Lipids of human atherosclerotic plaques and xanthomas: clues to the mechanism of plaque progression

Joseph H. Rapp, William E. Connor, Don S. Lin, Toshio Inahara, and John M. Porter

Section of Clinical Nutrition and Lipid Metabolism, Department of Medicine, and the Division of Vascular Surgery, Department of Surgery, The Oregon Health Sciences University, Portland, OR 97201

Abstract While the content of fatty streaks and fibrous plaques has been extensively studied in autopsied specimens, little is known about the lipid composition of advanced human atherosclerotic plaques requiring surgical removal. We have analyzed free cholesterol, cholesteryl ester, and the cholesteryl ester fatty acid content in 19 carotid and 7 femoral obliterative plaques obtained at endarterectomy. These were compared with values from each subject's plasma and from xanthomas removed from eight patients. The total cholesterol content was 75.1 mg/g dry weight for carotid plaques, 56.0 mg/g for femoral plaques, and 106.8 mg/g for xanthomas. The free cholesterol content was 56.6% and 50.4% of the total cholesterol for carotid and femoral plaques, respectively, while the free cholesterol of xanthomas was only 25.5%. The fatty acids of cholesteryl esters were analyzed in an attempt to identify the site of their esterification, i.e., within plasma or within plaque. This can be determined using the ratio of linoleic acid (18:2) to oleic acid (18:1) in the cholesteryl ester. The ratios were 0.36 for xanthoma, 1.62 for carotid plaque, 1.73 for femoral plaque, and 2.51 in plasma. These data emphasize two chemical changes occurring with evolution of the atherosclerotic process: *1*) The cholesteryl ester fatty acid composition of the plaque becomes increasingly similar to that of plasma, and *2*) there is a continuing increase in the percentage of free cholesterol. These alterations reflect a decreased metabolic efficiency within atherosclerotic lesions and may initiate events that enhance plaque progression. **Rapp, J. H., W. E. Connor, D. S. Lin, T. Inahara, and J. M. Porter.** Lipids of human atherosclerotic plaques and xanthomas: clues to the mechanism of plaque progression. *J. Lipid Res.* 1983. **24:** 1329–1335.

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Atherosclerotic plaques and xanthomas both represent lesions of lipid storage usually associated with elevated plasma cholesterol levels (1). Both lesions occur commonly in familial hypercholesterolemia where their severity and rate of progression are closely related (2, 3). Fatty streaks, probably the earliest lesion of atherosclerosis, and xanthomas have similar lipid compositions (4–7). Their structures are also similar being predominantly cellular in composition (i.e., foam cells). In contrast, advanced atherosclerotic plaques consist predominantly of acellular material (5). This loss of cellularity could be a determinant of the metabolic changes in the advanced plaque as compared to earlier atherosclerotic lesions.

The type of fatty acid esterified to cholesterol appears to be specific for the site of esterification. Monounsaturated fatty acids, primarily oleic acid (18:1 n-9), are the preferred substrate for intracellular cholesterol esterification by the acylcoenzyme A:cholesterol acyltransferase system (ACAT) (8, 9). In humans oleic acid can be either newly synthesized or obtained from dietary sources. Cholesterol esterification in plasma involves the enzyme lecithin:cholesterol acyl transferase (LCAT) which transfers fatty acids from the 2-position of lecithin to cholesterol (10). As the 2-position usually carries a polyunsaturated fatty acid, most commonly linoleic acid (18:2 n-6), cholesteryl linoleate is the most common cholesteryl ester. Linoleic acid cannot be synthesized by humans and must be obtained exclusively through the diet. A predominance of cholesteryl oleate within plaque would most likely imply local esterification, while high levels of polyunsaturated cholesteryl ester (i.e., cholesteryl linoleate) within the plaque implies infiltration and deposition of cholesteryl ester from plasma. Other possibilities for the origin of plaque cholesteryl linoleate may exist. Fatty acids for cholesteryl ester synthesis could originate from plaque phospholipids. However, LCAT activity in the lesions of experimental atherosclerosis is not thought to be significant (11).

