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Research Article

Aortic atheromatous plaques regress slowly in cholesterol-fed rabbits that have been returned to normal laboratory diet. To delineate metabolic factors potentially responsible for persistence of atherosclerosis under these conditions, the physical, chemical, and metabolic characteristics were determined for lipoproteins of d less than 1.006 g/ml; such lipoproteins are thought to be the major determinant of progression of atherosclerotic lesions in cholesterol-fed rabbits. At the time of return to a normal laboratory diet regimen after 3 mo of feeding with cholesterol-enriched laboratory diet, plasma cholesterol concentrations were 2,275 +/- 252 mg/dl, mostly attributable to cholesteryl ester-rich very low density lipoproteins (VLDL). On the hypercholesterolemic diet, fractional catabolic rates of plasma clearance of 125I-labeled VLDL were reduced (0.011 +/- 0.002 vs. 0.151 +/- 0.015 h-1), but the total VLDL catabolic rate was increased considerably (17.1 +/- 2.2 vs. less than 1.2 +/- 0.4 mg of protein/kg X d), because of the expansion of the endogenous pool of cholesteryl ester-rich VLDL. The total catabolic rate of VLDL was maintained above estimated control values (5.8 +/- 0.7 mg protein/kg X d) even 10 wk after return of the rabbits to a normal chow regimen, an effect attributable to continued high rates of cholesteryl ester-rich VLDL synthesis in liver. Accumulation of cholesteryl ester-rich VLDL into aortic tissue persisted at a high rate. Thus the persistence […]

Metabolism of Very Low Density Lipoproteins after Cessation of Cholesterol Feeding in Rabbits

A Factor Potentially Contributing to the Slow Regression of Atheromatous Plaques

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Abstract

Aortic atheromatous plaques regress slowly in cholesterol-fed rabbits that have been returned to normal laboratory diet. To delineate metabolic factors potentially responsible for persistence of atherosclerosis under these conditions, the physical, chemical, and metabolic characteristics were determined for lipoproteins of $d < 1.006$ g/ml; such lipoproteins are thought to be the major determinant of progression of atherosclerotic lesions in cholesterol-fed rabbits. At the time of return to a normal laboratory diet regimen after 3 mo of feeding with cholesterol-enriched laboratory diet, plasma cholesterol concentrations were 2,275±252 mg/dl, mostly attributable to cholesteryl ester-rich very low density lipoproteins (VLDL). On the hypercholesterolemic diet, fractional catabolic rates of plasma clearance of ^{125}I-labeled VLDL were reduced (0.011±0.002 vs. 0.151±0.015 h^{-1}), but the total VLDL catabolic rate was increased considerably (17.1±2.2 vs. <1.2±0.4 mg of protein/kg·d), because of the expansion of the endogenous pool of cholesteryl ester-rich VLDL. The total catabolic rate of VLDL was maintained above estimated control values (5.8±0.7 mg protein/kg·d) even 10 wk after return of the rabbits to a normal chow regimen, an effect attributable to continued high rates of cholesteryl ester-rich VLDL synthesis in liver. Accumulation of cholesteryl ester-rich VLDL into aortic tissue persisted at a high rate. Thus the persistence of aortic atheromatous lesions after cessation of cholesterol feeding was attributable in part to continued high rates of hepatic production of cholesteryl ester-rich VLDL and its persistent delivery into the aortic wall.

Introduction

Atherosclerotic vascular disease is the leading cause of mortality in the Western industrial countries. In view of the prevalence of atherosclerosis, mechanisms by which atherosclerotic lesions may regress are of particular interest. Regression of established atheromatous lesions has been studied in several species exposed to modified diets or drugs (1) including rabbits previously fed cholesterol-supplemented diets. The rabbit responds very rapidly to changes in dietary cholesterol with increased concentrations of plasma cholesterol, changed lipoprotein profiles, and the appearance of extensive atheromatous lesions in the aorta. The extent of regression of aortic atheromatous lesions in cholesterol-fed rabbits returned to normal laboratory diet regimens is controversial (2, 3). Judging from determinations of cholesterol in the entire aorta, little or no regression occurs in the first 3 mo after cessation of the high cholesterol diet (4–6). In fact, paradoxical increases in aortic cholesterol content may occur after cessation of the atherogenic diet (7). Although mechanisms responsible for the slow regression of aortic atheromatous lesions in previously cholesterol-fed rabbits have been shown to include a slow rate of return of plasma cholesterol concentrations to normal levels (2), factors responsible for the persistent hypercholesterolemia and the persistent atherogenesis have not been elucidated.

Associations have been reported between the frequency of occurrence of atheromatous lesions and the appearance in plasma of cholesterol-fed dogs of very low density lipoprotein, (VLDL) which is enriched in cholesteryl esters and which migrates electrophoretically in the β-position (8). VLDL obtained from hypercholesterolemic rabbits is a powerful stimulant of cholesterol ester formation in cultured cells (9). Furthermore, labeled VLDL from hypercholesterolemic rabbits accumulates in rabbit aortic atheromatous lesions both in vitro (10) and in vivo (11). Hence VLDL in plasma of continuous cholesterol-fed rabbits appears to be potentially atherogenic.

The present study was designed to identify metabolic factors potentially responsible for the slow regression of aortic plaques in cholesterol-fed rabbits returned to normal laboratory diet, by analysis of plasma concentrations, composition, kinetics of clearance, and aortic uptake of the potentially atherogenic lipoprotein, VLDL. Our results demonstrate increased flux of VLDL-protein through plasma and enhanced aortic accumulation of VLDL-protein and cholesterol in association with intake of a cholesterol-enriched diet. The observed abnormalities including aortic accumulation of VLDL-protein persisted for at least 10 weeks after return of the hypercholesterolemic rabbits to a normal laboratory diet.

