Slowly Miscible Cholesterol Pools in Progressing and Regressing Atherosclerotic Aortas[1] (37418)

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Abundant evidence exists from previous investigations to warrant the statement that the lipids of atherosclerotic plaques are physically and chemically heterogeneous.

Histologic studies of atherosclerotic plaques in various species such as the pigeon (1), rabbit (2, 3), squirrel monkey (4), cebus monkey (5), rhesus monkey (6, 7) and man (8, 9) have shown the physical heterogeneity of lipid pools by describing amorphous lipid, spherulites, spherical droplets, perifibrous lipid, and sterol crystals in the lesion.

Reports on the chemical analysis of distinct lipid fractions in atherosclerotic plaques either studied after microdissection (10) or after homogenation and ultracentrifugation (11) describe characteristic lipid compositions for each fraction. Smith, Evans and Downham (12) described morphologically and chemically two types of lipid in human atherosclerotic plaques. One type, fine extracellular droplets termed perifibrous lipid, was described aligned along collagen and elastin fibers. The lipid was chemically distinct with a cholesterol ester fatty acid composition of 28% oleic and 40% linoleic. Another type was in the form of intracellular coarse lipid droplets and had a higher cholesterol ester concentration composed of fatty acids which were 48% oleic and 14% linoleic.

Eisenberg *et al.* (11) have shown by fractionation methods that the cholesterol contained within aortas of cholesterol-fed rabbits can be separated into metabolically heterogeneous pools. Additionally, decreases in free and esterified cholesterol concentrations of atherosclerotic plaques seen after lesion regression suggest that there are at least two pools of cholesterol in the plaque (13, 14).

The study presented here was undertaken in order to characterize the cholesterol pools in progressing and regressing plaques and to investigate the miscibility of these pools with plasma cholesterol.

Materials and Methods. White Carneau pigeons from the Bowman Gray School of Medicine Research Farm and ranging in age from 30 to 35 mo at the termination of the study were used. One group of five pigeons fed a control, cholesterol-free, grain diet of Purina Pigeon Pellets was used as a source of grossly normal aortic tissue and naturally occurring aortic plaques. A second group was five pigeons fed an atherogenic diet of 0.5% cholesterol, 10% lard, and 89.5% Purina pellets for 12 mo and provided a source of diet-aggravated plaques. A third group was three pigeons fed the atherogenic diet for 12 mo, then fed control diet for 16 mo. The arterial lesions of this group were termed regressed plaques.

Arterial cholesterol was labeled *in vivo* by giving an initial iv and subsequent im injections twice daily with 5 μCi of [1,2-^{3}H] cholesterol (New England Nuclear Corp., Boston, MA) as a saline:ethanol 7:3 (v/v) suspension. Blood samples were obtained 2, 4, 6, and 10 days after the initial isotope injection and serum cholesterol specific activities were calculated. Serum cholesterol concentrations were measured on isopropanol extracts by an autoanalyzer adaptation of the method of Block, Jarrett and Levine (15). Radioactivity was measured on an aliquot of the extract using a Beckman DPM-100 liquid

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scintillation counter.

After a 10 day equilibration period of aortic and plasma [^{3}H] cholesterol, the birds were killed by decapitation. The aortas from the brachiocephalic bifurcation to the origin of the celiac artery were removed, cleaned of adventitial lipid and washed in saline to remove any traces of blood. The vessels were opened longitudinally, rinsed with saline and dissected into areas of grossly normal aorta and plaque. Arterial segments with fatty streaks or flecks were not analyzed.

All similar tissues for each group were pooled and homogenized in 0.25 *M* sucrose using a glass tissue homogenizer. The aortic homogenates were centrifuged for 40 min at 140,000*g* and 4° using a Beckman Model L2-65B ultracentrifuge with a type 65 rotor. Centrifuged homogenates were fractionated arbitrarily into a 2 cm top, 5 cm middle (infranatant) and pellet by slicing the tubes and drawing off the fractions. All pellets were washed three times with 0.25 *M* sucrose and the washes were discarded.

The fractions were extracted three times with chloroform:methanol 2:1 (v/v) and the lipids were separated by thin-layer chromatography. Free and esterified cholesterol concentrations and radioactivity were determined by the methods previously outlined for serum.

Aliquots of the pellet fractions were extracted for elastin with 0.1 *N* NaOH in a Precision Model 25 shaking water bath at 98° and at 100 oscillations/min for 45 min (16).