Although the lipid content of fibrous plaques has been analyzed, to our knowledge, a series of exclusively

Abbreviations: TLC, thin-layer chromatography; GLC, gas–liquid chromatography; LCAT, lecithin:cholesterol acyltransferase; ACAT, acylcoenzyme A:cholesterol acyltransferase.

obliterative human plaques has not been examined. It has been generally assumed that the cholesterol content of these advanced plaques is predominantly cholesteryl ester. However, as plaques develop, their ability to esterify cholesterol may be impaired, allowing free cholesterol to accumulate (12). Since the injection of free cholesterol crystals is irritating to tissues, stimulating an inflammatory response with the local deposition of collagen (13), their accumulation in plaques could play a part in the evolution of the occlusive atherosclerotic lesion.

In order to determine *1*) if cholesteryl ester in advanced plaque is derived directly from plasma; and *2*) if the free cholesterol content of plaque increases as the disease becomes more advanced, we have assayed occlusive carotid and femoral plaques for cholesteryl ester fatty acids and for free and esterified cholesterol content. These have been compared to analogous values from plasma, xanthoma, and plaques at different stages of atherosclerosis as noted in the literature.

METHODS

Advanced atherosclerotic plaques were obtained from 19 patients with angiographically demonstrated carotid artery stenosis who underwent carotid endarterectomy and from seven patients with severe claudication who had femoral endarterectomies. Endarterectomy specimens consisted of only the portions of artery wall that were involved with the atherosclerotic process. The dissection plane included intima and the innermost layers of media when these were obviously diseased. Xanthomas were obtained from eight patients who elected to have their surgical removal. Six of those xanthomas were tuberous xanthomas of the skin obtained from various locations and two were tendon xanthomas. All patients provided informed consent according to protocols approved by the Human Research Committee of the Oregon Health Sciences University.

Tissues were processed immediately after surgical removal. The tissues were washed with physiologic saline to remove thrombi and blood, then blotted dry. Each sample was minced, weighed, freeze dried, reweighed, and extracted by grinding with a mortar and pestle in four aliquots of chloroform–methanol. Lipids were extracted from plasma by the chloroform–methanol procedure of Folch, Lees, and Sloane Stanley (14).

Aliquots of the extracts were plated on silica gel G thin-layer chromatography (TLC) plates after [4-^{14}C]cholesterol and cholesteryl [^{14}C]stearate were added as internal standards. The plates were developed in hexane–chloroform–ether–acetic acid 80:10:10:1. After spraying with Rodamine G, the lipid bands were visualized with ultraviolet light. The free cholesterol and cholesteryl ester bands were removed and extracted with ether. Cholesteryl ester was saponified with alcoholic KOH and the cholesterol was extracted with hexane. The cholesteryl ester fatty acids were recovered by acidifying the aqueous phase and re-extracting with hexane. Cholesterol content was determined by gas–liquid chromatography (Hewlett Packard 7610A, Avondale, PA) on a 3.8% SE-30 glass column. Cholestane and stigmasterol were used as internal standards, according to the method of Miettinen, Ahrens, and Grundy (15).

The fatty acids were methylated with boron trifluoride–methanol and relative concentrations were determined by gas–liquid chromatography (Hewlett Packard 5830A, Avondale, PA) with a coiled-glass column packed with Supelco 10% SP2330 on chromosorb, WAW GA 2537 A (100/120 mesh) (16). The column temperatures were programmed from 180°–200°C at a rate of 2°C/min. Fatty acid standards (Supelco, Inc., Bellefonte, PA) were run daily.

For comparison, a series of samples were also analyzed by a gas–liquid chromatograph (Perkin-Elmer Sigma 3B, Norwalk, CT) equipped with glass capillary column (Supelco SP 2330, 30 meters). The results of the two methods were identical for all major peaks. We have utilized the packed column data in this report for most of the results.

