Cholesterol turnover in lipid phases of human atherosclerotic plaque[1]

Saul S. Katz,[2] Donald M. Small, Frank R. Smith, Ralph B. Dell, and DeWitt S. Goodman

Department of Medicine, Royal Victoria Hospital, Montreal, Quebec H3A 1A1,[3] Biophysics Division, Department of Medicine, Boston University School of Medicine, Boston, MA,[4] and Departments of Medicine and Pediatrics, Columbia University College of Physicians and Surgeons, New York, NY

Abstract The turnover of free cholesterol in atheromatous plaque lipid phases was studied in a patient undergoing peripheral vascular surgery. [^{14}C]Cholesterol was injected intravenously 139 days prior to surgery, and [^{3}H]cholesterol was injected 12 days pre-op. The plasma cholesterol specific radioactivity decay curves were determined from the times of isotope injection until surgery. At surgery, atheroma, skin, muscle, and tendon were obtained. Lipid phases of plaque homogenate were isolated by density gradient centrifugation. The top layer of the gradient, layer 1, contained the cholesteryl ester oil droplet phase, layer 2 was enriched in phospholipid bilayer phase, layer 3 contained cholesterol monohydrate crystals and the pellet, layer 4 had more dense plaque components such as collagen and elastin. The tissue:plasma specific radioactivity ratios on days 12 and 139 respectively were muscle, 0.86, 2.47; skin, 0.74, 1.20; tendon, 0.18, 1.45; total plaque, 0.22, 1.39; plaque layer 1, 0.31, 1.50; layer 2, 0.22, 1.53; layer 3, 0.08, 0.61; and layer 4, 0.20, 0.88. Thus, plaque atheroma, which contains physically distinct forms of cholesterol, had correspondingly different rates of cholesterol turnover. Cholesterol solubilized in liquid oil droplets (layer 1) and liquid crystalline phospholipid bilayers (layer 2) had specific radioactivity values similar to those of tendon cholesterol, and represented tissue cholesterol that was undergoing slow equilibration with the plasma cholesterol pool. Pellet cholesterol (layer 4), which is probably connective tissue-associated, had lower specific radioactivity values, well below those of plasma cholesterol even after 5 months. Crystalline cholesterol (layer 3) had the lowest specific radioactivity values of all tissues and plaque fractions. Therefore, cholesterol in the crystalline state is relatively inert. Since crystalline cholesterol can account for over 40% of plaque free cholesterol, resistance to mobilization of this lipid may be an important obstacle to plaque regression.—Katz, S. S., D. M. Small, F. R. Smith, R. B. Dell, and D. S. Goodman. Cholesterol turnover in lipid phases of human atherosclerotic plaque. *J. Lipid Res.* 1982. **23:** 733–737.

Supplementary key words cholesterol pools • cholesterol monohydrate crystals

The turnover time of cholesterol in human atherosclerotic plaques has been estimated to be over 400 days (1). Although this rate is very slow, especially compared to cholesterol in most other tissues, the demonstration of some equilibration of plaque cholesterol with plasma suggests that it may be possible to mobilize the large cholesterol deposits of plaques, thereby promoting plaque regression. However, cholesterol in human atheroma is physically heterogenous (2, 3), and therefore previously measured turnover rates may not be applicable to all the lipid phases of plaques. Further, studies of animal atherosclerosis have also suggested metabolic heterogeneity of cholesterol in the lesion (4–6).

Human atheroma contains free cholesterol in at least four lipid phases (7): *i*) solubilized in cholesteryl ester oil droplets; *ii*) solubilized in phospholipid bilayers; *iii*) crystallized as cholesterol monohydrate; and *iv*) associated with non-lipid tissue components such as elastin or collagen. Because of the different physical properties of these lipid phases, one would predict that the more liquid phases, cholesteryl ester droplets and phospholipid bilayers, would exchange with plasma cholesterol more rapidly, while cholesterol bound to protein and especially crystalline cholesterol might turn over much more slowly. In order to explore this question, we have used density gradient centrifugation of plaque homogenate to isolate the different lipid phases of surgically resected human atheroma (7) in a patient previously injected with radioactive cholesterol. By injecting two different isotopes at different times, information was obtained about the relative turnover rates of plaque and tissue free cholesterol compared to that of cholesterol in plasma.

