Characterization of very low density lipoprotein from Watanabe heritable hyperlipidemic rabbits

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Abstract We investigated the properties of very low density lipoprotein (VLDL) from two types of Watanabe heritable hyperlipidemic (WHHL) rabbits: one with a high incidence of coronary atherosclerosis (type 1), and the other with a low incidence (type 2). When incubated with mouse peritoneal macrophages, VLDL from type 1 WHHL rabbit (type 1-VLDL) stimulated cholesteryl ester synthesis 10.5-fold more than VLDL from the type 2 WHHL rabbit (type 2-VLDL) did. Moreover, a similar difference was seen in the stimulation of cholesteryl ester synthesis in peritoneal macrophages isolated from the WHHL rabbits. The mass ratios of cholesterol to protein in type 1- and type 2-VLDL were 5.69 and 2.05, respectively. Agarose gel electrophoresis of type 1-VLDL showed beta mobility, and that of type 2-VLDL showed pre-beta mobility. No difference was seen between the sizes of VLDL particles of the two types. The amount of apolipoprotein E in type 1-VLDL was greater than that in type 2-VLDL. ▪▪ In conclusion, the difference between type 1 and type 2 WHHL rabbits is at least partly due to the presence in type 1 animals of VLDL particles rich in cholesteryl esters and apolipoprotein E, particles which are very similar to β-VLDL in conformation. —**Ishii, K., T. Kita, M. Yokode, N. Kume, Y. Nagano, H. Otani, Y. Yamamura, S. Murayama, Y. Morimoto, Y. Teranishi, and C. Kawai.** Characterization of very low density lipoprotein from Watanabe heritable hyperlipidemic rabbits. *J. Lipid Res.* 1989. **30:** 1–7.

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Homozygous Watanabe heritable hyperlipidemic (WHHL) rabbits resemble human hyperlipidemic subjects in having an accumulation of cholesterol-rich particles in very low density lipoprotein (VLDL) and intermediate density lipoprotein classes, as well as in low density lipoproteins when maintained on a low fat diet, tendon xanthomas, severe atherosclerotic lesions of the aorta, and a genetic defect in the LDL receptors of all tissues (1–8). Although these human subjects die from coronary atherosclerosis between the ages of 3 and 30 years, the incidence of the disease in homozygous WHHL rabbits at an early age (4–12 mon) has been low (9, 10).

Recently, Watanabe, Ito, and Shiomi (11) found that WHHL rabbits could be classified into those with a high incidence (designated type 1 in this article) and a low incidence (designated type 2) of coronary atherosclerosis. After selective breeding, the incidence of coronary atherosclerosis rose from 21.8% to 58.9%, the development of coronary atherosclerosis occurred earlier, the mean serum lipid levels increased slightly, but the severity of aortic atherosclerosis showed no difference between the two types.

In cholesterol-fed animals and humans with type III hyperlipoproteinemia, elevated levels of plasma cholesteryl ester-rich lipoproteins, such as β-VLDL, lead to the deposition of large amounts of cholesteryl ester in macrophages throughout the body and eventually cause vascular disease (12, 13). Moreover, mouse peritoneal macrophages and human monocyte-macrophages take up β-VLDL and are transformed into foam cells in vitro (14–18). In WHHL rabbits, cholesterol-rich VLDL appears to play an important role in atherosclerosis (19). While studying the stimulative effect of cholesteryl ester synthesis by WHHL-VLDL, we noted that not all VLDL from WHHL rabbits strongly stimulated cholesteryl ester synthesis.

In this study, we focused on the properties of VLDL particles either from type 1 and type 2 WHHL rabbits

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and cholesterol-fed rabbits using an in vitro macrophage system. The results indicated that the properties of VLDL from type 1 WHHL rabbit were similar to those of β-VLDL. By analyzing VLDL, WHHL rabbits can be classified while they are alive and can be efficiently bred.