Methods

Dietary regimens and experimental design. New Zealand rabbits (3–3.5 kg) were obtained from a single source (Eldridge Laboratory Animals, St. Louis, MO) and housed individually in stainless steel cages. Food and water were available ad libitum. Four groups of recipient rabbits were used: Group 1 was fed a 2% wt/wt cholesterol-enriched diet for 11–13 wk, then returned to normal laboratory diet, and studied after 0, 4, and 10 wk of normal feeding of laboratory diet. Group 2 was fed normal laboratory diet, and studied at an interval corresponding to those for group 1. Group 3 was fed a cholesterol-enriched diet for 12 wk with a schedule initiated so that the end of the diet period coincided with the 10 wk of return to normal laboratory diet of group 1. Group 4 was fed normal laboratory diet and studied at intervals, corresponding to those for group 3. For statistical analysis, the data from groups 2 and 4 (i.e., rabbits fed normal laboratory diet) were combined in view of the lack

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of any apparent differences between results in the two groups. A donor group of rabbits was fed the cholesterol-enriched diet for ~12 wk and used for harvesting cholesteryl ester-rich VLDL after fasting for 18 h.

Characterization of lipoproteins and plasma lipids. Lipoprotein and plasma triglycerides, cholesteryl esters, and cholesterol were assayed enzymatically with commercially available kits (Bio-Dynamics, Boehringer Mannheim Corp., Indianapolis, IN and Wako Chemical Company, Dallas, TX). Protein was assessed by the method of Lowry et al. (12) with bovine serum albumin used as a standard. Phospholipids in plasma were first extracted. Plasma and lipoprotein phospholipid phosphorous were assayed by the method of Fiske and Subbarrow (13).

Electrophoretic mobility of lipoproteins was assessed with agarose gels (1% wt/vol) stained with Fat Red O (Corning Medical and Scientific, Palo Alto, CA). Apolipoprotein composition was determined with 3–27% wt/vol sodium dodecyl sulfate-polyacrylamide gels (Integrated Separation Systems, Newton, MA). Apolipoproteins were stained with Coomassie blue with migration compared to that of molecular weight standards of albumin, ovalbumin, chymotrypsin and cytochrome *c*. Gels lanes were scanned with a LKB 2202 Ultrascan laser densitometer (LKB Instruments, Inc., Gaithersburg, MD) and the relative content of apolipoproteins in cholesteryl ester-rich VLDL was determined from the area under the curve of the scans as calculated by digital planimetry. VLDL was sized by high performance liquid chromatography performed on a Varian model 5000 (Varian Associates, Inc., Palo Alto, CA) equipped with a TSK guard column (TSK America Inc., North Bend, WA) (7.5 × 1,000 mm) and a TSK GS5000PW column (7.5 × 6,000 mm) and with a mobile phase of sodium chloride (0.15 M) and sodium azide (0.02% wt/vol). Eluent from the column was monitored continuously at 280 nm with a Varian UV-50 detector. Distribution of cholesterol in eluted fractions was determined enzymatically with modified commercially available assay kits (Wako Chemical Company).

Preparation of donor lipoproteins. Kinetics of plasma clearance of ^{125}I-labeled cholesteryl ester-rich VLDL were evaluated at three intervals with freshly prepared lipoproteins used for each experiment. Plasma was obtained by bleeding from the abdominal aorta of a pentobarbital-anesthetized hypercholesterolemic donor rabbit which had been fasted for 16 h. The VLDL fraction was obtained by ultracentrifugation in a Beckman L8-55 (Beckman Instruments, Inc., Palo Alto, CA) with the use of Quick-Seal tubes (Beckman Instruments, Inc.) in a 60Ti rotor. Plasma (containing 1 mM EDTA) was centrifuged at 50,000 rpm for 18 h at 14°C. The infranatant fraction was centrifuged again in EDTA (1 mM)/sodium chloride (0.15 M) (d = 1.006 g/ml, pH 8.4). All lipoproteins were used within 2 wk after the plasma had been obtained.

Radioiodination. Cholesteryl ester rich-VLDL was radioiodinated with ^{125}I or ^{131}I with the iodine monochloride technique as described by McFarlane (14). Free iodide and radiolabeled-lipoprotein were separated by gel filtration through Sephadex G25 (0.9 × 30.0 cm) followed by dialysis against EDTA (1 mM)/sodium chloride (0.15 M) (pH 8.4) with three changes over 24 h. 84% of radioactivity in ^{125}I-labeled VLDL was precipitable by tetramethyl urea. Thus the particle was labeled predominantly in apolipoprotein B. As assessed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis, the percentage of apoprotein labeling was 77% for apolipoprotein B, 22% for apolipoprotein E, and negligible for the apolipoprotein C's. Greater than 94% of radioactivity was precipitable by trichloroacetic acid (10% wt/vol) for all preparations of radioiodinated tracers.

^{125}I and ^{131}I (as Na^{125}I and Na^{131}I carrier-free) were obtained from the Amersham Corp., Arlington Heights, IL.

Plasma clearance of radioiodinated lipoproteins. Plasma clearance kinetics of ^{125}I-labeled cholesteryl ester-rich VLDL were determined at 0, 4, and 10 wk after animals had been returned to normal feeding of laboratory diet. At each interval, a group of rabbits maintained on normal diet was studied concurrently. At week 10, another group which had been maintained on a continuous cholesterol-enriched diet for 12 wk was studied as well.

At week 0 and 4 for the hypercholesterolemic animals returned to normal chow, ^{125}I-labeled cholesteryl ester-rich VLDL was administered in 1 ml of EDTA-saline (4 × 10^6 cpm, 50 μg of protein, i.v.). At week 10, ^{125}I-labeled VLDL was administered in 1.2 ml of EDTA-sodium chloride (33 × 10^6 cpm, 300 μg of protein, i.v.). All other experimental groups received the same quantity of the identical lipoprotein.