METHODS

Study subject; cholesterol turnover study

The patient, a 66-year-old white male, underwent aorto-femoral bypass surgery for peripheral vascular

disease. Written informed consent was obtained for the study reported here. This patient was previously identified as Subject #26 [Table 1, ref. (8)] in a report of the results of long-term cholesterol turnover studies in 54 subjects. His mean serum cholesterol and triglyceride levels (n = 46) were 163 mg/dl and 105 mg/dl, respectively, during the course of the study.

One hundred and thirty-nine days prior to surgery, the patient was injected intravenously with 51.1 μCi of [4-^{14}C]cholesterol (New England Nuclear, Boston, MA) that had been complexed in vitro with the subject's own serum lipoproteins. The specific radioactivity of serum total cholesterol was determined in samples collected serially thereafter. The methods used have been described in detail previously (9, 10). Twelve days prior to surgery, 51.4 μCi of [1,2-^{3}H]cholesterol was administered intravenously in a similar manner. The specific radioactivity of serum total cholesterol for both isotopes was determined daily thereafter.

The ^{14}C specific radioactivity data were analyzed by a weighted, least-squares technique described before (10, 11) to determine the parameters of a three-pool mammillary model which would provide the best fit. The model used has been discussed in detail in previous reports (8, 10).

Tissue samples

At surgery, a segment of atherosclerotic aorta was obtained, as well as biopsies of muscle, skin, and tendon. Atheroma from a gruel-containing plaque was finely minced on a glass slide, and then homogenized in distilled water using a Kontes Duall glass-glass homogenizer (Kontes Co., Vineland, NJ). The portion of atheroma remaining on the slide was examined by polarizing microscopy to identify lipid phases (12, 13). A small aliquot of homogenate was taken for chemical analysis and the remainder was placed on a sucrose density gradient chosen, as previously described (7), to isolate plaque lipid phases: 1) d 1.00 g/ml, to isolate the cholesteryl ester droplets; 2) d 1.028 g/ml, to isolate phospholipid bilayer phase; and 3) d 1.054 g/ml, to isolate cholesterol monohydrate crystals. More dense plaque components were expected to pellet at the bottom of the density gradient.

Chemical analyses

Lipid of biopsied tissue, plaque homogenate, and density gradient fractions were extracted according to Folch, Lees, and Sloane Stanley (14). The chemical compositions of plaque and its fractions were determined by quantitative TLC as previously described (3). Free cholesterol was then isolated from each lipid extract by preparative TLC using the solvent system hexane–diethyl ether–acetic acid 70:30:1 (v/v/v), and the amount of the isolated lipid was measured by quantitative TLC. Ra-

dioactivity was determined by liquid scintillation counting for 100 min for each sample, using the dual-channel, simultaneous equation method to assay for both ^{14}C and ^{3}H in a given sample.

RESULTS

Parameters of cholesterol turnover

Fig. 1 shows the plasma cholesterol turnover curve as determined from the plasma [^{14}C]cholesterol specific radioactivity data. From these data the parameters of the three-pool model of body cholesterol turnover were determined as follows: production rate (PR) = 1.17 g/day; the size of the rapidly turning over pool 1 (M_1) = 21.4 g; the rate constants for transfer between pool 2 or 3 and pool 1, in days^{-1}: k_{12} = 0.129, k_{21} = 0.033, k_{13} = 0.027, and k_{31} = 0.049. The minimum values for the more slowly turning-over pools 2 and 3 were calculated as: M_2 = 5.6 g, and M_3 = 38.8 g.