MATERIALS AND METHODS

Animals and materials

Female DDY mice (25–30 g), and Japanese White male and female rabbits (3–3.5 kg) were purchased from Shimizu Laboratories (Kyoto, Japan). Homozygous WHHL rabbits were raised both in Kobe and Kyoto. Mice were fed mouse laboratory chow (MF; Oriental Yeast Co. Ltd., Tokyo, Japan) and rabbits were fed rabbit laboratory chow (RC4; Oriental Yeast Co. Ltd., Tokyo, Japan). [1-^{14}C]Oleic acid (52.6 mCi/mmol) and cholesteryl [1,2,6,7-^{3}H(N)]oleate (79.8 Ci/mmol) were obtained from New England Nuclear (Boston, MA). Bovine serum albumin (BSA, catalog No. A 4378) was obtained from Sigma Chemical Co. (St. Louis, MO). Lactalbumin hydrolysate (catalog No. 670-1800) was obtained from GIBCO (Grand Island, NY). Fetal calf serum (FCS), obtained from MA. Bioproduct Co. (Walkersville, MD), was heat-inactivated (56°C, 30 min) before use. Dulbecco's modified Eagle's (DME) medium (catalog No. 05915) and Dulbecco's phosphate-buffered saline (PBS, catalog No. 05913) were purchased from Nissui Seiyaku Co., Ltd. (Tokyo, Japan). Liquid paraffin oil (catalog No. 26114) was obtained from Nakarai Chemicals Co. Ltd., (Kyoto, Japan). Plastic Petri dishes were obtained from Falcon, Division of Becton, Dickinson & Co. (Oxnard, CA). Other materials were obtained as previously described and were reagent grade (19).

Preparation of mouse and rabbit macrophage monolayers

Mouse peritoneal cells were harvested from unstimulated mice in PBS as described by Edelson and Cohn (19, 20). Peritoneal cells from WHHL rabbits pretreated with liquid paraffin oil were harvested in PBS as described by Gontijo and Wayne (21). The fluid, from either 20 to 40 mice (3 to 6 × 10^6 cells/mouse) or one rabbit, was pooled and the cells were collected by centrifugation (400 g, 10 min, 4°C) and washed once with 30 ml of PBS. The cells were resuspended in DME medium containing 10% (v/v) FCS, penicillin (100 U/ml), and streptomycin (100 μg/ml) at a final concentration of 2 to 3 × 10^6 cells/ml. In the case of rabbit, 0.2% lactalbumin hydrolysate was used instead of 10% FCS. Aliquots (1 ml) of this cell suspension were dispensed into plastic Petri dishes (35 × 10 mm) and

then incubated in a humidified CO$_2$ (5%) incubator at 37°C (19). After 2 hr, each dish was washed twice with 2 ml of DME medium without serum to remove the non-adherent cells. After the monolayers were incubated for 18 hr at 37°C in 1 ml of DME medium containing 10% FCS, the cells were washed with 2 ml of DME medium. Then 0.6 ml DME medium with lipoproteins was added to macrophage monolayers and incubated under the conditions described.

Lipoproteins

Blood was obtained from normal 6- to 8-month-old Japanese White and 6-month-old WHHL rabbits. VLDL (d < 1.006 g/ml) was isolated by ultracentrifugation from plasma prepared with anticoagulant EDTA (22). β-VLDL was isolated from Japanese White rabbits fed with 2% cholesterol and 10% corn oil diet for more than 14 days (12). All animals were fasted overnight prior to obtaining blood (22, 23), and in some cases the plasma from two or more normal Japanese White rabbits was pooled to obtain larger quantities of VLDL fractions.

Electron microscopy

Electron microscopic examination was performed according to the method described by Forte and Nordhausen (24). In brief, VLDL, filtered through a 0.45-μm filter, was dialyzed against 500 volumes of 3 mM sodium phosphate buffer, pH 7.4, and was then combined with the same volume of 2% sodium phosphotungstate, pH 7.2, to make the final concentration of 250 μg protein/ml in 1% sodium phosphotungstate. A small aliquot of the mixture was overlaid on a Formvar-carbon-coated grid and was examined under an electron microscope (Hitachi, H-7000).

Assays

The amounts of [^{14}C]oleate-albumin (9,255 cpm/nmol) incorporated into cellular cholesteryl [^{14}C]oleate by cell monolayers were measured as described by Brown et al. (25) with minor modifications (15). Protein determinations were made by the method of Lowry et al. with BSA as a standard (26). All data represent an average of duplicate incubations. Cholesterol concentrations were determined by enzymatic methods (27). Apolipoprotein E was assayed by electroimmunoassay as described by Laurell (28).