The pellet digests were centrifuged and the elastin residue was washed three times with distilled water. The residue was dehydrated with three changes of absolute ethanol and delipidated with three changes of chloroform:methanol (2:1), two changes of acetone and two changes of ether.

The lipid solvents used in dehydration and delipidation were pooled and analyzed for free and esterified cholesterol and radioactivity as described.

The complete delipidation of elastin was confirmed by treating the final product with elastase (Nutritional Biochemicals Corp., Cleveland, OH), delipidating the hydrolyzate, and analyzing for cholesterol.

Results. As many studies have indicated, the lipid fraction which increases to the greatest extent in an atherosclerotic plaque is esterified cholesterol (13, 17, 18). Unfortunately in this experiment a technical error during analysis resulted in loss of several esterified cholesterol fractions; therefore, only the nonesterified or free cholesterol concentrations and specific activities will be considered.

In fractionated normal aorta from grain-fed pigeons, 95–97% of the total arterial free cholesterol was found associated with the middle and pellet fractions (Table I). The cellular materials which sediment under the conditions used in this experiment should include proteinaceous structures such as membranes, fibrillar proteins and cellular organelles. Since most of the free cholesterol of normal aorta is incorporated into mem-

TABLE I. Cholesterol Concentrations in Fractionated Normal Aorta and Naturally-Occurring Atherosclerotic Plaques in White Carneau Pigeons.

	Fraction	Cholesterol[a]	Sp act[b]	Elastin-bound cholesterol[a]	Sp act	Elastin-bound cholesterol[c]
Normal aorta	Top	0.01	23,620			
	Middle	0.09	23,390			
	Pellet	0.21	25,930	0.01	29,730	3
Plaque	Top	2.51	10,130			
	Middle	0.59	14,480			
	Pellet	1.92	5130	0.25	4760	13

[a] mg free cholesterol/g aorta wet weight.

[b] dpm/mg cholesterol.

[c] Expressed as a percentage of the pellet cholesterol.

brane structures (11, 19) it is reasonable that the bulk of the free cholesterol pool of a nonaffected artery is bound in the membrane fraction of the pellet. Only 3% of the arterial free cholesterol in normal artery was localized in the top fraction. The top fraction or nonbound cholesterol pool presumably represented that cholesterol which exists in the artery as intra- and extracellular droplets.

The cholesterol specific activities of each of the three fractions were similar indicating a reciprocative miscibility with one another and with plasma cholesterol.

Naturally occurring plaques had large increases in cholesterol concentration in all three fractions in comparison with the levels of the respective fractions of normal aorta. The dramatically increased cholesterol level in the top fraction amounted to 50% of the total arterial cholesterol.

A comparison of the specific activities of the three aortic fractions showed a large decrease in the pellet fraction in comparison with the top and middle fractions. This lower cholesterol specific activity implies the presence of a very slowly miscible pool or pools of cholesterol associated with the bound fraction.

The analysis of elastin-bound cholesterol in normal and plaque fractions showed a large increase in elastin-bound cholesterol of plaque. In fact, 13% of the free cholesterol associated with the pellet was attributed to binding with elastin while only 3% of the pellet cholesterol was associated with the elastin of normal aorta. Approximately 5% of the total free cholesterol of the entire plaque was associated with elastin.

The elastin-cholesterol specific activities of both normal aorta and aortic plaques were similar to the specific activities of their respective pellets, indicating the elastin-bound cholesterol pool was miscible with other pools of cholesterol.

Table II shows the cholesterol levels in normal aorta and in diet-aggravated plaques. In the grossly normal artery most of the cholesterol was associated with the middle and pellet fractions while less was nonbound. Slightly more cholesterol was present in the top fraction of the aortas from cholesterol-fed pigeons than in aortas from the grain-fed birds possibly arising from microscopic lesions not seen grossly. The specific activities of each of the cholesterol fractions were comparable and seemingly indicate a miscibility with one another and with plasma cholesterol.

The diet-aggravated plaques had large increases in the cholesterol concentration of all three fractions when compared with the cholesterol levels in normal aorta. This increment in cholesterol concentration was especially noticed in the top fraction which contained approximately 40% of the total arterial cholesterol. The higher nonbound cholesterol fraction had a specific activity similar to that of normal aorta and was threefold higher than the bound cholesterol specific activity. The lowered specific activity of the bound cholesterol implied less miscibility of this fraction with the other pools of cholesterol.

TABLE II. Cholesterol Concentrations in Fractionated Normal Aorta and Diet-Aggravated Atherosclerotic Plaques in White Carneau Pigeons.