Radiolabeled lipoproteins were injected into the left marginal ear vein of recipient rabbits. Blood samples from the vein of the opposite ear were obtained in tubes containing EDTA (1.5 mg/ml) at selected intervals. Radioactivity in whole plasma aliquots and trichloroacetic acid-soluble fraction was determined with an Isoflex gamma counter (Micromedic, Horsham, PA).

To determine whether cholesteryl ester-rich VLDL from continuously cholesterol-fed rabbits was a suitable tracer for use in animals returned to normal laboratory diet, VLDL was isolated from a cholesterol-fed rabbit and from a hypercholesterolemic rabbit that had been returned to normal laboratory diet for 10 wk. These lipoproteins were radiolabeled with ^{131}I and ^{125}I, respectively. Radiolabeled VLDL preparations were then injected simultaneously into continuously cholesterol-fed rabbits and hypercholesterolemic animals that had been returned to normal laboratory diet for 10 wk.

After correction for spillover of ^{131}I radioactivity into the ^{125}I channel, radioactivity was expressed as counts per minute per milliliter of plasma. The calculated radioactivity present at 0 min was estimated from the injected counts divided by the calculated plasma volume and regarded as the 100% value. Radioactivity at each subsequent interval was expressed as a percentage of the zero time value.

Kinetics of plasma clearance of ^{125}I- and ^{131}I-labeled lipoproteins were analyzed by the method of Matthews (15) as described previously (11). Total catabolic rates were calculated as the product of the fractional catabolic rate and pool size in plasma. In rabbits in which endogenous pools were below limits of detection (<1 mg/dl for lipoprotein protein), it was not possible to calculate total catabolic rates definitively. Consequently, estimates were based on an assumed plasma concentration of 1 mg/dl. These estimates, although subject to error, provide reasonable boundary values for kinetic parameters. The parameters were so dramatically different from those in other groups that they are included for comparison.

24 h after injection of the radiolabeled lipoprotein, blood samples taken from the central ear artery were placed into tubes containing EDTA (1.5 mg/ml). 3-ml samples of plasma were fractionated by sequential ultracentrifugation with density cuts (g/ml) d < 1.006, 1.006–1.063, d > 1.21. Each centrifugation was performed with Beckman Quick-Seal tubes in a 40Ti rotor centrifuged at 40,000 rpm for 18 h at 14°C in a Beckman L8-55 ultracentrifuge. Cholesterol content and radioactivity were determined for each density cut and expressed as percentages in each fraction of total counts.

Determination of plasma volume. Calculation of fractional catabolic rate required an accurate assessment of plasma volume for calculation of the concentration of radioactivity present in plasma immediately after injection of radiolabel. This consideration is particularly pertinent to the present study because plasma volume may change after cholesterol feeding in rabbits (16). Two common methods of determination of plasma volume involve use of Evans blue or ^{125}I-labeled albumin. Neither was used in this study because the hyperlipidemic plasma represents a source of error in the spectrophotometric measurement of dye and because long-term housing of radioactive animals is logistically difficult. Instead, a vascular tracer, transferrin, was labeled with the positron-emitting isotope gallium-68 (^{68}Ga) providing advantages attributable to its short physical half-life (68 min). Approximately 0.5 mCi of generator-produced ^{68}Ga coupled to transferrin was injected into the marginal ear vein of the rabbit and blood samples taken at 5 and 15 min from the vein of the opposite ear. Plasma radioactivity was determined with a Beckman gamma counter. After correction for radioactive decay of ^{68}Ga, the activity in plasma was extrapolated to 0 time and plasma volume calculated. Calculated plasma volume of rabbits maintained on normal laboratory diet was 28±2 ml/kg (n = 4) compared with 33±2 ml/kg (n = 4) for the hypercholesterolemic group.

Preparation of aortic and hepatic tissues. 24 h after injection of the radiolabeled lipoproteins in the experiments performed 10 wk after return to a normal laboratory diet regimen, the rabbits were killed with an

overdose of pentobarbital. Visceral and thoracic cavities were opened rapidly and irrigated with cold saline. Aortas were exposed from the arch to the bifucation and removed. Extraneous tissue was dissected free and the aortas cut to expose the intimal surface. This procedure required ~15 min. Five aortic segments were prepared: arch and upper thoracic, thoracic, upper, mid- and lower abdominal as described previously (11). Segments were blotted lightly and weighed. Samples were then frozen in 12 × 75-mm test tubes. Radioactivity of ^{125}I and ^{131}I in the still frozen samples was determined simultaneously with an Isoflex gamma counter. After radioactivity had been determined, the tissues were thawed and homogenized in sodium chloride (0.15 M, pH 7.4) with a Polytron (Brinkmann Instruments, Westbury, NY). Homogenized tissue was extracted according to the Bligh and Dyer procedure (17). The organic phase was removed and prepared for determination of cholesterol. The aqueous phase was incubated with trichloroacetic acid (10% wt/vol) for 1 h at 4°C followed by centrifugation at 3,000 rpm for 30 min in a Beckman J6 centrifuge. The supernatant fraction was decanted and radioactivity of ^{125}I and ^{131}I determined in both infranate and supernate.

Spill-over of ^{131}I radioactivity into the ^{125}I channel was corrected in all measurements. To calculate mass of lipoprotein-protein present in aorta segments, the median specific activity of lipoprotein-protein was calculated from the area under the specific radioactivity–time–plasma clearance curves. Areas were determined with a Hewlett Packard 9864A digitizer (Hewlett-Packard Co., Palo Alto, CA).

