Analysis of the physical state of cholesteryl esters in arterial-smooth-muscle-derived foam cells by differential scanning calorimetry

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The physical state of cholesteryl esters (CE) in the arterial-smooth-muscle-derived foam cells may contribute to the documented reduction in CE hydrolysis. The physical state of CE may also provide a potential enhancing mechanism for increased CE accumulation. To explore these concepts, we therefore examined the influence of alterations in CE and triacylglycerol (TG) content and their fatty acid composition on the thermotropic behaviour of these lipids by differential scanning calorimetry (d.s.c.). After exposure to cationized LDL (cLDL) or after infection with herpes simplex virus type I (HSV), smooth-muscle cells accumulated significant amounts of CE. The CE/TG ratio was significantly higher in cells treated with cLDL compared with HSV infection. TG content was unaffected by either treatment. However, the fatty acid profile of both CE and TG was significantly different between treatment groups, with the polyunsaturated fatty acid/saturated fatty acid (PUFA/SFA) ratio being significantly higher in cLDL-treated cells than in HSV-infected cells. The d.s.c.-generated thermograms of intact cells revealed that neutral lipids of both treatment groups were in the isotropic-liquid state, similar to the state of lipids derived from 'fatty streak' types of atherosclerotic lesions. Differences in the thermograms between HSV-infected and cLDL-treated cells can be ascribed to differences in the CE content and the fatty acid composition of CE and TG (PUFA/SFA ratio). Polarizing optical microscopy revealed the presence of isotropic lipids in both groups. Biochemical and physicochemical data confirm the lysosomal localization of engorged CE, and indicate that the cellular isotropic CE in these foam cells are in a physical state which favours enzymic hydrolysis.

INTRODUCTION

Atherosclerotic vascular disease is characterized by intimal thickening, a consequence of smooth-muscle-cell proliferation, and the accumulation of lipid and protein (Ross, 1981). Vascular injury induced by hyperlipoproteinaemic states or viral or chemical mutagens can induce smooth-muscle-cell migration to regions where they may transform, proliferate, accumulate CE, degenerate into foam cells, and thus initiate lesion development.

Metabolism of CE within the arterial wall may be dependent upon its physical state. Non-esterified and esterified cholesterol exists in several physical states, depending on the type of arterial lesions. Fatty streaks are characterized by a single phase of CE in either an isotropic-liquid state or an anisotropic (disordered), smectic (layered) liquid-crystal state (Lundberg, 1985). Advanced gruel plaques, which have a necrotic lipid-rich core, are characterized by three phases, consisting of a cholesterol monohydrate crystal, an oily CE phase or a lamellar phospholipid liquid-crystalline phase which is saturated with non-esterified and esterified cholesterol (Small, 1977*a,b*, 1988). In either case, smooth-muscle cells accumulate TG/CE inclusions and evolve eventually into foam cells. The physicochemical nature of the CE (an ordered or disordered state) can influence the metabolic cycle and eventual clearance of CE from the cell (Small, 1977*a,b*; Glick *et al.*, 1983, 1987). These observations are important, since the CE in the ordered, smectic, liquid-crystalline state is hydrolysed more slowly than CE in disordered, liquid droplets (Adelman *et al.*, 1984; Glick *et al.*, 1983, 1987), which would,

therefore, have a significant effect on the flux of cholesterol between the lesion and the plasma (Small & Shipley, 1974).

Several tissue-culture models have been used as models of atherosclerotic foam cells, including smooth-muscle cells (Goldstein *et al.*, 1977; Wolfbauer *et al.*, 1986; Pomerantz & Hajjar, 1989), macrophages (Brown & Goldstein, 1983; Snow *et al.*, 1988) and hepatoma cells (Rothblat *et al.*, 1977). Each cell type has been shown to contain an active metabolic CE cycle, i.e., CE is continually hydrolysed by lysosomal CE hydrolase, re-esterified by cytoplasmic fatty acyl-CoA:cholesterol acyltransferase, and then hydrolysed by cytoplasmic CE hydrolase.

In previous studies we found that arterial smooth-muscle cells from human, bovine or avian tissue infected by herpes virus accumulated substantial quantities of CE (Hajjar *et al.*, 1985, 1987; Hajjar, 1986) because of (1) reduced lysosomal and cytoplasmic CE-hydrolytic activities (Hajjar *et al.*, 1985, 1987) and (2) decreased translation of mRNA transcripts in the cell (Hajjar *et al.*, 1989). Similarly, lipid loading of arterial smooth-muscle cells *in vitro* can be achieved by exposure to cationized low-density lipoproteins (cLDL) (Goldstein *et al.*, 1977; Pomerantz & Hajjar, 1989); such CE-enriched cells also displayed reduced CE-hydrolytic activities (Pomerantz & Hajjar, 1990). Arterial cells infected with herpes virus or exposed to modified LDL have been shown to produce a lipid profile similar to the human arteriopathy (Goldstein *et al.*, 1977; Hajjar *et al.*, 1985, 1987; Pomerantz & Hajjar, 1989).

