans and apolipoproteins B and A-I in human aortas. Chemical and immunological analysis of lesion-free aortas of children and adults. *Arteriosclerosis.* **7:** 333–340.
6. Schaffner, T., K. Taylor, E. J. Bartucci, K. Fischer-Dzoga, J. H. Beeson, S. Glagov, and R. W. Wissler. 1980. Arterial foam cells with distinctive immunomorphologic and histochemical features of macrophages. *Am. J. Pathol.* **100:** 57–80.
7. Brown, M. S., and J. L. Goldstein. 1983. Lipoprotein metabolism in the macrophage: implications for cholesterol deposition in atherosclerosis. *Annu. Rev. Biochem.* **52:** 223–261.
8. Steinberg, D. 1983. Lipoproteins and atherosclerosis. A look back and a look ahead. *Arteriosclerosis.* **3:** 283–301.
9. Goldstein, J. L., Y. K. Ho, M. S. Brown, T. L. Innerarity, and R. W. Mahley. 1980. Cholesteryl ester accumulation in macrophages resulting from receptor-mediated uptake and degradation of hypercholesterolemic canine β-very low density lipoproteins. *J. Biol. Chem.* **255:** 1839–1848.
10. Koo, C., M. E. Wernette-Hammond, and T. L. Innerarity. 1986. Uptake of canine β-very low density lipoproteins by mouse peritoneal macrophages is mediated by a low density lipoprotein receptor. *J. Biol. Chem.* **261:** 11194–11201.
11. Hoff, H. F., and J. W. Gaubatz. 1982. Isolation, purification, and characterization of a lipoprotein containing apoB from the human aorta. *Atherosclerosis.* **42:** 273–297.
12. Goodman, D. S. 1969. Hydrolysis and formation of cholesterol esters in rat liver. *Methods Enzymol.* **XIV:** 522–537.
13. Forte, T., and A. V. Nichols. 1972. Application of electron microscopy to the study of plasma lipoprotein structure. *Adv. Lipid Res.* **10:** 1–41.
14. Folch, J., M. Lees, and G. H. Sloane Stanley. 1957. A simple method for the isolation and purification of total lipides from animal tissues. *J. Biol. Chem.* **226:** 497–509.
15. Ylä-Herttuala, S., and T. Nikkari. 1985. Effect of postmortem time on the biochemical composition of coronary arteries. *Atherosclerosis.* **56:** 1–10.
16. Kates, M. 1972. Techniques of lipidology: isolation, analysis and identification of lipids. *In* Laboratory Techniques in Biochemistry and Molecular Biology. T. S. Work and E. Work, editors. North-Holland Publishing Company, Amsterdam, London. 355–356.
17. Nelson, G. J. 1972. Quantitative analysis of blood lipids. *In* Blood Lipids and Lipoproteins. G. J. Nelson, editor. Wiley-Interscience, New York, London, Sydney, Toronto. 25–74.
18. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193:** 265–275.
19. Egusa, G., D. W. Brady, S. M. Grundy, and B. V. Howard. 1983. Isopropanol precipitation method for the determination of apolipoprotein B specific activity and plasma concentrations during metabolic studies of very low density lipoprotein and low density lipoprotein apolipoprotein B. *J. Lipid Res.* **24:** 1261–1267.
20. Tikkanen, M. J., R. Dargar, B. Pfleger, B. Gonen, J. M. Davie, and G. Schonfeld. 1982. Antigenic mapping of human low density lipoprotein with monoclonal antibodies. *J. Lipid Res.* **23:** 1032–1038.
21. Tikkanen, M. J., C. Ehnholm, R. Bütler, S. G. Young, L. K. Curtiss, and J. L. Witztum. 1986. Monoclonal antibody detects Ag polymorphism of apolipoprotein B. *FEBS Lett.* **202:** 54–58.
22. Tikkanen, M. J., T. G. Cole, K-S. Hahm, E. S. Krul, and G. Schonfeld. 1984. Expression of apolipoprotein B epitopes in very low density lipoprotein subfractions. Studies with monoclonal antibodies. *Arteriosclerosis.* **4:** 138–146.
23. Solakivi-Jaakkola, T., T. Nikkari, J. Viikari, H. K. Åkerblom, L. Räsänen, M. Uhari, M. Dahl, P-L. Lähde, E. Pesonen, M. Pietikäinen, and P. Suoninen. 1985. Atherosclerosis precursors in Finnish children and adolescents. VI. Serum apolipoproteins A-I and B. *Acta Paediatr. Scand. Suppl.* **318:** 119–125.
24. Ehnholm, C., H. Garoff, K. Simons, and H. Aro. 1971. Purification and quantitation of the human plasma lipoprotein carrying the Lp[a] antigen. *Biochim. Biophys. Acta.* **236:** 431–439.
25. Ehnholm, C., M. Lukka, T. Kuusi, E. Nikkilä, and G. Utermann. 1986. Apolipoprotein E polymorphism in the Finnish population: gene frequencies and relation to lipoprotein concentrations. *J. Lipid Res.* **27:** 227–235.
26. Stalenhoef, A. F. H., M. J. Malloy, J. P. Kane, and R. J. Havel. 1984. Metabolism of apolipoproteins B-48 and