Livers were excised and weighed. A segment from each was extracted according to the Bligh and Dyer procedure (17). The organic phase was removed and dried under nitrogen. Extracted material of hepatic and aortic tissue was then resuspended in isopropyl alcohol. Total cholesterol was determined enzymatically with commercial test kits (Boehringer Mannheim).

Statistical analysis. Values are means±standard error of the mean. Student's *t* test (two-tailed) was used to determine statistical significance with a probability level of <5%.

Results

Concentrations of plasma lipids. Three experimental groups were used including rabbits fed normal laboratory diet, a group fed a cholesterol-enriched diet for 12 wk, and rabbits fed a cholesterol-containing diet for 12 wk followed by normal laboratory diet for 10 wk. As has been documented previously, cholesterol feeding elevated plasma cholesterol concentrations grossly (Fig. 1 *A*). Hyperphospholipidemia was present in the cholesterol-fed (576±75 mg/dl) compared to values in normal laboratory diet-fed animals (94±22 mg/dl). After return of animals to normal laboratory diet, plasma cholesterol concentrations declined only slowly. 10 wk after return to normal laboratory diet, plasma cholesterol concentrations were 351±35 mg/dl, which was still grossly elevated compared with values in concurrent controls (80±11 mg/dl). Hyperphospholipidemia regressed slowly also, but plasma concentrations at 10 wk were no longer significantly greater than those in controls (178±28 compared with 98±21 mg/dl). No changes were observed in plasma concentrations of triglycerides during the period of return to normal laboratory diet.

Most of the increased plasma cholesterol in continuously cholesterol-fed animals was in the $d < 1.006$ g/ml fraction (Table I). No cholesterol was observable in the $d > 1.063$ g/ml plasma fraction; results which are in agreement with the work of Roth et al. (18). 10 wk after hypercholesterolemic animals had been returned to normal laboratory diet, cholesterol was still not detectable in the $d > 1.063$ g/ml fraction (Table I).

Chemical and physical characterization of VLDL. VLDL was characterized over the period of return to normal laboratory diet by chemical composition, apoprotein composition, electrophoretic mobility, and size distribution. VLDL harvested from continuously cholesterol-fed rabbits was enriched in cholesteryl esters (57.67±0.54%) with only a small percentage of triglyceride present (3.12±0.78%). Despite reduced plasma cholesterol concentrations after 4 wk of return to normal diet, the chemical composition was not significantly different from that observed at week 0. At week 10, changes in chemical composition of VLDL were evident. There were a relative depletion of cholesteryl esters and an increase in triglyceride and protein. The relative contents of free cholesterol and phospholipids were not significantly different for any of the harvested VLDLs (Table II).

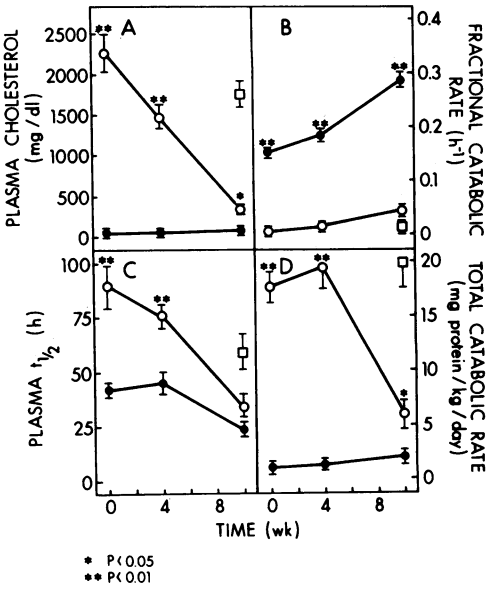

Figure 1. (*A*) Plasma cholesterol concentrations; (*B*) fractional catabolic rate of plasma clearance of ^{125}I-labeled VLDL; (*C*) half time of elimination of ^{125}I-labeled VLDL; and (*D*) total catabolic rate of cholesteryl ester-rich VLDL; in group fed normal diet (●, $n = 7$), cholesterol-fed group (□, $n = 4$), and the hypercholesterolemic group returned to a normal diet regimen (○, $n = 4$). The time axis represents the number of weeks after the hypercholesterolemic animals had been returned to normal laboratory diet. At week 10, a group of continuously cholesterol-fed animals was studied concomitantly with the normal diet fed and previously cholesterol-fed rabbits. Values were measured 0, 4, and 10 wk after the hypercholesterolemic group had been returned to a normal diet regimen and are means±standard errors. *$P < 0.05$; **$P < 0.01$.

Table I. Cholesterol Content in Fractions of Plasma

Diet group	Density fractions (g/ml) <1.006	1.006–1.063	>1.063
	mg/dl		
Normal laboratory diet (7)	ND	30±14	20±5
Cholesterol-fed (4)	998±84	458±66	ND
Hypercholesterolemic animals returned to normal diet (4)	164±31	242±49	ND

(*n*) Number of animals in each group; ND, not detectable. Hypercholesteremic group had been on normal diet for 10 wk before determinations were obtained.

Table II. Percent Composition of Very Low Density Lipoproteins at Selected Intervals after Return of Animals to a Normal Laboratory Diet Feeding Regimen

Weeks after return to normal diet	Protein	Cholesteryl esters	Free cholesterol	Triglycerides	Phospholipid
	%	%	%	%	%
0	6.15±0.53	57.17±0.54	14.72±0.68	3.12±0.98	17.99±0.42
4	7.20±0.51	58.91±1.50	13.73±0.33	4.60±0.86	15.56±0.61
10	9.95±1.41	47.07±3.56	11.83±0.76	15.04±3.09	16.10±0.45

Values are the means±standard errors of four observations in each case.