Miscellaneous

Agarose gel electrophoresis of lipoproteins, SDS polyacrylamide gel electrophoresis, and isoelectric focusing analysis of apolipoproteins were performed as previously described (7, 19, 29).

RESULTS

As shown in **Fig. 1**, the d < 1.006 g/ml lipoproteins (VLDL) from cholesterol-fed and type 1 WHHL rabbits (type 1-VLDL) produced a marked stimulation in cholesteryl [^{14}C]oleate synthesis in mouse peritoneal macrophages. The dose response curve showed saturation kinetics with half-maximal stimulation occurring at 10–20 μg protein/ml. VLDL from control rabbits fed commercial rabbit show, which had no cholesterol, showed no significant effect on cholesteryl ester synthesis. On the other hand, VLDL from type 2 WHHL rabbits (type 2-VLDL) stimulated cholesteryl ester synthesis, but was much less potent than type 1-VLDL.

The stimulative effects of type 1- and type 2-VLDL (75 μg/ml) on cholesteryl [^{14}C]oleate synthesis in the mouse peritoneal macrophages were about 148- and 14-fold higher than that of the control VLDL, i.e., 26.6, 2.52, and 0.18 nmol/mg protein per 6 hr, respectively (**Table 1**). We also examined the stimulative effects of VLDL from type 1 and type 2 WHHL rabbits on cholesteryl ester synthesis in peritoneal macrophages from homozygous WHHL rabbits. The difference observed between these two lipoproteins in the stimulation of cholesteryl ester synthesis was similar to that seen in mouse peritoneal macrophages (**Fig. 2**).

On agarose gel electrophoresis, type 1-VLDL migrated to the same position as β-VLDL, whereas the mobility of type 2-VLDL was nearly the same as that of the control VLDL (**Fig. 3**). This suggested that the properties of type 1-VLDL were similar to those of β-VLDL. As shown in Table 1, the mass ratios of cholesterol to protein in type 1-VLDL and β-VLDL are higher than that of type 2-VLDL.

No remarkable differences in particle size were observed (**Fig. 4**). Analysis by high performance liquid chromatography showed similar results (data not shown). Apolipoprotein components examined by 10% SDS-polyacrylamide gel electrophoresis (**Fig. 5**) showed that,
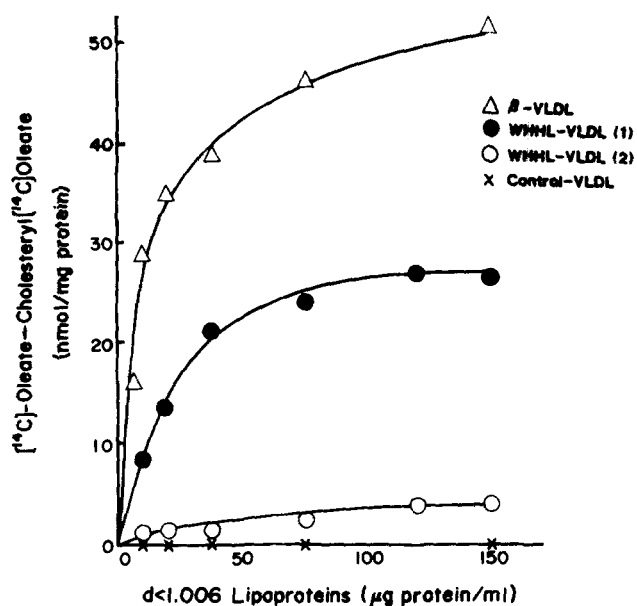

Fig. 1. Cholesteryl ester synthesis in mouse macrophages incubated with different concentrations of d < 1.006 g/ml lipoproteins from cholesterol-fed, control, type 1 WHHL, and type 2 WHHL rabbits. Each monolayer received 0.6 ml of DME medium containing 0.2 mM [^{14}C]oleate-2.4 mg/ml BSA and the indicated concentrations of d < 1.006 g/ml lipoproteins obtained from one of the following animals: (●) type 1 WHHL rabbit; (○) type 2 WHHL rabbit; (△) cholesterol-fed rabbit; (X) control rabbit. After incubation for 6 hr at 37°C, the cellular contents of cholesteryl [^{14}C]oleate were determined. The content of cholesteryl [^{14}C]oleate in the cells incubated without d < 1.006 g/ml lipoproteins was 0.18 n mol/mg protein. Points are the mean of duplicate cultures.

