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Chemical Composition and Physical State of Lipid Deposits in Atherosclerosis

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Summary

The composition, morphology, and physical properties of lipids in atherosclerotic lesions from human aortas were studied in order to elucidate the factors for the accumulation of cholesterol and its esters in the vessel wall. Lesions were classified histologically into 3 groups: fatty streak, fibrous plaque, and advanced plaque. The relative lipid composition of the lesions was plotted on the phase diagram of the 3 major lipids: cholesterol, cholesteryl ester, and phospholipid. Early fatty streaks had compositions within the 2-phase zone with a cholesterol–phospholipid liquid crystalline phase and a cholesteryl ester oily phase. Advanced fatty streaks and fibro-fatty plaques fell within the 3-phase zone with excess free cholesterol. Advanced plaques also had an average lipid composition within the 3-phase zone, but with a larger excess of free cholesterol. From the lipid-chemical point of view there is a continuous progression from early fatty streaks through advanced fatty streaks and fibro-fatty plaques to advanced plaques.

In fatty streaks the cholesteryl esters accumulate in the form of isotropic and anisotropic droplets. The latter are in the smectic liquid crystalline state with the molecules arranged in layers and have surfaces that are spherical and smooth. Fibrous and advanced plaques showed beside droplets also amorphous lipids and cholesterol monohydrate crystals. Some of the amorphous lipids were solid up to about 45°C and exhibited a smectic phase at cooling, indicating cholesteryl esters as the major component. The transition temperatures of high-melting cholesteryl esters, e.g. palmitate, are depressed by low-melting ones. Most of the triglycerides are present in the cholesteryl ester droplets and abolish the cholesteric liquid crystalline phase.

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Introduction

The accumulation of cholesterol and its esters is a characteristic feature of atherosclerosis. The normal cholesterol homeostasis of pheripheral cells has been elucidated in detail [1], but the mechanism behind the abnormal accumulation of lipids in arterial tissue is still unclear.

The earliest chemical change in the pathogenesis of the atherosclerotic lesion is an accumulation of cholesteryl esters in the form of intracellular liquid or liquid crystalline droplets. The cholesteryl esters present in the early lesion of fatty streak are synthesized by the arterial cells from plasma-derived free cholesterol [2]. The accumulation of cholesteryl esters is preceded by an increase in the molar ratio between free cholesterol and phospholipids [3], which may be a decisive factor for the initiation of atherosclerosis.

The question whether the fatty streak can progress to a more advanced type of lesion is still controversial. In contrast to the fatty streak, the fibrous plaque contains high levels of extracellular lipids, mainly cholesteryl esters with a composition similar to that for cholesteryl esters of LDL [4]. Numerous apoB-containing particles in the size-range of LDL and VLDL can be extracted from such lesions [5]. The most severe lesions may have a core of 'amorphous' lipid, containing cholesterol monohydrate crystals [6]. The formation of cholesterol crystals in the plaque may proceed either by supersaturation of the lipid phases or by hydrolysis of cholesteryl esters.

From the physicochemical point of view the atheroma lipids may exist in any of 3 states; liquid, liquid crystalline or crystalline, depending on their relative concentrations, lipid–lipid interactions, and transition temperatures [7,8]. The appearance of different lipid phases in atherosclerotic plaques can be explained on the basis of the phase behaviour of the lipid classes found in the lesions [9].

This paper is a study of the chemistry, morphology, and physical chemistry of lipids in human atherosclerotic lesions. The aim was to elucidate factors which can contribute to the deposition of lipid in the lesions. The main interest was attached to the cholesteryl esters, because they may play an important part in the progression of the plaque.

Materials and Methods

Sample selection and preparation

The upper abdominal parts of 21 aortas were obtained at autopsy within 24 h of death from 14 male and 7 female subjects, 42–81 years of age. Specimens for freeze-etching and scanning electron microscopy were obtained from surgically resected arterial tissue. The luminal surface was thoroughly washed free of blood

and the adventitia was carefully removed. Lesions were examined under a dissecting microscope and blocks were cut from representative regions for histological examinations. A combined macroscopic and microscopic grading was made and the atherosclerotic lesions were divided into 3 groups; stage I, fatty streaks were superficial, yellow or yellow-grey lesions; stage II, atheroma and/or fibro-fatty plaques were raised firm lesions with a gray fibrous cap overlaying a yellow lipid-rich center; stage III, complicated lesions had additional changes such as hemorrhage, ulceration, or calcification [10]. The lesions were dissected with a scalpel, minced and dried by lyophilization and in vacuo over P_2O_5. Lesions of the same type from individual aortas were pooled together to give enough material for lipid analysis and calorimetric measurements.

Lipid isolation and analysis

Lipids were extracted twice from the dried homogenates in 10 volumes of chloroform/methanol (2 : 1, v/v) containing 0.001% 4-methyl-2,6-*tert*-butylphenol (BHT) as an antioxidant, after which a Folch procedure [11] was carried out. Total lipids were obtained by weighing dried extracts. Lipid groups were isolated from total lipids by preparative thin-layer chromatography (TLC) on silica gel plates with petroleum ether/benzene/acetic acid (30 : 8 : 1) as developing solvent. Detection was carried out by spraying co-developed standard lipids with 0.2% 2′,7′-dichlorofluorescein in ethanol. The cholesteryl ester, triglyceride and total phospholipid spots were scraped off and the silica removed by eluting the lipid with chloroform/methanol (1 : 2, v/v) through a sintered glass funnel. Cholesteryl esters from selected samples were separated into high-, medium- and low-melting types by fractionated crystallization from chloroform solutions by adding methanol/ethanol mixtures. In order to demonstrate the existence of different lipid phases, density gradient centrifugation was performed giving a top layer of neutral lipids and a bottom layer of polar lipids [12].