Fig. 1 also shows the calculated specific radioactivity curves for cholesterol in each of the side pools (pools 2 and 3). As discussed previously (8, 10), pool 2 consists of cholesterol that equilibrates at an intermediate rate
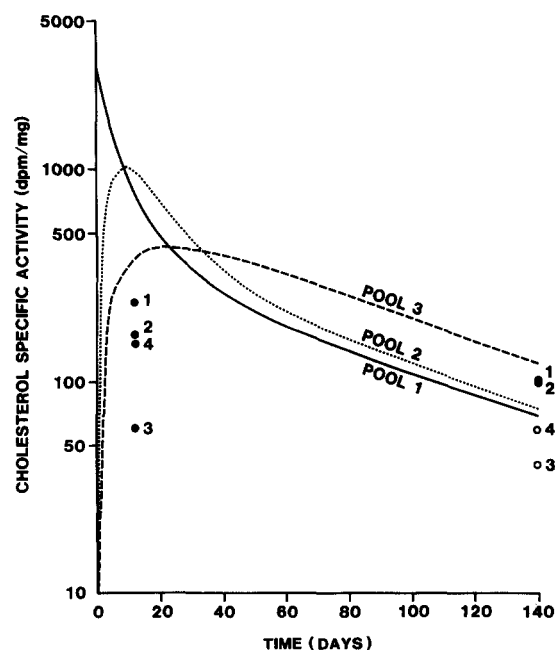

Fig. 1. Cholesterol specific radioactivity versus time curves for each of the compartments of the three-pool model of body cholesterol turnover. The curve for pool 1 represents the computed best fit for a three exponential equation to the measured plasma cholesterol specific radioactivity data. The curves for pool 2 and for pool 3 were calculated for a three-pool model from the parameter values derived from the plasma data (15). The measured specific radioactivity values for cholesterol in the four different plaque fractions are plotted as points for days 12 (the ^{3}H data) and 139 (the ^{14}C data).

with plasma cholesterol, whereas pool 3 comprises the most slowly turning-over compartment of exchangeable body cholesterol in the three-pool model.

Atheroma lipid composition

The lipid compositions of the atheroma and its density gradient fractions are shown in **Fig. 2.** Layer 1, d < 1.00 g/ml, contained many isotropic droplets by polarizing microscopy, and was 69.3% cholesteryl ester, 14.7% cholesterol, 8.6% triglyceride, and 7.4% phospholipid. The composition approximated the cholesteryl ester phase of pure lipid systems. Layer 2, d < 1.028 g/ml, was enriched in phospholipid bilayer phase, and was 40.5% cholesterol, 35.8% phospholipid, 21.7% cholesteryl ester, and 2.1% triglyceride. Layer 3, d < 1.054 g/ml, contained many rhomboidal plate crystals and had the highest relative amount of free cholesterol, 61.2%. It also contained 23.1% phospholipid and 15.7% cholesteryl ester. The composition of the pellet was 48.2% cholesteryl ester, 31.6% cholesterol, 18.2% phospholipid, and 2.1% triglyceride. The distribution of free cholesterol in the density gradient fractions was 11% layer 1, 21% layer 2, 41% layer 3, and 27% layer 4.

Tissue and plaque cholesterol specific radioactivity

By normalizing the ^{3}H and the ^{14}C plasma cholesterol data to the same injected dosage, the use of two isotopes, injected at two different times, enabled us to obtain tissue specific radioactivity data representing two time points along the turnover curves shown in Fig. 1. Thus, the [^{14}C]cholesterol specific radioactivity values for tissue cholesterol represented values at 139 days after the start of the turnover study, whereas the tissue [^{3}H]cholesterol values could be considered as representing those at 12 days after the start of the turnover study. The radioactivity of the plaque layers ranged from 16.3 cpm ± 5% to 61.3 cpm ± 3% for [^{3}H]cholesterol and from 12.4 cpm ± 7% to 56.6 cpm ± 3% for [^{14}C]cholesterol.

Fig. 1 shows the specific radioactivity values of cholesterol in each of the four isolated density gradient plaque homogenate fractions, for each of the two time points. Large differences were seen among the specific radioactivity values for the four plaque fractions. At both time points, cholesterol in layer 3 (cholesterol monohydrate crystals) showed very low radioactivity values, considerably below those seen in other plaque fractions or computed for the three pools of the model.