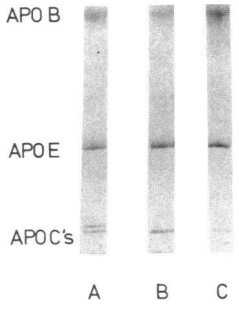

Figure 3. Apoprotein composition of VLDL during return to normal laboratory diet. Gradient (3-27% wt/vol) sodium dodecyl sulfate-polyacrylamide gels stained with Coomassie blue. Apolipoproteins of VLDL obtained from rabbit plasma after return to a normal diet regimen at week 10 (lane *A*), 4 (lane *B*), 0 (lane *C*).

Changes in electrophoretic mobility of plasma VLDL on agarose gels were noted after hypercholesterolemic rabbits had been returned to normal laboratory diet. During the interval of cholesterol feeding, plasma VLDL migrated exclusively in the β position. After animals had been returned to normal diet electrophoretic mobility of plasma VLDL became greater, but migration was still not in the pre-β position even after 10 wk (Fig. 2).

VLDL harvested from continuously cholesterol-fed rabbits contained predominantly apolipoprotein E (66%) and apolipoprotein B (28%), and small quantities of apolipoprotein C's (16%). As a function of the duration of feeding of normal diet, the VLDL was relatively depleted of apolipoprotein E with an increased presence of apolipoprotein C (28% at week 10; Fig. 3).

Distribution of subfractions of VLDL was characterized after return to normal laboratory diet by high performance liquid chromatography. VLDL from rabbits at time of return to normal diet was resolved into two major subfractions. A large sized subfraction (FxI) eluted in the void volume at 23 min and the other (FxII) eluted at 29 min (Fig. 4 *A*). Distribution of cholesterol was principally (>95%) in FxII although the cholesterol content of FxI was clearly discernible (Fig. 4 *D*). At week 4, FxI was barely detectable by optical density at 280 min (Fig. 4 *B*). It did not contain significant amounts of cholesterol (Fig. 4 E). FxII eluted at the same position. By week 10, FxI was not discernable by either technique. FxII eluted somewhat more slowly (31 min; Fig. 4 *C* and *F*).

Plasma clearance of radiolabeled lipoproteins. Kinetics of plasma clearance of ^{125}I-labeled VLDL were altered after 12 wk of cholesterol feeding compared to kinetics in normal chow-fed animals. Half time of elimination (ln 2/β) was prolonged greatly after cholesterol feeding (Fig. 1 *C;* see also Fig. 5 *B*). After 24 h, 7% of initially injected ^{125}I was present in the plasmas of rabbits fed laboratory diet. In contrast, 36% was present in the cholesterol-fed group. Fractional catabolic rate of ^{125}I-labeled VLDL was 0.011±0.004 h^{-1} for the hypercholesterolemic group after ~12 wk of intake of the cholesterol-enriched diet (Fig. 1 *B*). In the concurrently studied normal diet-fed group, the fractional catabolic rate was 0.151±0.012 h^{-1}. Although the fractional catabolic rate was decreased, the highly elevated plasma concentrations of VLDL protein indicated that total catabolic rates remained high in these groups (17.1±2.2 compared with <1.2±0.4 mg of protein/kg·d for cholesterol and normal laboratory diet-fed groups). Because cholesteryl ester-rich VLDL is not present in detectable quantities in rabbits fed laboratory diet, the total catabolic rate was estimated only by assigning an upper boundary on an assumed VLDL pool size. Thus the estimated rate cannot be taken as definite. Nevertheless, if errors are present they would be likely to magnify the already large differences between the calculated values among groups. Over the 24 h during which kinetics of plasma clearance were determined, a steady concentration of lipoprotein in plasma was assumed supported by the lack of significant changes in plasma cholesterol concentrations. Judging from an assumed steady state for rates of synthesis and degradation over the course of the experiment, the elevated concentrations of cholesteryl ester-rich VLDL appeared to reflect increased rates of synthesis as demonstrated previously (11). In addition to differences in kinetics

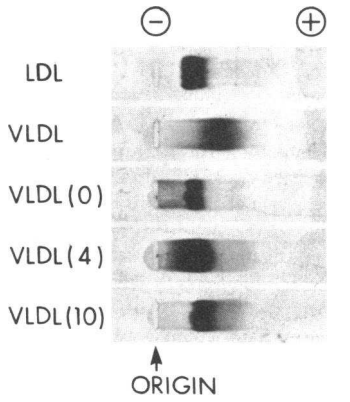

Figure 2. Electrophoretic mobility on agarose gels of plasma VLDL in animals returned to a normal laboratory diet regimen. Lipoproteins were visualized after staining with Fat Red O. VLDL and LDL harvested from normolipidemic rabbits were used to identify β and pre-β mobility. VLDL fractions from rabbits returned to normal diet are shown 0, 4, and 10 wk after discontinuation of the cholesterol-enriched diet.

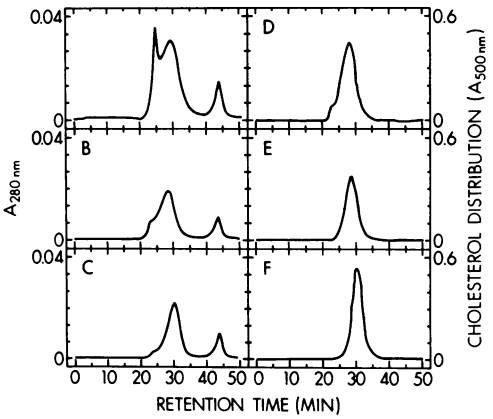

Figure 4. High performance liquid chromatography subfractionization by size of plasma VLDL after reinstitution of a normal chow regimen. Absorbance at 280 nm (A_{280nm}) was recorded for VLDL obtained from rabbit plasma after animals had been returned to a normal laboratory diet regimen at (*A*) 0, (*B*) 4, and (*C*) 10 wk. The distribution of cholesterol within the subfractions is illustrated in *D, E,* and *F.*

of plasma clearance, differences in redistribution of ^{125}I between lipoprotein fractions were observed (Table III). 24 h after injection of ^{125}I-labeled VLDL, very little redistribution of ^{125}I-label was found, i.e., 90±3% of ^{125}I remained in the $d < 1.006$ g/ml fraction in rabbits fed cholesterol for 12 wk. In contrast, in rabbits fed normal laboratory diet there was substantial redistribution into other fractions with only 40±1% remaining in the $d < 1.006$ g/ml fraction after 24 h (Table III).