Quantitative TLC was used to measure the cholesteryl esters, triglycerides, cholesterol, and phospholipids. Precoated silica gel plates (E. Merck, AG) were cleaned by treatment with chloroform/methanol (2 : 1) and activated for 30 min at 110°C. Samples of about 50 μg were applied and the plates were developed first to half the width in chloroform/methanol/acetic acid/H_2O (50 : 25 : 8 : 4) and then to the top in petroleum ether/benzene/acetic acid (30 : 8 : 1). Densitometric quantification was performed after detection by spraying with 50% sulphuric acid and heating at 180°C for 20 min. A reference mixture was applied on each plate containing equal amount of cholesteryl oleate, triolein, cholesterol, and egg lecithin. The molar ratio of free cholesterol to phospholipid was obtained by measuring phosphate [13] and cholesterol by an enzymatic method [14].

Fatty acid composition of the cholesteryl ester, triglyceride, and phospholipid classes was determined by gas–liquid chromatography after alkaline methanolysis [15]. The fatty acid methyl esters were purified by TLC on silica gel plates with hexane/diethyl ether (95 : 5) as solvent. A 30-m long, 0.3-mm ID glass capillary column coated with 1,4-butanediol succinate (BDS) was operated at a temperature of 190°C.

Histological methods

Blocks were fixed in formalin and sectioned by a freezing microtome. Sections were alternately stained with oil red O-hematoxylin and examined with ordinary light microscope or examined unprocessed with a polarizing microscope equiped with a hot stage. The freeze-etching was carried out with an apparatus manufactured by Balzers. The tissue was thoroughly minced with fine scissors. The mince was homogenized at 22°C in 5 ml phosphate-buffered saline with a tissue grider at 1 200 rpm for 2 min. The homogenate was centrifuged at 20 000 *g* for 30 min at 22°C. The fatty streak lipid droplets floated on the top of the tubes and were carefully drawn off and suspended in a small volume of buffer. Isolated fatty streak lipid globules and model system cholesteryl ester globules were affixed to copper discs and rapidly frozen from 37°C into liquid freon and nitrogen. The fractured specimens were etched at −100°C for 1 min. Artery specimens chosen for scanning electron microscopy were freeze-sectioned and air-dried in a dessicator over silica gel. The specimens were coated with a layer (20 nm) of gold and examined in a Jeol 35 scanning electron microscope at a beam voltage of 20 kV.

Physical methods

Polarizing microscopy was used to identify physical states of plaque and isolated lipids. The temperature was varied ($1°C \cdot min^{-1}$) with a heating and cooling stage. Sections were examined unprocessed. Cholesteryl ester in liquid crystalline state was identified from its reversible liquid–liquid crystalline transitions. Cholesterol monohydrate crystals were identified from the rhomboidal plate structure with corner angels of 80° [16]. The lyotropic phospholipid lamellar liquid crystalline phase was identified from the typical myelin-like figures.

The differential thermal analysis (DTA) was conducted with a Fisher Model 370 apparatus. The lipid samples were weighed into aluminium pans, which were then sealed with aluminium lids. The heating and cooling rate was $5°C \cdot min^{-1}$. Each sample was scanned several times in the temperature range of interest.

Results

Lipid composition

Total lipid (TL) values ranged from 6.7–25.6% of the dry weight of the aortic tissue, with mean values of 10.3 (±2.3), 11.9 (±1.6), and 22.3 (±3.5)% for stages I, II, and III, respectively. There was a slight increase in TL values with age for stage I and II, while those of stage III were almost unvaried with age. The correlations were weak for all 3 stages. The free cholesterol (FC) content of the lesions showed positive correlations with the amount of cholesteryl esters (CE) (correlation coefficients, $r = 0.20$, 0.68 and 0.76 for stages I, II, and III, respectively). The CE/phospholipid (PL) molar ratios were well correlated with stage I CE/FC weight ratios ($r = 0.69$), while the corresponding correlation coefficients for stages II and III were very low. The sphingomyelin/phosphatidylcholine ratios showed positive correlations with age, FC/PL ratios, and severity of atherosclerosis.

The relative lipid compositions of the atherosclerotic lesions in terms of the 3

major lipid groups FC, CE, and PL are illustrated in Fig. 1. Two of the fatty streaks fall within the 2-phase region (zone III, lamellar PL–FC, and oily CE phase) and the 3 others on the borderline between the 2- and 3-phase regions (zone IV, the phases of zone III plus crystalline FC). All of the stage II lesions lie within the 3-phase zone. The stage III lesions lie further up in the 3-phase region and can be predicted to contain even more FC crystals than the stage II lesions.