Table 1 shows the tissue:plasma cholesterol specific radioactivity ratios for [^{3}H]- and for [^{14}C]cholesterol in muscle, skin, tendon, plaque, and in the four fractions of the plaque homogenate. At day 12, the specific radioactivity values for muscle and skin cholesterol were 86% and 74%, respectively, of that of plasma. Tendon and plaque cholesterol equilibrated at a much slower rate
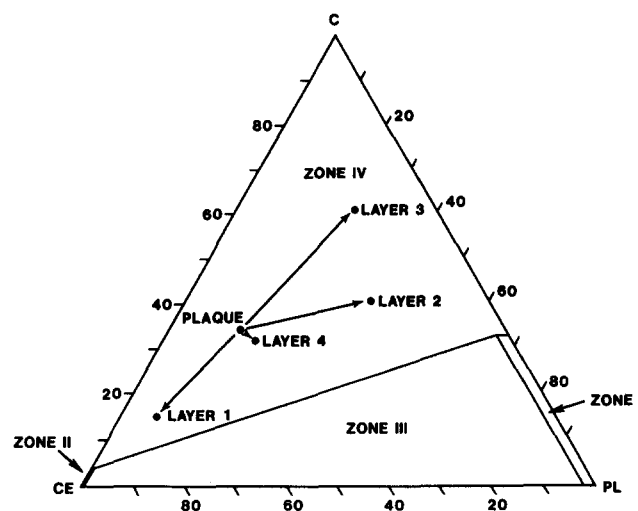

Fig. 2. Lipid compositions of plaque and its isolated lipid phases are plotted on the phase diagram of cholesterol-phospholipid-cholesteryl ester. Zone I is a one-phase zone of phospholipid bilayers, and zone II is a one-phase zone of cholesteryl ester oil droplets. Zone III, a two-phase zone, has both oil droplets and phospholipid bilayers, and zone IV, a three-phase zone, contains in addition cholesterol monohydrate crystals.

with plasma cholesterol (specific radioactivity values at day 12 were, respectively, 18% and 22% as great as that of plasma cholesterol). In the isolated phases of the plaque, the oil phase in layer 1 had the highest cholesterol specific radioactivity at day 12, while the specific radioactivity of cholesterol in layer 3 was only 8% as great as that of plasma cholesterol.

By day 139, the specific radioactivity values of cholesterol in muscle, skin, tendon, and in the plaque oil phase and phospholipid bilayer phase exceeded that of cholesterol in plasma. In contrast, the specific radioactivity of cholesterol in the pellet fraction (layer 4) was only 88% as great as that of cholesterol in plasma at this time. Layer 3, the crystal-rich layer, had the lowest specific radioactivity (61% of that of plasma cholesterol). Small amounts of cholesteryl ester in layer 3 and low counts in layers 2 and 3 made comparisons of cholesteryl ester turnover in the various density gradient layers difficult.

DISCUSSION

Much of the lipid of human atheroma is accounted for by cholesterol (3). For example, total cholesterol made up 80% of the lipid of the plaque used in this study, with free cholesterol being 34% of the total lipids. However free cholesterol is not homogeneous within the plaque, but is distributed amongst several physically distinct compartments and exists in diverse physical states. These states are liquid (oil droplets), liquid crystalline (phos-

TABLE 1. Ratios of tissue:plasma cholesterol specific radioactivities

Day	Muscle	Skin	Tendon	Total Plaque	Plaque Layer			
					Layer 1[a]	Layer 2[b]	Layer 3[c]	Layer 4[d]
12[e]	0.86	0.74	0.18	0.22	0.31	0.22	0.88	0.20
139[f]	2.47	1.20	1.45	1.39	1.49	1.53	0.61	0.88

[a] Plaque homogenate density gradient layer d < 1.00 g/ml. The cholesteryl ester droplet phase.
[b] The phospholipid bilayer phase, d < 1.028 g/ml.
[c] Cholesterol monohydrate crystal phase, d < 1.054 g/ml.
[d] Pellet of connective tissue, cell nuclei, etc., d > 1.054 g/ml.
[e] Specific radioactivity ratios of [^{3}H]cholesterol.
[f] Specific radioactivity ratios of [^{14}C]cholesterol.