	Fraction	Cholesterol[a]	Sp act[b]	Elastin-bound cholesterol[a]	Sp act	Elastin-bound cholesterol[c]
Normal aorta	Top	0.12	10,350			
	Middle	0.07	12,030			
	Pellet	0.21	15,870	0.01	21,750	2
Plaque	Top	2.30	11,660			
	Middle	0.75	14,700			
	Pellet	2.83	4630	0.32	3380	11

[a] mg free cholesterol/g aorta wet weight.

[b] dpm/mg cholesterol.

[c] Expressed as a percentage of the pellet cholesterol.

TABLE III. Cholesterol Concentrations in Fractionated 16 mo Regressed Atherosclerotic Plaques in White Carneau Pigeon.

Fraction	Cholesterol[a]	Sp act[b]	Elastin-bound cholesterol[a]	Sp act	Elastin-bound cholesterol[c]
Top	0.64	7740			
Middle	0.54	11,190			
Pellet	6.75	2210	1.23	1480	18

[a] mg free cholesterol/g aorta wet weight.
[b] dpm/mg cholesterol.
[c] Expressed as a percentage of the pellet cholesterol.

The elastin-bound cholesterol of plaque comprised 11% of the pellet cholesterol whereas only 2% of the pellet cholesterol of normal artery was bound to elastin. Of the total plaque cholesterol, 5% was attributed to binding with plaque elastin. Elastin cholesterol specific activity of the plaque fractions was lower than the entire pellet and implicated some degree of lesser miscibility of this cholesterol pool with other pools.

Previously, in naturally occurring and diet-aggravated lesions, large increases were seen in the cholesterol concentration of the top fraction; but in fractionated regressed plaques (Table III) only 8% of the total arterial free cholesterol was localized in the top fraction. The bulk of the cholesterol in the regressed lesions was associated with the bound fraction.

The specific activity of the pellet fraction was markedly less than the top and middle fractions indicating the site of a slowly miscible cholesterol pool. The elastin-bound cholesterol of the regressed plaques constituted 18% of the pellet cholesterol and approximately 16% of the entire free cholesterol of the lesion.

Since the specific activity of the elastin cholesterol was nearly 50% lower than the specific activity of the total pellet, the elastin-bound cholesterol of these regressed plaques seemingly was less exchangeable with the cholesterol of other arterial pools and plasma.

The relative miscibilities of arterial and plasma cholesterol in progressing and regressing lesions were calculated for each group (Table IV). The specific activities of the arterial fractions were expressed as a percentage of the mean plasma cholesterol specific activity that was obtained during the 10 day period of isotope injections.

The mean plasma cholesterol specific activities for the grain-fed, cholesterol-fed, and grain-fed lesion regression groups were 103,780; 43,480; and 152,730, respectively.

In normal aorta, the free cholesterol specific activities of all three fractions were equivalent suggesting miscibility of arterial and plasma cholesterol pools. After comparing plaque cholesterol miscibility, the cholesterol of the diet-aggravated lesion showed relatively more miscibility with plasma cholesterol than the other two lesions. Cholesterol miscibility was less in the naturally occurring plaques and dramatically reduced in the regressed plaques.

TABLE IV. Relative Miscibility of Arterial Cholesterol Fractions and Plasma Cholesterol in Normal Aorta and Atherosclerotic Plaques.[a]

Fraction	Normal aorta	Plaque		
		Diet-aggravated	Naturally-occurring	Regressed
Top	23	27	10	5
Middle	23	34	14	7
Pellet	25	11	5	1

[a] Miscibility is expressed by a percentage value which represents the cholesterol specific activity of each fraction as a percentage of the mean plasma cholesterol specific activity.

In all cases the pellet was seen to be the site of the least miscible cholesterol pool.

Discussion. In this study we have separated the aortic cholesterol into three fractions which are metabolically homogeneous in normal aorta but heterogeneous in atherosclerotic plaques.

Previous reports based on ultrastructural and radioautographic findings (19, 20) have indicated that most of the free cholesterol and phospholipid of normal arteries are integral components of cellular membranes.

Several workers using ultracentrifugal methods have fractionated arterial homogenates from rabbits (11) and squirrel monkeys (19) fed diets supplemented with cholesterol. Pellet fractions were described as containing mainly connective tissue proteins such as collagen and elastin, cellular and nuclear membranes, and various fragmented cellular organelles. A middle or infranatant fraction was composed of membrane-bound vesicles which were formed during tissue homogenation and centrifugation. Portman, Alexander and Osuga (21) reported that the smooth vesicles produced during homogenation had some properties of microsomes. The top fraction has been described to be a fat-rich creamy layer of large fat globules and some myelin figures but no cell membranes. Any lipids seen in such creamy or top fractions were in nonmembraneous forms.