In order to verify the phase behaviour of the atheroma lipids as predicted from Fig. 1, chemical analysis of lipid phases isolated by sucrose density gradient centrifugation was performed. The lipids separated into 2 or 3 layers. Stage I lipids gave an oily CE phase floating on the top and a lamellar PL–FC phase at density 1.03 g/ml. Stages II and III gave further an essentially pure FC phase at density 1.06 g/ml. The floating CE phases were found to contain 76.5 (±3.5)% CE, 11.4 (±0.4)% FC, 10.6 (±2.5)% triglycerides (TG), and 1.5 (±0.4)% PL. The PL–FC lamellar phases contained 60.8 (±3.1)% PL, 29.8 (±1.5)% FC, 7.8 (±1.2)% CE, and 1.6 (±0.4)% TG. In Fig. 1 the positions in the phase diagram of the 3 phases are indicated by arrows.

The fatty acid content of the separated lipid classes CE, TG and PL are given in Table 1. They showed striking differences between all 3 groups. The 2 major fatty acids in CE (CEFA) were oleic and linoleic with the former dominating in stage I

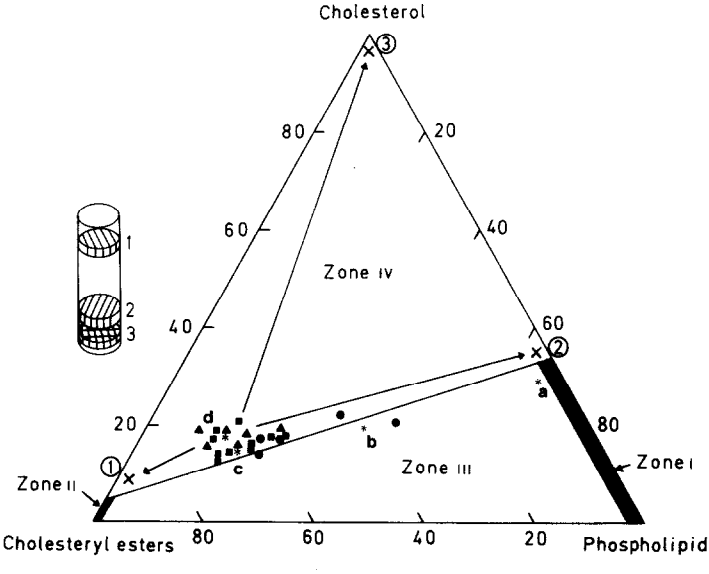

Fig. 1. Phase diagram with the relative lipid compositions in terms of cholesterol, phospholipid, and cholesteryl ester (after Small et al. [9]). Zone I, phospholipid bilayers with up to 33% cholesterol (w/w), and 2% cholesteryl ester. Zone II, a cholesteryl ester oil or liquid crystal phase with up to 4% cholesterol. Zone III, 2-phase region in which the phospholipid–cholesterol bilayer phase and the cholesteryl ester phase coexist. Zone IV, a 3-phase region with the phases of zone III plus cholesterol monohydrate crystals. The positions of normal intima, fatty streak, fibrous plaque and advanced plaque are shown by a, b, c, and d. Arrows indicate the advanced plaque (1) cholesteryl ester oily phase; (2) phospholipid–cholesterol bilayer phase; (3) cholesterol monohydrate crystals.

TABLE 1

FATTY ACID COMPOSITION OF THE CE, TG AND PL GROUPS FROM STAGE I (FATTY STREAK), II (FIBROUS AND LIPID PLAQUE) AND III (COMPLICATED LESION)

Values are means ± SE.

	Percentage of total fatty acids						
	16:0	16:1	18:0	18:1	18:2	20:4	Other
Stage I							
CE	14.2± 1.5	5.9±1.6	1.3±0.4	34.1±5.0	29.5±4.7	5.3±2.1	9.7±2.5
TG	32.7± 0.8	3.9±1.2	6.2±2.8	34.5±5.0	7.4±3.9	3.1±2.0	12.2±3.4
PL	40.9±10.8	–	17.9±4.1	20.9±7.2	6.6±5.4	4.9±2.0	8.8±2.3
Stage II							
CE	13.9± 2.0	4.6±0.8	1.2±0.1	27.5±4.3	37.5±5.8	6.8±2.2	8.5±1.9
TG	27.8± 3.2	4.1±1.3	6.2±3.1	37.5±3.8	10.2±3.6	2.4±1.3	12.1±3.2
PL	37.5± 8.1	–	18.8±5.8	18.8±6.8	6.8±3.7	6.3±3.1	11.8±2.7
Stage III							
CE	13.4± 4.1	4.4±1.4	1.3±0.5	28.2±3.6	36.5±5.4	7.5±2.1	8.7±2.1
TG	28.7± 3.7	3.6±1.4	6.8±2.2	41.4±2.8	8.2±4.1	1.6±0.6	9.7±2.6
PL	40.6±11.3	–	15.4±3.7	22.3±3.5	8.8±4.5	5.9±2.8	7.0±1.7

and the latter in stages II and III. The percentages of palmitic acid were also quite high in CE, but much lower than in TG and PL. In fact this acid was the major one in PL while oleic acid was the most abundant in TG. Noteworthy is also the high stearic acid values in PL and the much lower linoleic acid percentages in TG and PL than in CE. Significant differences between the different stages could only be discerned regarding the CE values, with cholesteryl oleate (18 : 1) dominating in stage I and cholesteryl linoleate (18 : 2) in stages II and III. The 18 : 1/18 : 1 + 18 : 2 ratio was highest for stage I (0.54 ± 0.01), while the values for stages II and III were 0.41 ± 0.06, and 0.42 ± 0.05, respectively. The ratios between the saturated, high-melting CE (palmitate + stearate) and the major fatty streak CE, oleate, were 0.45, 0.55, and 0.52 for stages I, II, and III, respectively.