Because the physical state of CE in arterial-smooth-muscle-

Abbreviations used: CE, cholesteryl esters; cLDL, cationized low-density lipoprotein; d.s.c., differential scanning calorimetry; HSV, herpes simplex virus type I; LDL, low-density lipoprotein(s); PBS, phosphate-buffered saline, pH 7.4; PUFA, polyunsaturated fatty acids; SFA, saturated fatty acids; TG, triacylglycerols; DMEM, Dulbecco's modified Eagle's medium; MOI, multiplicity of infection.

derived foam cells produced by viral infection or exposure to modified LDL may influence the rate of CE hydrolysis and subsequent efflux, we analysed the phase behaviour of CE in arterial-smooth-muscle-derived foam cells by differential scanning calorimetry (d.s.c.) and polarizing optical microscopy.

EXPERIMENTAL

Materials

Cholesterol, CE and triolein (trioleoylglycerol) were obtained from Applied Science Laboratories (State College, PA, U.S.A.). BSA (fatty-acid-free, fraction V) was purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Dulbecco's modified Eagle's medium (DMEM), penicillin/streptomycin and Fungizone (amphotericin B) were obtained from Gibco (Grand Island, NY, U.S.A.). Fetal-bovine serum (heat-inactivated) was purchased from Hyclone (Logan, UT, U.S.A.). *NN*-Dimethylpropane-1,3-diamine was bought from Kodak Chemicals (Rochester, NY, U.S.A.), and 1-ethyl-3-(3-dimethylaminopropyl)carbodi-imide hydrochloride was purchased from Aldrich Chemicals (Milwaukee, WI, U.S.A.). β -Sitosterol was purchased from Alltech Associates (Deerfield, IL, U.S.A.). Cholesteryl heptadecanoate and fatty acid methyl esters (standards) were obtained from Nu-Chek Prep (Elysian, MN, U.S.A.). All organic solvents (h.p.l.c. grade) and other biochemicals (reagent grade) were obtained from Fisher Scientific (Springfield, NJ, U.S.A.).

Preparation of LDL, cLDL and apo-cLDL

Plasma LDL were isolated by preparative ultracentrifugation of pooled donor plasma in a Beckman 55.2 rotor at densities between 1.019 and 1.063 g/ml, NaBr being used to adjust the density (Havel *et al.*, 1955). Cationized LDL (cLDL) was prepared from LDL by covalent attachment of *NN*-dimethylpropane-1,3-diamine to aspartate and glutamate residues of native LDL using 1-ethyl-3-(3-dimethylaminopropyl)carbodi-imide hydrochloride as a catalyst at pH 6.5 (Goldstein *et al.*, 1977). Demonstration of successful derivatization of LDL to cLDL was accomplished by differential migration of native and cLDL at pH 8.6 by agarose-gel electrophoresis. Lipoproteins were dialysed against 12 litres of 0.154 M-NaCl/0.05 M-EDTA/10 mM-Hepes, pH 7.4, for 48 h before concentration by ultracentrifugation. Lipoproteins were added to tissue-culture media just before initiation of experiments.

Isolation and culture of rabbit aortic smooth-muscle cells

Smooth-muscle cells from rabbit thoracic aorta were isolated and cultured as previously described (Hajjar *et al.*, 1982). All experiments were performed on cells between passages 2 and 4. Cells were identified as arterial smooth-muscle cells by their 'hill-and-valley' configuration at confluence by phase-contrast microscopy. Smooth-muscle cells were induced to accumulate lipid by exposure to either cLDL or by infection with HSV. Confluent cultures in T-25 cm² or T-75 cm² flasks (Costar, Cambridge, MA, U.S.A.) were incubated with cLDL (100 μ g/ml) in 2 ml of DMEM containing 10% (v/v) fetal-bovine serum for 6 or 7 days. Fresh media containing cLDL was added every 3 days. Alternatively, T-25 cm² or T-75 cm² flasks were infected by HSV at a multiplicity of infection (MOI) of 1.0 for 3 or 4 days (Hajjar *et al.*, 1987, 1989). Prior to analysis, cells were harvested and washed three times in phosphate-buffered saline (PBS, pH 7.4) containing 0.1% BSA, followed by washing in protein-free PBS. For cells in which d.s.c. was performed, cells were scraped into PBS, pelleted, and resuspended in 0.5–1.0 ml of PBS. Cells were stored for no longer than 2–3 h before d.s.c. analysis. For biochemical analysis, parallel flasks of cells were

extracted into hexane/propan-2-ol (3:2, v/v) (Hara & Randin, 1978).