Plasma clearance of ^{125}I-labeled VLDL was assessed again 4 and 10 wk after the group of hypercholesterolemic rabbits had been returned to a normal diet regimen (Fig. 1). At 4 wk there was some reduction in the fractional catabolic rate compared to values at week 0 (0.019±0.004 vs. 0.011±0.004 h^{-1}) although the total catabolic rate remained high (19.8±2.4 mg of protein/kg·d). At 10 wk, the fractional catabolic rate had increased (0.043±0.008 h^{-1}) and the total catabolic rate was reduced (5.8±0.7 mg of protein/kg·d; Fig. 1 *D*). However, values remained significantly different from those estimated in normal diet-fed animals. In keeping with these trends of reversal, redistribution of ^{125}I after administration of ^{125}I-labeled VLDL to rabbits was noted 4 and 10 wk after cessation of cholesterol diet with progressively less radioactivity in the $d < 1.006$ g/ml fraction (80±3% and 70±3%, respectively, Table III).

To evaluate the suitability of using ^{125}I-labeled cholesteryl ester-rich VLDL harvested from a continuously cholesterol-fed rabbit as the tracer for VLDL during the period of return to normal laboratory diet, a crossover kinetic experiment was performed. VLDL was harvested from a continuously cholesterol-fed rabbit and from one returned to normal diet for 10 wk. VLDL from each was radioidinated with ^{131}I and ^{125}I, respectively. A mixture of the two radiolabeled VLDL preparations was injected into cholesterol-fed rabbits as well as into animals which had been returned to normal diet for 10 wk. Both components of the mixture of ^{125}I- and ^{131}I-labeled VLDL exhibited similar kinetics of plasma clearance in cholesterol-fed rabbits (fractional catabolic rates of 0.015 and 0.014 h^{-1}, respectively; Fig. 5 *B*). In rabbits returned to normal diet for 10 wk the plasma clearance of VLDL harvested from a cholesterol-fed animal was slower than that of the VLDL obtained from rabbits returned to normal diet (fractional catabolic rates of 0.031 and 0.021 h^{-1}, respectively; Fig. 5 *A*). Although significant, the magnitude of the difference was modest. Thus the VLDL harvested from cholesterol-fed rabbits was regarded as a tracer appropriate for characterization of kinetics of plasma clearance of VLDL during the interval in which hypercholesterolemic rabbits were returned to normal diet regimens.

Tissue cholesterol content. After cholesterol feeding aortic tissue cholesterol content was increased greatly in all sections. Tissue cholesterol content was even higher in hypercholesterolemic rabbit aortas 10 wk after the animals had been returned to a normal laboratory diet regimen (Fig. 6).

Although it was not possible to quantify the percentage of intimal area that was covered by definable atherosclerotic lesions, gross visual inspection showed more extensive areas of intima covered by atherosclerotic plaques in hypercholesterolemic rabbits returned to normal diet compared with regions in the group fed a cholesterol diet for 12 wk. This impression was supported by quantitative determinations of cholesterol.

Cholesterol feeding resulted in larger "fatty" livers with increased concentrations of cholesterol (Fig. 7). Marked reductions of hepatic mass and hepatic concentration of cholesterol were noted 10 wk after animals had been returned to a normal laboratory diet regimen compared with values in the continuously cholesterol-enriched diet-fed group, although hepatic cholesterol concentrations were significantly greater than those in normal diet-fed animals ($P < 0.01$).

Aortic accumulation of radiolabeled VLDL. Accumulation of cholesteryl ester rich-VLDL protein over 24 h was considerably higher in aortas of cholesterol-fed animals than in aortas from control animals (Fig. 8). 89±3% of ^{125}I present in aortas of cholesterol-fed rabbits was precipitable by trichloroacetic acid. Only

Table III. Redistribution of ^{125}I in Plasma Density Fractions 24 h after Administration of ^{125}I-labeled VLDL

Weeks after return to normal laboratory diet	Recipient groups	Density fractions (g/ml)		
		<1.006	1.006–1.063	>1.063
		%	%	%
0	RNC (4)	90±3	8±2	3±1
	NC (4)	40±1	20±1	29±1
4	RNC (4)	80±3	15±3	3±1
	NC (4)	45±4	21±2	32±3
10	RNC (4)	70±3	27±3	2±1
	NC (7)	58±2	22±7	19±1
	CF (4)	92±4	7±3	1±1

Numbers for each fraction represent the percentage of radioactivity in the sum of all fractions 24 h after injection of ^{125}I-labeled VLDL.
(*n*) Number of animals in each group.
RNC, group previously fed cholesterol-enriched diet and studied at the times indicated after return to normal laboratory diets. NC, group fed normal laboratory diet ("chow") and studied concurrently with RNC group. CF, group fed cholesterol diet continuously for 12 wk and studied at week 10.