We have examined samples of fractionated normal pigeon aorta and plaque by electron microscopy (data not presented). These fractions were comparable to those used in the present study and were found to contain similar components as described above.

The bulk of the free cholesterol in fractionated normal aorta samples was associated with protein structures which sedimented at $d = 1.0312$.

Both naturally occurring and diet-aggravated plaques had large increases in cholesterol concentrations of all three fractions. The prominent increments seen in the cholesterol in the top fractions most probably appear histologically as intracellular and extracellular droplets. Apparently the unbound fraction is more miscible with plasma than the pellet cholesterol pool as indicated by the higher specific activity of the former. The regressed plaques showed a dramatic decrease in cholesterol concentration in the nonbound fraction in comparison with the cholesterol-aggravated and the naturally occurring plaques. Conceivably, this nonbound cholesterol pool constitutes the bulk of the lipid capable of regression. Theoretically, the limits of regression of atherosclerotic plaques may depend upon the size of the nonbound pool of cholesterol. These biochemical findings are in agreement with the histologic appearance of regressed lesions of pigeons (13) which indicate the disappearance of lipid from foam cells and from large extracellular lipid droplets.

The decrease in cholesterol content of plaques during regression suggests the presence of a two pool system (13). One pool is rapidly miscible and described by the rapid decrease in plaque cholesterol seen through 4 mo regression. The other is a slowly miscible pool and described by a plateaued arterial cholesterol level seen through 16 mo regression.

In this study, the bulk of the cholesterol in the 16 mo regressed lesions was associated with the bound fraction. The morphologic description of the regressed pigeon plaque indicates the lesion to be predominantly collagen, smooth muscle cells, fibrocytes, and cholesterol crystal clefts. The slowly miscible cholesterol pool remaining in the regressed lesions and described metabolically in this study may be synomymous with the prominent sterol clefts in regressed lesions.

A comparison of the relative miscibilities of arterial and plasma cholesterol was greatest in the cholesterol-aggravated plaques, less in the naturally occurring plaques and least in the regressed plaques. This implies that the cholesterol exacerbated plaques which occur in the short time interval of 12 mo may contain a more labile form of cholesterol than contained in a naturally occurring lesion developed during a longer time interval. The regressed lesions void of large amounts of lipid constituting a labile cholesterol pool, had a low degree of miscibility with plasma cholesterol.

Certainly one of the most discernible events in the atherosclerotic plaque is a

splitting or fragmentation of the internal elastica at the base of the lesion. Concurrent with observations of sterol crystals aligned along fragmented elastic fibers (1, 13) elastin recently has been implicated to be an important site of cholesterol binding (22, 23). Lipid deposition in human atherosclerotic plaques has been indicated to result from a lipid, possibly low density lipoprotein cholesterol, interaction with an altered elastin protein (23). We have shown that elastin is an important site for cholesterol binding, however, this may account for only 10–20% of the bound cholesterol in the lesion.

The pellet fraction of regressed plaques was least miscible with plasma cholesterol than the other two types of plaques. An identification of specific compounds and any structural interactions that may act to bind cholesterol would further characterize this slowly miscible pool of lipid. Conceivably collagen may be important in a yet undefined manner, since morphologic and biochemical data indicate very large increases in the collagen content of regressed lesions (13, 14).

Summary. Cholesterol pools were characterized metabolically in normal aorta and aortic plaques in White Carneau pigeons with naturally occurring, cholesterol-aggravated, and regressed atherosclerosis. Ultracentrifugation methods were used to separate *in vivo* [^{3}H] cholesterol labeled aortic homogenates into top (nonbound), middle, and pellet (bound) fractions.

Naturally occurring and cholesterol-aggravated plaques had 40–50% of the total arterial cholesterol in the unbound pool whereas regressed lesions had only 8%. The unbound pool was described as the cholesterol capable of leaving a lesion after regression.

The bound fraction of all plaques was the site of a slowly miscible cholesterol pool, and an increased amount of elastin-bound cholesterol.

The relative miscibility of plaque cholesterol pools with plasma was the greatest in cholesterol-aggravated lesions, less in naturally occurring lesions and dramatically reduced in regressed lesions.

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