pholipid bilayers), solid (cholesterol monohydrate crystals), and probably protein-associated (collagen, elastin). The distribution of cholesterol in the plaque lipid phases separated by density gradient centrifugation was oil 11%, phospholipid bilayers 21%, crystals 41%, and pellet 27%. The 41% cholesterol found in the crystal-rich layer is probably a minimum estimation, since some cholesterol crystals were seen by polarizing microscopy contaminating the other layers, and since ideal phase separation in the absence of protein interaction would yield a crystal layer containing 74% of plaque free cholesterol (7). We sought to determine the influence of the physical state of cholesterol in plaques on its rate of turnover.

The use of two isotopes of cholesterol, injected at two different times, provided information about the turnover rate of tissue free cholesterol at both an early and at a late time point during the course of a long-term turnover study. Twelve days after injection of [^{3}H]cholesterol, muscle and skin cholesterol were almost equilibrated with plasma cholesterol, while the slower turnover of tendon and plaque cholesterol was shown by their 4-fold lower specific radioactivity values. Large differences in the cholesterol specific radioactivity values of the different lipid phases of the plaque were evident by 12 days. The highest specific radioactivity was found in the liquid oil phase, followed by the liquid crystalline phospholipid phase, and then the protein-associated cholesterol of the pellet. Crystalline cholesterol had the lowest specific radioactivity.

By day 139, the specific activity curves of cholesterol in muscle, skin, tendon, and total plaque had crossed over the plasma curve, and the specific radioactivity values for cholesterol in these tissues exceeded that of plasma cholesterol. The value for plaque cholesterol was, however, a composite of the heterogeneous values seen in the four different plaque lipid phases. Thus, while the specific radioactivity values of cholesterol in the oil and phospholipid bilayer phases were approximately 1.5 times that of plasma cholesterol at day 139, the pellet and crystalline cholesterol specific radioactivities were only 88% and 61%, respectively, of that of plasma cholesterol.

These observations indicate that cholesterol was turning over at exceedingly slow rates in these phases of the plaque. Furthermore, since the crystal-rich fraction of plaque was somewhat contaminated by both the oil and phospholipid bilayer phases, some or all of the labeled cholesterol may have been derived from these phases. The true crystalline cholesterol specific radioactivity would therefore be even lower than the values shown in Table 1, and it is possible that there is almost no turnover of crystalline cholesterol in the plaque.

In addition to the constraints to turnover of molecules in a crystalline lattice, physical barriers might inhibit equilibration between crystals and plasma. These barriers would include a) the fibrous capsule around the atheroma as well as fibrous stroma around clusters of crystals, b) the location of the crystals deep within the atheroma, and c) the relative avascularity of the necrotic core of the plaque. The relative importance of the physical state of crystalline cholesterol vis-a-vis these structural factors is not known. In addition, during the 20-week period of this study, the size of one or more plaque lipid phases may have changed, and thus steady conditions in regard to these phases may not have been obtained. For example, new crystal formation, or an increased rate of crystal growth may have occurred after the [^{3}H]cholesterol was given, and may thus account for the higher specific radioactivity on day 12 than on day 139 in layer 3 of the plaque (Fig. 1). Finally, pool size as well as turnover rate of cholesterol in the different lipid phases could have played a role in the measured specific radioactivities, since the larger amount of free cholesterol in the crystalline phase would have contributed to the low specific radioactivity in this phase. However, pool size cannot be the entire explanation for the lower specific radioactivity in plaque cholesterol crystals, since the rate of equilibration is much less than would be expected by the differences in the mass of cholesterol in the plaque fractions.

These data suggest that plaque cholesterol in the crystalline state is relatively inert, and may be extremely difficult or impossible to mobilize in an in vivo situation.

Thus, cholesterol associated with plaque proteins (found in the pellet layer), and particularly cholesterol crystals, may constitute important obstacles to plaque regression. Accordingly, the appearance of a crystalline cholesterol phase may represent a critical event in the development of a significant and persistent atheromatous plaque. Further studies are needed to explore the implications of these observations in relation to other factors that contribute to and influence plaque progression and regression.█

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