although the apolipoprotein (apo) B contents were almost the same, the apoE contents increased in β-VLDL and type 1-VLDL. As the yields of VLDL were different in each group, the apoE contents were compared by their ratios to VLDL protein (**Table 2**). This ratio was high in both β-VLDL and type 1-VLDL, and was low in type 2-VLDL, as well as in control VLDL. The qualitative difference of apoE was examined by isoelectric focusing

TABLE 1. Stimulation of cholesteryl ester synthesis in mouse peritoneal macrophages by lipoproteins from control, WHHL, and cholesterol-fed rabbits

Source of Lipoproteins	Lipoprotein Fraction Added to Medium	Cholesteryl [^{14}C]Oleate	Mass Ratio Cholesterol/Protein
		nmol/mg protein	
	None	0.18	
Control rabbit	VLDL[a]	0.18	0.82
WHHL rabbit (Type 1)	VLDL[a]	26.6 ± 0.34	5.69
WHHL rabbit (Type 2)	VLDL[a]	2.52 ± 0.44	2.05
Cholesterol-fed rabbit	β-VLDL[a]	47.2 ± 3.16	13.6

Each monolayer received 0.6 ml of DME medium containing 0.2 mM [^{14}C]oleate with 2.4 mg/ml albumin in the presence of 75 μg/ml protein of the indicated lipoprotein fraction. After incubation for 6 hr at 37°C, the cellular content of cholesteryl [^{14}C]oleate was determined. Each data point represents the mean ± SD of values from three animals.
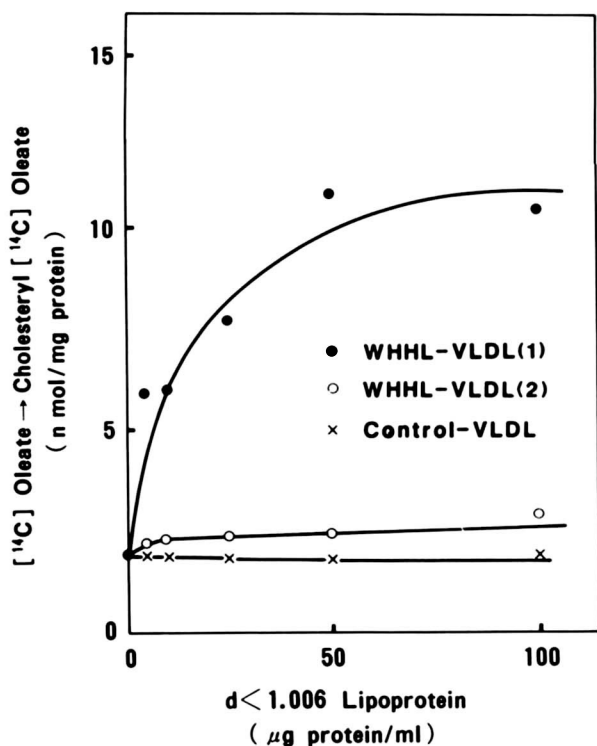
[a]Density < 1.006 g/ml.

Fig. 2. Cholesteryl ester synthesis in peritoneal macrophages from WHHL rabbit incubated with different concentrations of d < 1.006 g/ml lipoproteins from control, type 1 WHHL, and type 2 WHHL rabbits. Each monolayer received 0.6 ml of DME medium containing 0.2 mM [^{14}C]oleate-2.4 mg/ml BSA and the indicated concentrations of d < 1.006 g/ml lipoproteins obtained from one of the following animals: (●) type 1 WHHL rabbit; (○) type 2 WHHL rabbit; (X) control rabbit. After incubation for 6 hr at 37°C, the cellular contents of cholesteryl [^{14}C]oleate were determined. The content of cholesteryl [^{14}C]oleate in the cells incubated without d < 1.006 g/ml lipoproteins was 1.9 n mol/mg protein. Points are the mean of duplicate cultures.