Morphological and physical characteristics of lesion lipids

Most of the accumulated lipid in stage I lesions was present as intracellular droplets in fatty streaks. These droplets consist of CE with only a small amount of PC, PL, and TG [7,12]. At body temperature (37°C) they are present in 2 forms, distinguished by their optical properties, isotopic (true liquid) and anisotropic (liquid crystal). At cooling the isotropic droplets change to anisotropic ones. The anisotropic droplets have a positive sign of birefringence indicating a smectic liquid crystalline state. The similarity between fatty streak droplets and artificially preparated CE droplets is demonstrated in Figs. 2 and 3. The internal structure of the droplets are lamellar (Fig. 4) and the surface texture of the lesion droplets appears essentially spherical and smooth (Fig. 5). At cooling below body temperature both

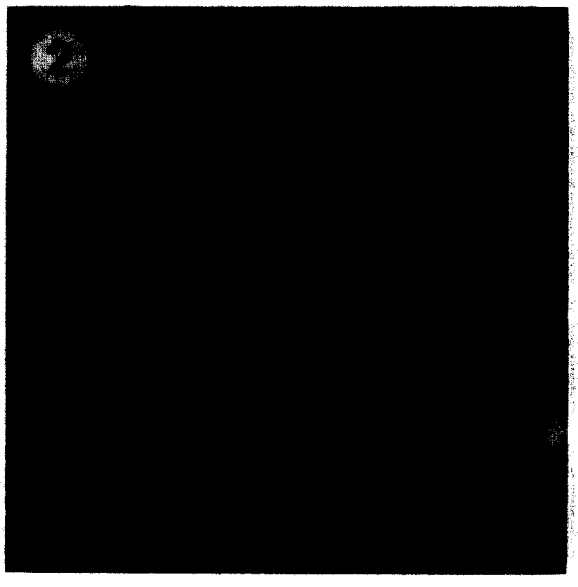

Fig. 2. Anisotropic liquid crystalline droplets in early fatty-streak lesions from aortic intima examined with polarizing light microscope, 37°C, ×1000.

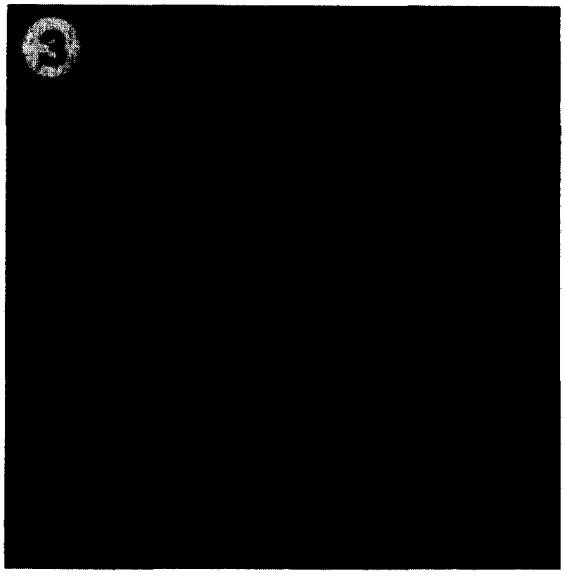

Fig. 3. Anisotropic cholesteryl oleate droplets mechanically disperged in water, 37°C, × 400.

fatty streak droplets as well as CE model droplets slowly crystallize, showing that they both are in a thermotropic liquid crystalline state. The advanced fatty streaks showed large clusters of lipid-filled cells (Fig. 6) and beginning cell disintegration.

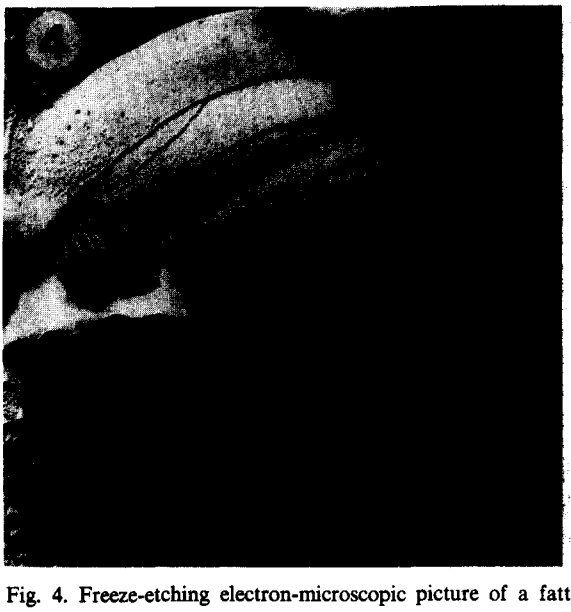

Fig. 4. Freeze-etching electron-microscopic picture of a fatty-streak lipid droplet isolated by sucrose gradient centrifugation, ×20000.