Although identification of most fatty acids could be achieved by this methodology, the peaks of three pairs of fatty acids overlapped. These were 18:3 (n-3) and 20:1 (n-9); 20:4 (n-6) and 22:1 (n-11); and 22:4 (n-6) and 24:1 (n-9). Separation of these mono- and polyunsaturated fatty acids was achieved by TLC of the fatty acid methyl esters on silver nitrate plates as described by Dudley and Anderson (17). After the samples were extracted with hexane, they were then analyzed by gas–liquid chromatography as described above. These determinations were also verified by glass capillary column GLC.

Comparisons of the fatty acid composition of plasma, xanthoma, and atherosclerotic plaque were carried out with the paired *t* test using the Bon Ferroni correction factor for multiple comparisons (18). The means, standard deviations, and confidence limits are listed with each result.

RESULTS

Cholesteryl ester fatty acid composition

Oleic (18:1) and linoleic (18:2) acids were the most common fatty acids found in the cholesteryl esters of all plasma and tissues (see **Table 1** and **Table 2**). Together they amounted to more than 60% of the total fatty acids in plaque, xanthoma, and plasma. However,

TABLE 1. The fatty acid composition of cholesteryl esters from plasma, atheromatous plaques and xanthoma

Fatty Acid	Plasma n = 14	Carotid Plaque n = 11	Femoral Plaque n = 7	Xanthoma n = 8
		percent of total fatty acids		
14:0	0.7 ± 0.5	0.9 ± 0.6	1.2 ± 0.6	0.8 ± 0.3
14:1 (n-9)	0.2 ± 0.3	0.4 ± 0.6	0.6 ± 0.5	0.2 ± 0.2
15:0	0[a]	0.1 ± 0.1	0.2 ± 0.2	0.2 ± 0.2
16:0	12.0 ± 1.1	12.7 ± 1.7	16.2 ± 3.9	7.6 ± 2.0
16:1 (n-9)	3.8 ± 1.1	4.6 ± 1.3	3.3 ± 1.9	7.6 ± 1.9
17:0	0	0	0	0
18:0	1.4 ± 0.6	1.5 ± 0.5	2.7 ± 2.1	1.4 ± 1.0
18:1 (n-9)	20.2 ± 2.5	25.5 ± 4.5	23.5 ± 3.1	47.8 ± 4.4
18:2 (n-6)	50.0 ± 5.2	38.1 ± 6.7	40.0 ± 8.9	17.4 ± 3.5
18:3 (n-6)	0.7 ± 0.9	0.6 ± 0.3	0.6 ± 0.5	0.4 ± 0.7
18:3 (n-3)	0.1 ± 0.2	0.4 ± 0.3	0.3 ± 0.4	1.1 ± 1.3
20:1 (n-9)	0	0.2 ± 0.3	0.3 ± 0.4	1.3 ± 1.4
20:0	0	0	0	0
20:3 (n-6)	0.4 ± 0.5	2.4 ± 0.8	1.2 ± 0.9	3.5 ± 1.7
20:4 (n-6)	8.1 ± 2.5	8.3 ± 2.3	5.5 ± 2.6	4.3 ± 1.4
22:1 (n-11)	0	0	0	0
20:5 (n-3)	0.3 ± 0.3	0.7 ± 0.6	0.3 ± 0.3	0.5 ± 0.5
22:0	0	0	0	0
22:4 (n-6)	0	0.7 ± 0.7	0.1 ± 0.1	1.2 ± 0.8
24:1 (n-9)	0	0.2 ± 0.2	0	0.5 ± 0.6
22:5 (n-3)	0	0.2 ± 0.2	0	0.5 ± 0.5
22:6 (n-3)	0.2 ± 0.2	0.6 ± 0.3	0.1 ± 0.1	0.6 ± 0.8
24:0	0	0.1 ± 0.3	0	0

[a] Amounts listed as zero were below 0.05%.

the relative amounts of these two key fatty acids were unique to each source. In plasma, linoleic acid predominated and accounted for 50.0% of the cholesteryl ester fatty acids. In femoral and carotid plaques, linoleic acid contributed 40.0% and 38.1%, respectively, while in xanthomas it was only 17.4% of cholesteryl ester fatty acids. The reverse order of concentration occurred for oleic acid in the three different tissues. The oleic acid content of xanthoma cholesteryl ester was the highest of the samples studied, 47.8% of total fatty acids; in plasma it was 20.2%; while in carotid and femoral plaques it was 25.5% and 23.5%, respectively.