Fig. 3. Agarose gel electrophoresis of VLDL. Aliquots (25 μg of protein) of VLDL from type 1 (A), type 2 (B), cholesterol-fed (C), and normal (D) rabbit were subjected to agarose gel electrophoresis and stained with Fat Red 7B.

(IEF) followed by densitometer analysis. As the amount of apoE obtainable from normal rabbit was very low, we used apoE obtained from a cholesterol-fed normal rabbit as a substitute. Major isoforms determined by IEF electrophoresis were E-3,4,5,6 in β-VLDL, E-4,5,6 in type 1-VLDL, and E-5,6 in type 2-VLDL (**Fig. 6**). In addition, the amounts of apoCs increased more in type 2-VLDL than in type 1- and β-VLDL.

DISCUSSION

Our results clearly indicate that VLDL from the two types of WHHL rabbits have marked differences in stimulating cholesteryl ester synthesis and suggest that the differences come from the properties of VLDL from type 1 WHHL rabbits which are quite similar to those of β-VLDL. Wakasugi et al. (30) analyzed lipoprotein compo-
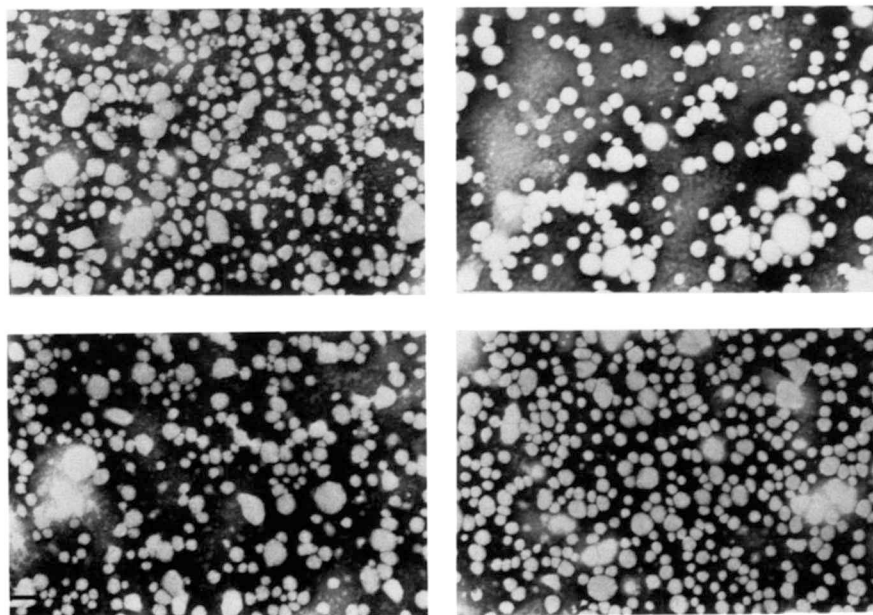