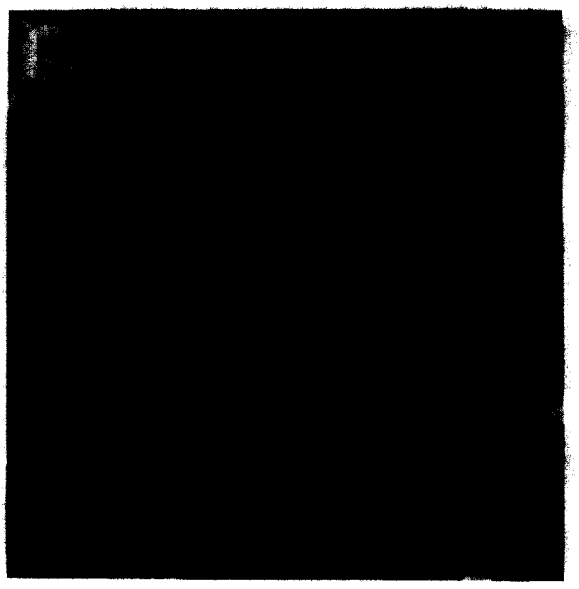

Fig. 5. Scanning electron micrograph of section with advanced fatty streak lesion showing lipid droplets, ×2000.

The intracellular droplets exhibited birefringence of the same type as the early fatty streaks (Fig. 7). Some of the stage I lesions contained besides fatty streak droplets also some 'perifibrous lipid' but crystalline lipid was never observed.

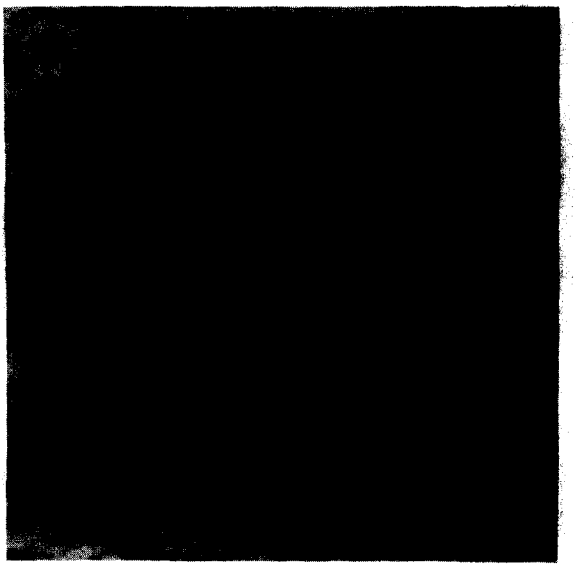

Fig. 6. Aorta, intima. Aggregates of lipid-filled cells in advanced fatty streak lesion, oil red O, ×200.

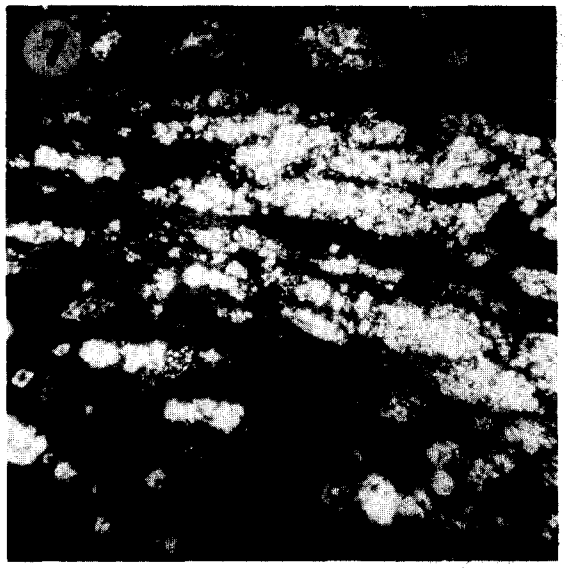

Fig. 7. Section from the same lesion as in Fig. 6. Large clusters of birefringent droplets. Polarizing microscope, 37°C, ×200.

The morphological appearance of the stage II lesions was more complex than that of stage I. They contained varying amounts of scattered and confluent lipid-filled cells, amorphous atheroma lipid of different extent and usually a lot of fine droplets

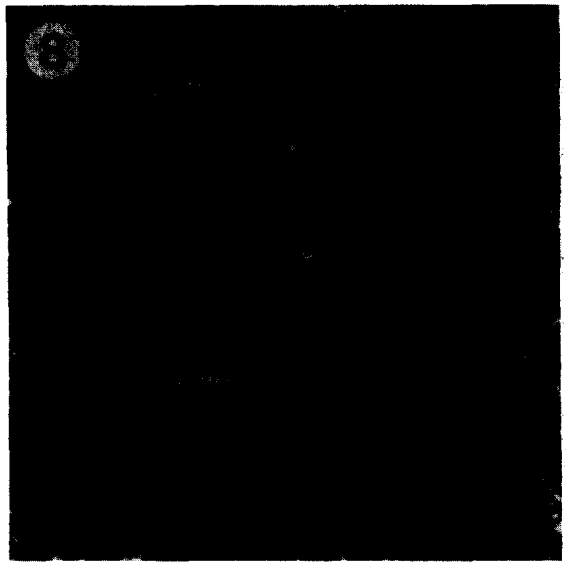

Fig. 8. Scattered, small, birefringent, low-melting droplets. Polarizing microscope, 25°C, ×400.