Table 2 summarizes the individual fatty acid data of Table 1. Polyunsaturated fatty acids are made up of two classes: the n-6 and the less common n-3 group. The n-6 fatty acids were predominantly linoleic (18:2 n-6), arachidonic (20:4 n-6), and two intermediaries in the synthetic pathway from linoleic to arachidonic, dihomo-gamma linolenic (18:3 n-6) and eicosatrienoic (20:3 n-6). The n-3 fatty acids were mainly linolenic (18:3 n-3) and two biologically important longer chain fatty acids, eicosapentaenoic (20:5 n-3) and docosahexaenoic acid (22:6 n-3). In both plaques and xanthomas the amounts of n-3 fatty acids were increased over the amounts present in plasma.

The total polyunsaturated and monounsaturated fatty acids generally followed the trends set by 18:2 and 18:1 (Table 2). Xanthomas had a significantly different total polyunsaturated, monounsaturated, and saturated fatty acid composition compared to plaques and plasma ($P < 0.001$ for each category). Although femoral and carotid plaques contained lower levels of polyunsaturated fatty acids than plasma, the absolute difference was considerably smaller than that for plasma and xanthomas. The situation was more complex for saturated fatty acids. Femoral plaques had significantly higher levels of saturated fatty acids than plasma and carotid plaques, but each was increased over xanthoma.

An alternative way to express these tissue-plasma differences in cholesteryl ester fatty acids is to consider the ratios of linoleic acid to oleic acid (18:2 to 18:1) (**Table 3**). In plasma, this ratio was 2.51; in carotid and femoral plaques it was 1.62 and 1.73, respectively; while in xanthomas the ratio was 0.36. While the relative amounts of these two fatty acids in the tissues and plasma differed several-fold, the sum of 18:2 and 18:1 was similar in all samples.

Plaque and xanthoma cholesterol content

The total cholesterol and percentage of free cholesterol are shown for carotid and femoral plaques in **Table 4.** The total cholesterol was 75.1 ± 28.7 mg/g dry weight for carotid plaques and 56.0 ± 33.4 mg/g dry

TABLE 2. Summary of cholesteryl ester fatty acid composition

	Plasma	Carotid Plaque	Femoral Plaque	Xanthoma
		percent of total fatty acids		
Total polyunsaturated	59.5 ± 5.3	51.9 ± 6.9[a,b]	48.2 ± 7.0[a,c]	30.2 ± 4.2[c]
Total n-3	0.5 ± 0.4	1.8 ± 0.9[c,d]	0.9 ± 0.6[c,d]	2.7 ± 2.5[b]
Total n-6	59.1 ± 5.2	49.9 ± 6.7[a,c]	47.5 ± 7.1[a,c]	29.0 ± 4.1[c]
Total monounsaturated	24.7 ± 3.7	30.9 ± 5.2[a,b]	28.3 ± 4.2[a,b]	56.4 ± 3.2[c]
Total saturated	14.3 ± 1.8	15.3 ± 1.8[a,c,e]	20.2 ± 3.8[a,c]	10.7 ± 3.1[c]

[a] $P < 0.001$ vs. xanthoma.
[b] $P < 0.005$ vs. plasma.
[c] $P < 0.001$ vs. plasma.
[d] $P < 0.005$ vs. xanthoma.
[e] $P < 0.005$ vs. femoral.