Fig. 4. Transmission electron microscopy of VLDL from normal (left upper), cholesterol-fed (right upper), type 1 WHHL (left lower), and type 2 WHHL (right lower) rabbit. Lipoproteins were stained with 1% phosphotungstic acid, pH 7.2. Magnification 33,500x. The bar (left lower) represents 100 nm.
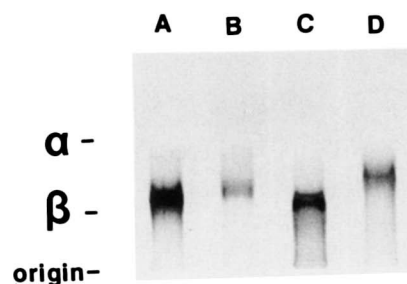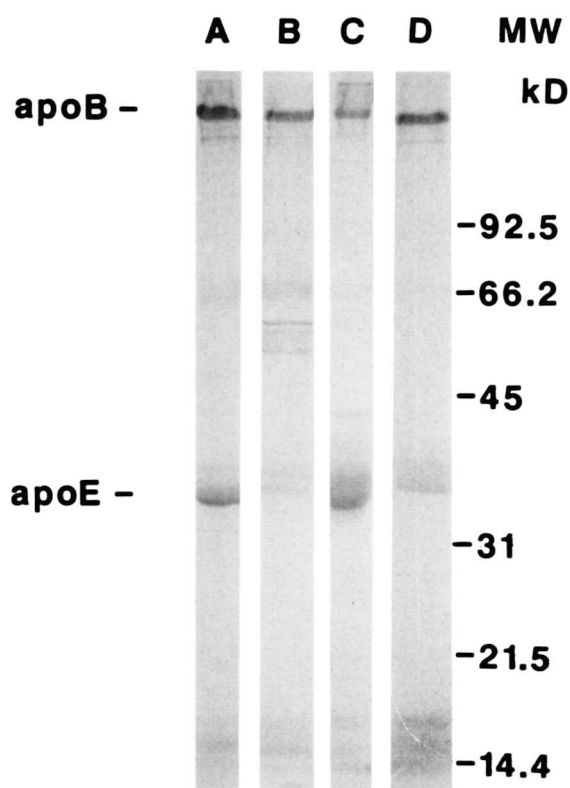
Fig. 5. SDS polyacrylamide gel electrophoresis of VLDL apolipoproteins from type 1 (A), type 2 (B) WHHL rabbits, cholesterol-fed (C), and normal (D) rabbits. Lipoproteins were delipidated by extraction with acetone–ethanol 1:1 (v/v), at −20°C. The apolipoprotein precipitate was dissolved in a buffer containing 10% glycerol, 5% 2-mercapteoethanol, 2.3% SDS, and 62.5 mM Tris-HCl(pH 6.8). After heating at 90°C for 3 min, 50 μg of each protein was applied and electrophoresis was carried out on 7% polyacrylamide gel. The gel was stained with Coomassie brilliant blue, destained, and photographed. Molecular weight markers are shown.

sitions in WHHL rabbits and concluded that they were the same as those in the control rabbits and were different from those in cholesterol-fed rabbits. Our findings showed that lipoprotein and apolipoprotein compositions in WHHL rabbits varied. The first difference was that the

cholesterol/protein ratio in type 1-VLDL was much higher than that in type 2-VLDL. In VLDL from cholesterol-fed dogs, Innerarity, Pitas, and Mahley (31) found a correlation between [¹⁴C]oleate incorporation into macrophages and the cholesterol/protein ratio of the VLDLs. The increased cholesterol/protein ratio in type 1-VLDL may play an important role in accelerating the accumulation of cholesteryl ester droplets.

Another difference was in apoE concentrations in VLDL. Havel et al. (32) analyzed lipoprotein composition in WHHL rabbits and reported that apoE increased in plasma and VLDL. Our results showed that the apoE level in type 1-VLDL increased more than that in type 2-VLDL (Fig. 5, Table 2). There is some evidence that apoE is the recognition factor for the uptake of β-VLDL by macrophages (33, 34). Therefore, the increase in the amount of apoE in type 1-VLDL may be responsible for the receptor-mediated binding and uptake of type 1-VLDL by macrophages.

We previously reported that WHHL-VLDL is recognized by the receptor for β-VLDL in mouse peritoneal macrophages (19). Koo, Wernette-Hammond, and Innerarity (35) and Ellsworth, Kraemer, and Cooper (36) have suggested that the uptake of β-VLDL by mouse peritoneal macrophages was mediated by a murine LDL receptor that cross-reacted with antibodies to bovine (35) or rat (36) LDL receptor, and its apparent molecular weight was 5000 less than that of the classical LDL receptor (37, 38). This receptor might be involved in the uptake of WHHL-VLDL.

The IEF pattern also showed that anionic C apolipoproteins were correspondingly less prominent in type 1-VLDL than in type 2-VLDL (Fig. 6). Havel et al. (32), noted that WHHL-VLDL contained less apoC than control VLDL. It is known that apoC-III retards the catabolism of chylomicron remnants in rat liver (37), and the degradation of β-VLDL by rat peritoneal macrophages was also retarded in the presence of apoC-III (38). Although the effect of apoC-III is uncertain, it might be implicated in the difference between type 1- and type 2-VLDL uptake by macrophages.