Fig. 9. Aorta, intima and inner media. Advanced lipid plaque with anisotropic droplets adjacent to a core with crystalline lipids, 37°C, ×200.

of 'perifibrous lipid'. Crystals of FC monohydrate are present in the central part of lipid plaques, which can be predicted from the phase diagram in Fig. 1. The 'fatty-streak globules' of stage II lesions had the same physical characteristics as the

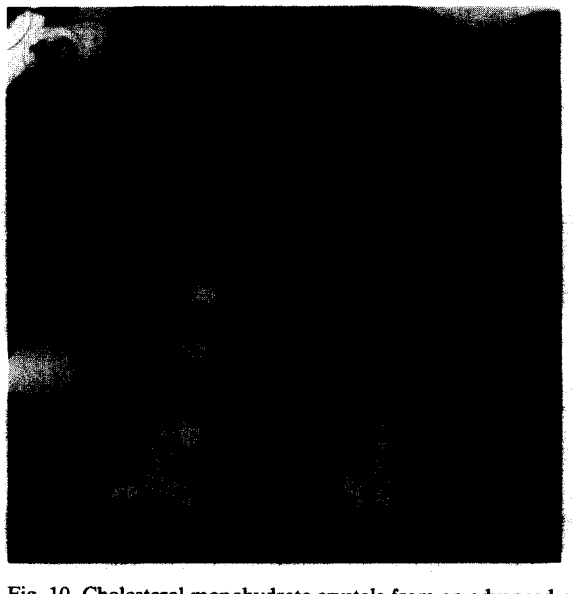

Fig. 10. Cholesterol monohydrate crystals from an advanced plaque. Polarizing microscope, 25°C, ×100.

stage I ones. The scattered small droplets of 'perifibrous lipid' appeared as small birefringent granules when examined by polarizing microscope at room temperature or below (Fig. 8). They typically melted at a lower temperature than the fatty streak globules and had melting points around 25°C.

Stage III lesions were necrotic plaques, which frequently ulcerated into the lumen. They contained abundant lipids in varying amounts of lipid-filled cells often around a necrotic region with crystalline and amorphous lipids, besides isotropic and anisotropic extracellular droplets (Fig. 9). As can be predicted from the phase diagram there are a lot of cholesterol monohydrate crystals, which can be isolated by gradient centrifugation (Fig. 10). The amorphous lipids melted between 40 and 50°C and on cooling they showed isotropic-smectic transitions before they slowly became solid again. In the regions between atheroma plaques there were many perifibrous, scattered lipid droplets.

When TL from stage I lesions were analyzed by DTA the curves were consistent.

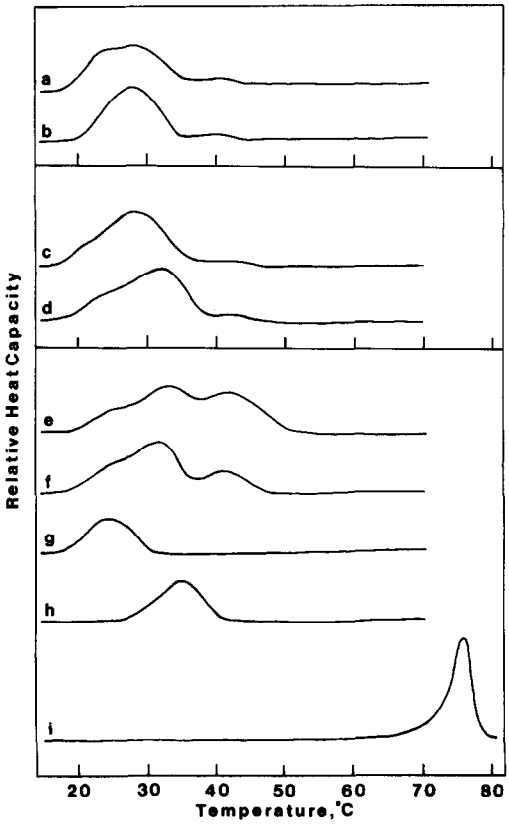

Fig. 11. Differential thermal analytic heating curves of lipids from atherosclerotic lesions; *a*, *c*, and *e*: total lipids from advanced fatty streak, fibrous plaque, and complicated lesion, respectively; *b*, *d*, and *f*: cholesteryl esters isolated from the total lipids of *a*, *c*, and *e*, respectively; *g*, *h*, and *i*: low-, medium- and high-melting cholesteryl esters fractionated by solvent crystallization.

The melting started at 20 (±2)°C and had a maximum at 28 (±3)°C. Most of the lipid were melted at 36 (±2)°C, but a small fraction remained solid up to 40 (±3)°C (Fig. 11*a*). On cooling, a broad liquid crystalline transition (liquid to smectic) appears at about 15°C. DTA traces of isolated CE showed that the major part of the melting energy of TL can be attributed to the CE fraction (Fig. 11*b*). On cooling, the CE fractions showed liquid crystalline transitions (smectic and cholesteric) at about 15°C.

The DTA traces of stage II TL were similar to those of stage I lipids but the individual variations were larger. Typically the melting began at 20 (±3)°C with a accentuated maximum at 28 (±2)°C. At 36 (±2)°C most of the TL were melted but a small portion was solid up to 42 (±3)°C (Fig. 11*c*). The isolated CE fractions showed melting maxima about 4°C higher than TL (Fig. 11*d*).