TABLE 3. The ratio of linoleic acid (18:2) to oleic acid (18:1) in the cholesteryl ester fraction of various tissues

Source	Ratio 18:2/18:1	Percentage of Total Cholesteryl Ester Fatty Acids Represented by 18:1 + 18:2
Xanthoma	0.36 ± 0.08[a]	65.2[a]
Early fatty streak	0.24[b]	66.0[b]
Advanced fatty streak	0.86[c]	65.8[c]
Fibrous plaque	1.19[d]	66.2[d]
Fibrous plaque	1.41[b]	70.9[b]
Fibrous plaque	1.32[c]	65.5[c]
Obliterative plaque		
Carotid	1.62 ± 0.46[a]	63.6[a]
Femoral	1.73 ± 0.44[a]	63.5[a]
Plasma	2.51 ± 0.62[a]	70.2[a]

[a] Present study.
[b] Reference 6.
[c] Reference 11.
[d] Reference 30.

weight for femoral plaques. This difference was not significant. The percentage of free cholesterol, however, was different between the two plaque series. In carotids, the percent of free cholesterol was 56.6 ± 6.0 while in the femorals it was lower (50.4 ± 5.5%) ($P < 0.025$). In xanthoma, the total cholesterol was 106.8 ± 60.4 mg/g dry weight. The percentage of free and esterified cholesterol was also fairly consistent from sample to sam-

TABLE 4. Cholestrol content of carotid and femoral atherosclerotic plaques

Subject	Free	Esterified	Total	% Free
		mg/g dry weight		
Carotid plaques (n = 19)				
1	99.0	66.0	165.0	60.0
2	50.6	40.9	91.5	55.3
3	51.0	40.9	91.9	55.4
4	29.6	29.1	58.7	50.4
5	23.2	28.7	51.9	44.7
6	20.0	12.5	32.5	61.5
7	54.0	24.0	78.0	69.2
8	26.8	27.1	53.9	49.7
9	48.2	36.6	84.8	56.8
10	20.5	19.6	40.1	51.1
11	31.1	26.6	57.7	53.9
12	49.6	23.4	73.0	67.9
13	45.6	40.8	86.4	52.8
14	66.2	43.8	110.0	60.2
15	41.4	29.2	70.6	58.6
16	40.1	35.8	75.9	52.8
17	40.3	28.5	68.8	58.6
18	40.0	28.4	68.4	58.5
19	39.5	28.0	67.5	58.5
Mean	43.0 ± 18.3	32.1 ± 11.5	75.1 ± 28.7	56.6 ± 6.0[a]
Femoral plaques (n = 7)				
1	12.3	12.8	25.1	49.0
2	43.2	60.0	103.2	41.9
3	29.7	32.6	62.3	47.7
4	9.0	9.9	18.9	47.6
5	13.9	11.4	25.3	54.9
6	45.6	40.8	86.4	52.8
7	41.4	29.2	70.6	58.6
Mean	27.9 ± 16.0	28.1 ± 18.5	56.0 ± 33.4	50.4 ± 5.5

[a] $P < 0.025$ vs. femoral.

TABLE 5. Percent of total cholesterol present as free cholesterol in xanthomas, atherosclerotic plaques, and plasma

Source	Percent of Free Cholesterol
Xanthomas	25.5[a]
Early fatty streaks	19.9[b]
Advanced fatty streaks	39.8[c]
Fibrous plaques	36.3[d]
Fibrous plaques	33.3[b]
Fibrous plaques	41.1[c]
Obliterative plaques	
Carotid	56.6[a]
Femoral	50.4[a]
Plasma	23[e]

[a] Present study.
[b] Reference 6.
[c] Reference 11.
[d] Reference 29.
[e] Reference 30.

ple for xanthoma, despite variations in the total cholesterol concentrations (data not shown). In contrast to the acellular atherosclerotic plaques, the percent of free cholesterol in xanthoma was only 25.5 ± 10.1 (**Table 5**).

DISCUSSION

While the lipid composition of atherosclerotic plaques is not a new subject, detailed analyses of obliterative lesions in well-defined, peripheral arteries by modern methodology have not been reported until the present study. The lipid content of these advanced plaques as compared to those of xanthomas and earlier atherosclerotic lesions demonstrates an impressive increase of free cholesterol and cholesteryl ester, especially cholesteryl linoleate. The accumulation of both these substances may represent a deterioration in the metabolic efficiency of the arterial wall which apparently occurs with plaque progression.