TABLE 2. Ratios of apoE concentration to protein concentration of VLDL

Lipoproteins	ApoE Concentration	Protein Concentration	Ratio of ApoE/Protein
	mg/dl	*mg/ml*	
Control VLDL	9.4	0.5	0.19
Type 1-VLDL	23.5	0.7	0.34
Type 2-VLDL	7.4	0.36	0.21
β-VLDL	62.0	1.3	0.48

VLDL samples were electrophoresed in the gel (containing 0.75% agarose, 3.5% dextran, and 3.5% anti-rabbit apoE serum) at 50 V for 15 hr. The height of each rocket was compared to a standard curve and apoE concentrations were calculated. As protein concentrations were different between experiments, one representative experiment is displayed.
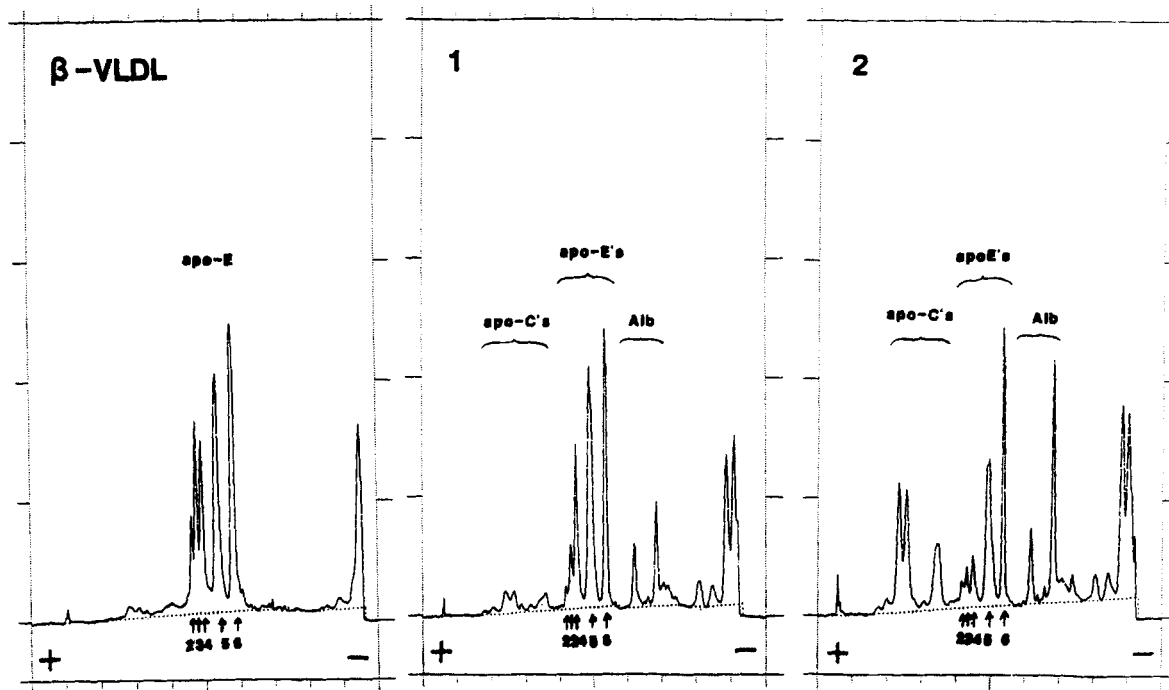
Fig. 6 Densitometric pattern of VLDL from cholesterol-fed (left), type 1 (middle), and type 2 (right) WHHL rabbits. VLDL apolipoproteins (200 μg protein) were obtained by acetone–ethanol 1:1 (v/v) delipidation of VLDL fractions at −20°C. Apolipoprotein E isoforms in apoVLDL were analyzed by isoelectric focusing on polyacrylamide gels. Focusing was carried out for 3,600 V-hr at 10°C. The proteins were fixed with 5% trichloroacetic acid and 5% sulfosalicylic acid, and stained with 0.1% Coomassie Brilliant Blue R-250. The gels were scanned at 570 nm with a densitometer.

In conclusion, type 1-VLDL may be the progenitor of the atheromatous plaques in coronary arteries and other peripheral arteries, like β-VLDL. Therefore, the existence of apoE and cholesteryl ester-rich VLDL particles plays an important role in the progression of coronary atherosclerosis in type 1 WHHL rabbits. ∎

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