After examination by DTA stage III TL and CE typically showed a double-peaked melting transition with maxima at 32 (±2)°C and 42 (±2)°C, respectively (Fig. 11*e*). On cooling, cholesteric and smectic transitions were exhibited between 18 and 12°C. In order to elucidate the contribution of the individual CE to the DTA curves, 3 representative samples were separated by solvent crystallization into low-, medium-, and high-melting ones. The low-melting fractions gave peak maxima at 25 (±2)°C (Fig. 11*f*) and had a mean fatty acid composition of 5% 16:1, 35% 18:1, 48% 18:2, and 6% 20:4 (rest 6%). The medium-melting fractions had peak maxima at 36 (±3)°C (Fig. 11*g*) with following fatty acids; 6% 16:0, 45.5% 18:1, 39% 18:2 and 3% 20:4 (rest 6.5%). The high-melting CE fractions gave a sharp melting transition at 75 (±3)°C (Fig. 11*h*) and were found to contain 87% 16:0, 2% 18:0, 4% 18:1, and 2% 18:2 (rest 5%).

Discussion

Fatty streak lesions are made up of foam cells filled with CE droplets stabilized by PLs and saturated with FC. The droplets occur in mixtures of 2 forms, anisotropic (smectic liquid crystalline) and isotropic (true liquid) ones; birefringence, interference colours, and internal structure of the anisotropic droplets are identical with liquid crystals of pure cholesteryl esters. The predominant CE fatty acid of the fatty streak is oleic acid. This is different from the CE in plasma lipoproteins, which are rich in polyenoic fatty acids. Based on this difference it seems likely that the CEs present in the early atherosclerotic lesion are synthesized intracellularly. The major pathway for CE droplet formation is thought to be the esterification of serum derived FC by acyl-CoA : cholesterol acyltransferase (ACAT) [17,18]. In this regard, CE-rich lipid inclusions which are isotropic or anisotropic have been observed in tissue culture cells. These droplets, which are similar to those seen in the fatty-streak lesions, were produced by incubating Fu5AH cells with hyperlipemic rabbit serum [19] or human lung fibrablasts with FC-rich vesicles [20]. These observations are consistent with the view that much of the cholesterol in the early atherosclerotic lesions is derived from extracellular lipoprotein either by uptake of the entire lipoprotein or by surface transfer of FC from lipoproteins to arterial cells.

Several authors have demonstrated directly that an increase in the microsomal FC

content produces increased ACAT activity [21,22]. Consistently the correlation between FC/PL (indicates membrane saturation with FC) and CE/FC (indicates activity of CE synthesis) found in this study for fatty streaks was positive and quite strong ($r = 0.70$). The correlation between FC and CE was weaker ($r = 0.20$) indicating that membrane saturation with FC is a more important factor for CE synthesis than the absolute FC concentration. This conclusion is in good agreement with recent cell-culture experiments showing that even a small increase in the FC/PL molar ratio can induce a considerable accumulation of CE [20].

When the CE solubility in the membrane system (about 5% [23]) is exceeded, the excess forms separate oleate-rich CE droplets falling within the 2-phase region (zone III in Fig. 4). Most of the TG appears to be present in these droplets [7,24] which is predictable from the complete mutual solubility of TG and CE above their melting point [25]. Small amounts of TG will abolish the cholesteric liquid crystalline phase [25], which explains why this phase has not been observed in the lipid droplets. TG also lowers the transition temperatures of CE, which explains the difference between the melting behaviour of CE as part of total lipid and in separated form. The solubilities of FC and PL in CE are quite low [23,26] and these lipids have a minor effect on the phase behaviour of the CE droplets.

The question of fatty-streak regression is important. Such a process should involve the hydrolysis of the intracellular CE droplets and removal of formed FC by an appropriate acceptor. Cell-culture experiments have indeed shown that such a CE depletion can take place in model systems [20,27]. It has been reported from a scanning electron microscopic study that a large proportion of CE-rich inclusions, especially from fibrous plaques, has surfaces that are disrupted with multiple pits of various sizes [28]. These defective forms, which have also been demonstrated in chylomicrons incubated with lipoprotein lipase [29], have been interpreted as signs of the inclusion being subjected to hydrolytic attack. However, the risk for artefacts is large when using scanning electron microscopy, because the vacuum dehydration during specimen preparation might rupture the surfaces of the lipid droplets. The scanning electron microscopic pictures of droplets from fatty streaks obtained in this study did not show defective forms, but the surfaces were essentially circular and smooth (Fig. 5). This study thus failed to demonstrate such possible marks of fatty-streak regression.