Downloaded from www.jlr.org by guest, on June 19, 2012

B-100 of triglyceride-rich lipoproteins in normal and lipoprotein lipase-deficient humans. *Proc. Natl. Acad. Sci. USA.* **81:** 1839–1843.
27. Manual of Laboratory Operations. Lipid Research Clinics Program. 1974. Lipid and lipoproteins analysis. DHEW Publication No. (NIH) 75-628. **1:** 1–81.
28. Ylä-Herttuala, S., O. Jaakkola, T. Solakivi, H. Kuivaniemi, and T. Nikkari. 1986. The effect of proteoglycans, collagen and lysyl oxidase on the metabolism of low density lipoprotein by macrophages. *Atherosclerosis.* **62:** 73–80.
29. Edelson, P. J., and Z. A. Cohn. 1976. Purification and cultivation of monocytes and macrophages. *In* In Vitro Methods in Cell-Mediated and Tumor Immunity. Vol. 2. B. R. Bloom and J. R. David, editors. Academic Press, New York. 333–340.
30. Hara, A., and N. S. Radin. 1978. Lipid extraction of tissues with a low-toxicity solvent. *Anal. Biochem.* **90:** 420–426.
31. Basu, S. K., J. L. Goldstein, R. G. W. Anderson, and M. S. Brown. 1976. Degradation of cationized low density lipoprotein and regulation of cholesterol metabolism in homozygous familial hypercholesterolemia fibroblasts. *Proc. Natl. Acad. Sci. USA.* **73:** 3178–3182.
32. Lee, D. M., and D. Downs. 1982. A quick and large-scale density gradient subfractionation method for low density lipoproteins. *J. Lipid Res.* **23:** 14–27.
33. Ehnholm, C., R. W. Mahley, D. A. Chappell, K. H. Weisgraber, E. Ludwig, and J. L. Witztum. 1984. Role of apolipoprotein E in the lipolytic conversion of β-very low density lipoproteins to low density lipoproteins in type III hyperlipoproteinemia. *Proc. Natl. Acad. Sci. USA.* **81:** 5566–5570.
34. Fainaru, M., R. W. Mahley, R. L. Hamilton, and T. L. Innerarity. 1982. Structural and metabolic heterogeneity of β-very low density lipoproteins from cholesterol-fed dogs and from humans with Type III hyperlipoproteinemia. *J. Lipid Res.* **23:** 702–714.
35. Clevidence, B. A., R. E. Morton, G. West, D. M. Dusek, and H. F. Hoff. 1984. Cholesterol esterification in macrophages. Stimulation by lipoproteins containing apoB isolated from human aortas. *Arteriosclerosis.* **4:** 196–207.
36. Basu, S. K., Y. K. Ho, M. S. Brown, D. W. Bilheimer, R. G. W. Anderson, and J. L. Goldstein. 1982. Biochemical and genetic studies of the apoprotein E secreted by mouse macrophages and human monocytes. *J. Biol. Chem.* **257:** 9788–9795.
37. Nicoll, A., R. Duffield, and B. Lewis. 1981. Flux of plasma lipoproteins into human arterial intima. Comparison between grossly normal and atheromatous intima. *Atherosclerosis.* **39:** 229–242.
38. Stender, S., and D. B. Zilversmit. 1981. Transfer of plasma lipoprotein components and of plasma proteins into aortas of cholesterol-fed rabbits. Molecular size as a determinant of plasma lipoprotein influx. *Arteriosclerosis.* **1:** 38–49.
39. Musliner, T. A., C. Giotas, and R. M. Krauss. 1986. Presence of multiple subpopulations of lipoproteins of intermediate density in normal subjects. *Arteriosclerosis.* **6:** 79–87.
40. Koren, E., P. Alaupovic, D. M. Lee, N. Dashti, H. U. Kloer, and G. Wen. 1987. Selective isolation of human plasma low-density lipoprotein particles containing apolipoproteins B and E by use of a monoclonal antibody to apolipoprotein B. *Biochemistry.* **26:** 2734–2740.
41. Zilversmit, D. B. 1973. A proposal linking atherogenesis to the interaction of endothelial lipoprotein lipase with triglyceride-rich lipoproteins. *Circ. Res.* **33:** 633–638.
42. Esterbauer, H., G. Jürgens, O. Quehenberger, and E. Koller. 1987. Autoxidation of human low density lipoprotein: loss of polyunsaturated fatty acids and vitamin E and generation of aldehydes. *J. Lipid Res.* **28:** 495–509.
43. Tikkanen, M. J., T. G. Gole, and G. Schonfeld. 1983. Differential reactivity of human low density lipoproteins with monoclonal antibodies. *J. Lipid Res.* **24:** 1494–1499.
44. Morton, R. E., G. A. West, and H. F. Hoff. 1986. A low density lipoprotein-sized particle isolated from human atherosclerotic lesions is internalized by macrophages via a non-scavenger-receptor mechanism. *J. Lipid Res.* **27:** 1124–1134.
45. Steinbrecher, U. P., S. Parthasarathy, D. S. Leake, J. L. Witztum, and D. Steinberg. 1984. Modification of low density lipoprotein by endothelial cells involves lipid peroxidation and degradation of low density lipoprotein phospholipids. *Proc. Natl. Acad. Sci. USA.* **81:** 3883–3887.
46. Heinecke, J. W., L. Baker, H. Rosen, and A. Chait. 1986. Superoxide-mediated modification of low density lipoprotein by arterial smooth muscle cells. *J. Clin. Invest.* **77:** 757–761.
47. Aulinskas, T. H., D. R. van der Westhuyzen, E. L. Bierman, W. Gevers, and G. A. Coetzee. 1981. Retroendocytosis of low density lipoproteins by cultured bovine aortic smooth muscle cells. *Biochim. Biophys. Acta.* **664:** 255–265.

Downloaded from www.jlr.org by guest, on June 19, 2012