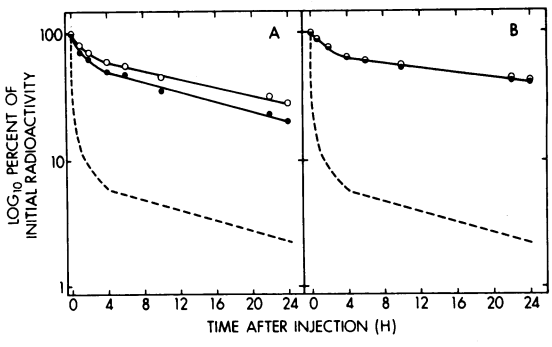

Figure 5. Plasma clearance kinetics of ^{131}I-labeled VLDL (○, values obtained from a cholesterol-fed rabbit) and ^{125}I-labeled VLDL (●, values obtained from a hypercholesterolemic rabbit that had been returned to a normal laboratory diet regimen for 10 wk). Lipoproteins (200 mg of protein, 8–11 × 10^{10} cpm each) were mixed immediately prior to injection into the marginal ear vein of (*A*) rabbits that had been returned to a normal laboratory diet regimen for 10 wk and (*B*) of cholesterol-fed animals. Plasma was obtained at the selected intervals indicated. Radioactivity in plasma aliquots was determined in fractions precipitated by trichloroacetic acid (10% wt/vol). Each point represents the average of two observations. For comparison, the dashed lines represent the plasma clearance of ^{125}I-labeled cholesteryl ester-rich VLDL in rabbits maintained on a normal laboratory diet.

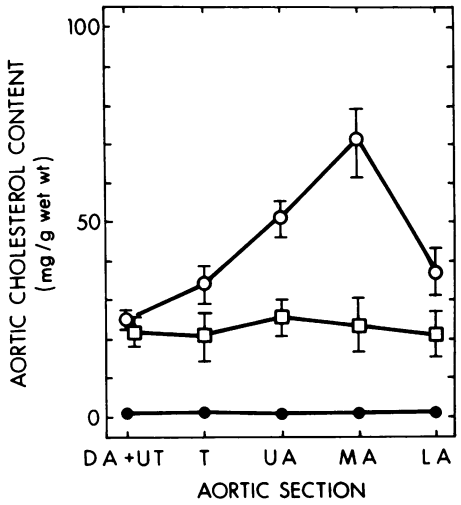

Figure 6. Aortic cholesterol content of aortic sections from normal laboratory diet-fed animals (●, n = 7), cholesterol-fed animals (□, n = 4), and hypercholesterolemic rabbits that had been returned to normal laboratory diet for 10 wk (○, n = 4). Values are means±standard errors. DA and UT, descending arch and upper thoracic; T, thoracic; UA, upper abdominal; MA, midabdominal; LA, lower abdominal.

26±4% was precipitable in aortas of laboratory diet-fed rabbits. When hypercholesterolemic animals were studied 10 wk after they had been returned to normal diet, less VLDL protein accumulated in aortic tissue than in aortas of continuously cholesterol-fed rabbits, but accumulation was greater than that observed in normal diet-fed animals (Fig. 8). A lower proportion of ^{125}I was trichloracetic acid-precipitable in hypercholesterolemic rabbits returned to normal diet (68±4%) compared with amounts precipitable in extracts from the cholesterol-fed group.

Discussion

Results obtained in this study imply that specific aspects of metabolism of cholesteryl ester-rich VLDL may account for the

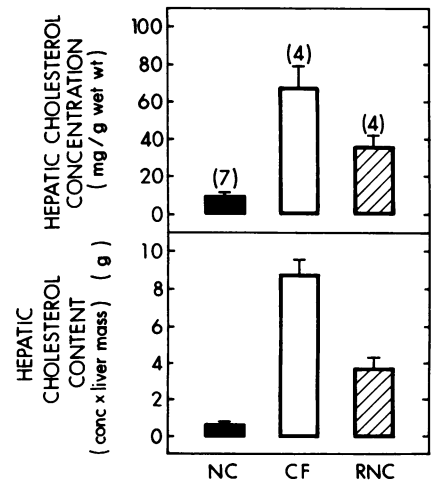

Figure 7. Hepatic cholesterol concentration (conc) and total cholesterol content from rabbits fed normal laboratory diet ("chow") (NC, ■), cholesterol-fed (CF, □) and hypercholesterolemic rabbits returned to normal laboratory diet for 10 wks (RNC, ▩). (n) Number of observations for each group. Values are means±standard errors.

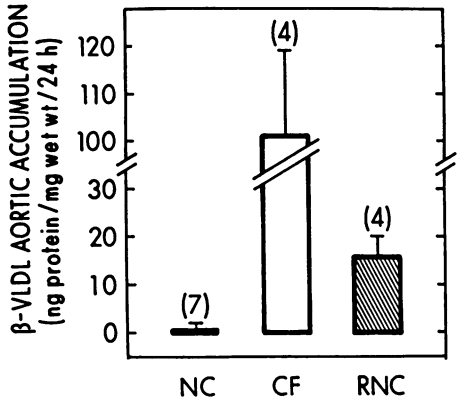

Figure 8. Accumulation of VLDL protein over 24 h in the descending arch/upper thoracic section of aortas from normal laboratory diet ("chow")-fed animals (NC, ■), cholesterol-fed (CF, □), and hypercholesterolemic animals returned to normal laboratory diet (RNC, ▩). All other sections showed a similar pattern of accumulation of lipoprotein-protein in the three groups of animals. (n) Number of observations for each group. Values are means±standard errors.

lack of regression of atheromatous lesions in rabbits subjected to 12 weeks of a cholesterol-enriched diet. Morphologically, the atheromatous plaques formed during cholesterol feeding are somewhat similar to fatty streaks in human aortas. Such fatty streaks exhibit intracellular lipid deposition within foam cells, with some extracellular lipid accumulation. It has been speculated that such lesions are precursors of the more complex fibrolipid plaques in nonhuman primates (19) and in humans (20). Because the fatty streak may represent an early phase of atheromatous plaque formation, it would be anticipated to be one of the lesions most susceptible to reversal. However, results from studies that have quantified the extent of regression in previously cholesterol-fed rabbits have been equivocal (2).