Plasma has been shown to be the major source of the cholesterol present in atherosclerotic lesions (19). However, in fatty streaks the LCAT-synthesized (plasma) linoleate ester of cholesterol exists only in small amounts (6). Most of the incorporated plasma cholesteryl esters are hydrolyzed and re-esterified via ACAT to cholesteryl oleate (7). In vitro the capacity of the membrane-bound ACAT system can be overwhelmed by large influxes of cholesteryl ester (20). Within fatty streaks in vivo, lowered ACAT efficiency is more likely due to decreasing membrane fluidity caused by the deposition of excess free cholesterol in cellular membranes (21). ACAT activity may be lost completely in advanced acellular plaque, since experimentally it is inactivated by cellular disruption (8).

Cholesteryl esters accumulating within the foam cells of xanthomas and fatty streaks aggregate into lipid droplets. There they may exist in a liquid crystalline configuration or in random order, dependent upon their fatty acid composition and their aggregate transition temperature (22). Unlike cholesteryl ester, free cholesterol forms plate-like, crystalline structures which are commonly seen in advanced atherosclerotic lesions (23, 24). The initial formation and continued presence of these crystals may have important consequences for plaque development.

Jackson and Gotto (21) have described an intracellular free cholesterol accumulation in fatty streaks. They suggest that initially excess free cholesterol is incorporated into cellular membranes. This decreases membrane fluidity and, therefore, generally reduces cellular metabolic efficiency. Decreasing ACAT activity may allow a further accumulation of nonesterified (free) cholesterol and continue the cycle of deteriorating metabolic efficiency. Eventually, the foam cells become supersaturated with free cholesterol (12). The precipitation of rigid free cholesterol crystals intracellularly could cause foam cell destruction and initiate the transformation of fatty streak into fibrous plaque. Intermediate lesions, fatty streaks with acellular cores, have been described histologically (6, 25).

While a degenerative process occurs commonly in atherosclerotic plaques, this has never been reported in xanthomas. Xanthoma cholesterol also originates from plasma (1) and accumulates to levels similar to advanced atherosclerotic lesions. Xanthomas also may have complex chemical characteristics (26). However, they retain their cellularity and ability to metabolize infiltrating plasma esters to cholesteryl oleate. Free cholesterol can accumulate in xanthomas and cholesterol crystals are often seen, but complete destruction of foam cells ap-

parently does not occur. Some innate cellular or mechanical characteristics of these lesions must allow xanthomas to better withstand the massive infiltration of plasma cholesterol.

Katz and colleagues (27) have calculated the metabolic activity of the separate physical states of cholesterol in atheromatous plaques. They found cholesteryl ester to have a slow but significant turnover while crystalline cholesterol was relatively inert. As a result, they proposed that a substantial mobilization of crystalline free cholesterol was unlikely to occur. There appears to be a sequestration of free cholesterol within plaque into an inaccessible pool. In the present study the free cholesterol content of advanced carotid and femoral lesions was 50% of the total cholesterol. This accumulation is remarkable, given that there is minimal de novo cholesterol synthesis (11) and that infiltrating plasma cholesterol is 75% esterified (28).

Crystalline free cholesterol may have a role in stimulating the inflammatory response that is characteristic of atherosclerosis. Experimentally, the injection of these crystals into the arterial wall (13) and other tissues (29) elicits an inflammatory response with the local production of collagen. With time, this chronic inflammation may be responsible for the scarring and calcification seen in advanced atherosclerotic lesions.

The loss of metabolic efficiency with a resultant accumulation of cholesterol linoleate and free cholesterol is only part of the puzzle of atherosclerotic plaque development. However, these accumulations may offer important clues to the mechanisms involved in the atherosclerotic process. Infiltration of plasma cholesteryl ester into the plaque demonstrates the intimate relationship of plasma lipids and the arterial wall. The increasing free cholesterol content may have a role in determining the extent and severity of the inflammatory response within the atherosclerotic lesion. Finally, as the free cholesterol content increases, the regression of these lesions may become more difficult, since once in its rigid crystalline form, solubilization and mobilization of cholesterol seems unlikely.

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