The relation between fatty streaks and advanced plaques is still obscure. Katz et al. [8] have reported a lesion intermediate between these 2 types. This lesion was found to lie within the 3-phase zone, but still had no FC crystals, which indicates supersaturation of the lipid phases with FC. The lipid values summarized in the phase diagram in Fig. 1 show no clearcut demarcation regions between advanced fatty streaks (marked with *b* in the diagram), medium lesions (*c*), and advanced lesions (*d*). This fact can be interpreted as a support for a simple progression between the different types of lesions. Some of the advanced fatty streaks lie within the 3-phase region, but did not show any FC crystals. These lesions may correspond to the intermediate lesions mentioned above. The transition from advanced fatty streaks to advanced lipid plaques is also supported by morphological observations. The advanced fatty streaks exhibited large clusters of lipid-filled cells and also

beginning cell disintegration (Fig. 6). The advanced lipid plaque also contained many lipid-filled cells, but typically it had a necrotic core with a lipid pool of droplets and solid lipids (Fig. 9). A plausible sequence for the formation of the atheroma plaque could be that metastable supersaturated FC crystallize and initiates cellular disruption including lysosomes, which release cholesteryl ester hydrolase attacking stored CE with further increase in FC monohydrate crystals. The idea that the increase in the proportion of FC in advanced plaques could be the result of hydrolysis of CE, and not of preferential uptake, has strong support; the correlation between the change in the proportion of FC and the change in CEFA composition is high [30], and labelled FC enter the amorphous centres of plaques from the plasma very slowly [31].

The extracellular, low-melting lipid granules recognized mainly in fibro-fatty lesions correspond to the 'perifibrous lipid' described by Smith et al. [4]. They were found to be considerably smaller and more uniform in size than the intracellular droplets, which is in agreement with a recent study using the fluorescent probe filipin to detect esterified cholesterol [32]. The extracellular lipid granules can appear in abundance (Fig. 8), but they seem not to cause necrosis. The extracellular localization and chemical composition of the 'perifibrous lipid' support the opinion that it is LDL-derived. In fact, apoprotein B (apo B), the major protein of LDL, has been identified in both normal [33] and atherosclerotic [34] intima. The quantitative significance of the insudation of intact LDL is, however, obscure and a recent study shows that the LDL concentration in interstitial fluid from fatty streaks is low [35]. It seems most likely that the 'perifibrous lipid' represents a line of development separate from that leading to complicated lipid plaques. Chemical (high in linoleic acid), physicochemical (low transition temperatures), and morphological (extracellular) evidence is in favour of this opinion.

The physical properties of the lipids in the advanced plaque are still controversial. Smith et al. [30] used the term 'amorphous lipid' for the solid lipid pool of advanced plaques, thereby avoiding to prejudge it to be FC crystals. Katz et al. [12] concluded that the solid lipid phase in atherosclerotic plaques is almost pure FC monohydrate crystals. Unlike this finding the present study demonstrates, by polarizing microscopy and DTA analysis, the existence of high-melting CEs besides FC monohydrate crystals in the core of advanced lipid plaques. Support for this issue is provided by Hillman and Engelman [36], who observed average melting temperatures of droplets and spots as high as 43.5°C. Apparent CE crystals have been observed in fibro-fatty plaques although they have been judged as artefacts [37]. Most of the high-melting CE melted between 40 and 50°C and exhibited a smectic liquid crystalline phase before it became crystalline on cooling. The melting curves of advanced plaque lipids typically showed 3 maxima; 2 below and 1 above body temperature. The CE can be fractionated into low-, medium- and high-melting ones. The high-melting esters were made up of almost pure cholesteryl palmitate, while the medium-melting ones contained oleate as major component. Both esters have melting points above body temperature: palmitate at 83.5°C and oleate at 51°C [38]. It has been shown in model systems that the transition temperatures of high-melting esters are depressed by low-melting CE and TG [25]. Obviously such a modulation

also occurs in atherosclerotic lesions, although the lipids are compartmentalized to some extent, and not in thermodynamic equilibrium as in model systems. A plausible explanation for the appearance of high-melting CE in advanced lipid plaques could be a preferential hydrolysis of low-melting esters. In fact it has been shown that smectic droplets will be metabolized less rapidly than the isotropic droplets [39] and that cholesteryl linoleate is hydrolyzed more rapidly by aortas than cholesteryl oleate [40]. This will result in an accretion of high-melting esters in regions where the hydrolytic activity is high.

However, the fact that solid CE can be observed in the core of advanced lipid plaques and in TL extracts from such plaques does not provide definite evidence for the existence of solid CE at body temperature in the living state. The biologically important CEs show a monotropic behaviour, which means that their melting points are higher than the liquid crystalline transitions. During freeze-sectioning of the tissue many of the CEs will crystallize to solids, whose melting points are higher than the liquid crystalline transition of the CE in the original lipid plaque. In spite of this reservation it seems likely that the high-melting CE in the core of lipid plaques is a sclerogenic factor [40] and an obstacle to the regression of the plaque.

The accumulation of FC and CE in atherosclerosis must depend on a slower net rate of efflux than influx of cholesterol in the vessel wall. It has been shown by cell-culture experiments that the net physicochemical transfer of FC from a donor particle to the cellular membranes will depend on the FC/PL molar ratio of the donor [19,20] and also that rate of FC exchange between lipoproteins and cells far exceeds that of receptor specific uptake [41]. A high concentration of cholesterol-rich lipoproteins may thus form a positive FC gradient driving the sterol to the esterification sites in the arterial wall. However, the cholesterol accumulation will also depend on local factors in the vessel wall. The turnover of the lipid molecules in the artery is dependent on their physical state. Molecules in crystalline or liquid crystalline states have restricted mobility and their metabolization is slower than in the liquid state. Thus the cooperation of physicochemical and metabolic factors would be of importance in the accumulation and regression of lipids in atherosclerosis.

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