The present study attempted to define the biochemical mechanisms responsible for lack of regression in the fatty streak stage in previously cholesterol-fed rabbits. In agreement with Gupta et al. (7), we noted an increase in aortic cholesterol content after hypercholesterolemic rabbits had been returned to normal laboratory diet for 10 wk, compared to values in animals fed the cholesterol-enriched chow continuously for 12 wk. To account for the lack of regression, we considered the possibility that the persistence of aortic atheromatous plaques in previously cholesterol-fed rabbits might be attributable to continued delivery of cholesterol, via cholesteryl ester-rich VLDL, and perhaps also intermediate density lipoprotein and low density lipoprotein (LDL) to the arterial wall. The metabolism of cholesteryl ester-rich VLDL was chosen for characterization because it appears rapidly and in large quantities in the plasma of cholesterol-fed rabbits. Cholesteryl ester-rich VLDL was also of particular interest because of the correlation between elevated concentrations of this lipoprotein in plasma and appearance of atheroma (8–11), and the fact that cholesteryl ester-rich VLDL is known to produce cholesterol loading in cells (9).

Feeding rabbits a high-cholesterol diet produced the expected hypercholesterolemia, accumulation of cholesteryl ester-rich VLDL in plasma, gross increases in hepatic and aortic cholesterol content, and aortic atherosclerotic plaques. 10 wk after cessation of cholesterol feeding and substitution of a virtually cholesterol-free diet, plasma concentrations of cholesterol remained high.

High rates of synthesis of cholesteryl ester-rich VLDL were remarkably persistent. Marked reductions of hepatic concentrations of cholesterol, albeit not entirely to normal, were evident suggesting that the continuing rapid flux of cholesteryl ester-rich VLDL after return to normal chow was attributable to continuing high rates of secretion of the lipoprotein by the liver.

In contrast to the case for liver, the cholesterol content of the aortic tissue continued to increase after animals had been returned to a normal laboratory diet regimen. In addition to increased cholesterol content, accumulation of VLDL protein was elevated over that in laboratory diet-fed rabbits even 10 wk after the cholesterol-fed animals had been returned to normal diet. Thus enhanced delivery of cholesteryl ester-rich VLDL to the aortic tissue appeared to continue to promote the deposition of cholesterol esters. The continuing rise in aortic cholesterol raises the additional possibility that rates of removal of cholesterol from the arterial wall were defective. The extent to which VLDL accumulated intra- or extracellularly is not clear, nor is it known whether cellular receptors for β-VLDL or LDL mediated intracellular accumulation and whether or not this changed over time.

The $d < 1.006$ g/ml plasma fraction of cholesterol-fed animals and type III hyperlipidemic subjects has been fractionated further using Sepharose 4B column chromatography (21–23). As demonstrated by the high performance liquid chromatographic separation, we found that rabbit cholesteryl ester-rich VLDL comprised two major fractions. The fraction eluted in the void volume does not represent aggregates of the small sized fraction because the two peaks have distinct chemical compositions. The larger sized fractions of cholesterol ester-rich VLDL possess very little protein, and consequently the vast majority of the radioactivity of ^{125}I-labeled VLDL is associated with the smaller sized fraction when prepared for donor lipoproteins of cholesterol-fed rabbits (data not shown). Only the small sized fraction is present in plasma of cholesterol-fed rabbits when they are returned to normal diet (23), probably reflecting the fact that the larger sized fraction is of intestinal origin and the smaller is synthesized in the liver. Radioiodination of whole VLDL attaches ~95% of the radioactivity to the smaller-sized fraction, and consequently the ^{125}I-labeled VLDL prepared from the cholesterol-fed rabbits would have traced primarily the smaller fraction. The crossover kinetics of plasma clearance experiments demonstrated that VLDL obtained from continuously cholesterol-fed rabbits was cleared more slowly from plasma of rabbits returned to normal laboratory diet for 10 wk than their own VLDL. Thus, use of both radiolabels simultaneously demonstrated that the apparent magnitude of cholesteryl ester-rich VLDL accumulation was affected by the source of donor ^{125}I-labeled VLDL in the hypercholesterolemic group that was returned to normal laboratory diet. However, because the effect was relatively small, the major conclusions of this study were confirmed using both labels, namely that excess aortic accumulation persisted even after 10 wk of feeding of normal laboratory diet.

The larger-sized VLDL fraction is the most potent in eliciting cholesterol ester deposition in cultured macrophages (21). It has been implicated as a mediator of atherogenic effects of cholesteryl ester-rich VLDL (24). However, in hypercholesterolemic rabbits returned to normal diet, marked deposition of cholesterol in aortic vessel wall persisted even though only the smaller sized cholesterol-enriched VLDL fraction was detectable in plasma.

The principal tissue site for catabolism of cholesteryl ester-rich VLDL is the liver (25). Uptake of VLDL in the liver is mediated by apolipoprotein E. This process is inhibited by apolipoprotein C (26, 27). However, VLDL harvested from cholesterol-fed animals, which has only trace amounts of apolipoprotein C, was cleared more slowly from plasma than VLDL harvested from rabbits returned to normal laboratory diet, which contains appreciable quantities of apolipoprotein C.

In summary, our results suggest that persistence of aortic atheromatous plaques in previously cholesterol-fed rabbits returned to a normal laboratory diet regimen is attributable in part to a continuing enhanced accumulation in the arterial wall of the atherogenic lipoprotein, cholesteryl ester-rich VLDL, possibly combined with an impaired reverse transport of cholesterol associated with diminution of the concentration of high density lipoprotein in plasma.

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