D.s.c.

D.s.c. experiments were conducted on a MicroCal MC-1 scanning calorimeter (MicroCal, Northampton, MA, U.S.A.), using matched 1 ml spectrometric cells. The experiments were performed with intact cells which had accumulated lipid either by exposure to cLDL or by infection with HSV. Intact cells were used because thermotropic transitions observed for isolated lipid inclusions are typically irreversible, whereas such thermotropic transitions are reversible when intact cells are used which contain the inclusions (Snow *et al.*, 1988). The cells were harvested as described above, suspended in PBS at a final concentration of 1–5 mg of CE/ml, then de-gassed for several minutes. Typically, 0.7 ml of the cell suspension and an equal volume of degassed PBS were loaded into the sample and reference spectrometric cells respectively and the samples were scanned at 1 °C/min. Enthalpies were calculated by using areas determined by planimetry. A buffer/buffer baseline was subtracted from the curves before performing these calculations. Internal temperature and power calculations were checked by scanning a suspension of dipalmitoyl phosphatidylcholine multilamellar vesicles in PBS (10 mg/ml). Values for the transition temperature and enthalpy associated with the gel $\leftrightarrow$ liquid crystal transition were taken to be 41.3 °C and 37 kJ (8.6 kcal)/mol respectively (Small, 1986).

Analytical and preparative procedures

The cholesterol and CE content was measured by g.l.c., using a 3% OV-17 100–200 mesh WHP column (Supelco). β -Sitosterol was used as an internal standard (Ishikawa *et al.*, 1975). Cell lipids were separated on silica-gel 60 (Merck) by sequential development in diethyl ether/acetic acid/light petroleum (100:3:97, by vol.), and diethyl ether/light petroleum (3:97, v/v) (Hajjar *et al.*, 1980). The fatty acid analyses of relevant lipid species were determined by derivatization to their corresponding fatty acid methyl esters by transmethylation using 14% BF₃ in methanol, followed by separation and quantification by g.l.c. using a Supelcowax 10TM fused-silica capillary column (30 m long, 0.53 mm internal diameter, 1.0 μ m film thickness; Supelco) employing cholesteryl heptadecanoate as an internal standard. Triacylglycerols (TG) were quantified by using the PeridoChrom TG assay kit obtained from Boehringer Mannheim, and was modified to a microassay previously described (Minor *et al.*, 1989). Triolein was used as a standard. Protein was determined by a method of Lowry *et al.* (1951), with BSA as standard. The physical state of the lipid droplets was also determined by polarizing optical microscopy.

Statistical analyses

Results of these experiments were analysed either by Student's *t* test or by analysis of variance. Differences between treatments were tested using the Newman–Keuls test.

RESULTS

Lipid analyses

Maximal accumulation of cholesterol and CE in arterial smooth-muscle cells was observed after a 3-day incubation period with an HSV infectivity level (MOI) of 1. HSV infection of arterial cells resulted in an almost 2-fold increase in non-esterified cholesterol and a 9-fold increase in CE (Fig. 1). Longer periods of time resulted in some loss of viability of the cells; shorter times of incubation with the virus resulted in submaximal levels of lipid accumulation. Similarly, cLDL-treated cells exhibited a 3-fold

Fig. 1. Neutral-lipid content of arterial smooth-muscle cells: effect of HSV infection and cLDL treatment

Confluent cultures of arterial smooth-muscle cells grown in T-25 cm² flasks were exposed to either HSV infection (MOI = 1.0, for 72 h) or cLDL (100 µg of protein/ml for 1 week with one media change). Cell lipids were extracted into hexane/propan-2-ol (3:2, v/v). After the addition of β -sitosterol (60 µg), portions of the cell lipid extracts were analysed for cholesterol (■) and CE (▒) by g.l.c. and for TG (□) content by enzymic assay. The protein content was assayed in cells digested in 0.2 M-NaOH. Results are expressed as µg/mg of cell protein unless otherwise specified (means $\pm$ S.E.M. for sextuplicate samples; **P* < 0.05).

increase in non-esterified cholesterol and a 60-fold increase in CE after 6–7 days of exposure at 37 °C (Fig. 1).

There were also striking differences in the pattern of fatty acids of the CE of HSV-infected and cLDL-treated cells. Interestingly, the principal CE fatty acid was linoleate. There were similar percentages of monounsaturated fatty acids in the CE of HSV-infected and cLDL-treated cells (30 as against 28%). However, the percentage of saturated fatty acids in HSV-infected cells was more than twice that in cLDL-treated cells (28 as against 13%), whereas the percentage of polyunsaturated fatty acids (PUFA) in HSV-infected cells was less than cLDL-treated cells (41 as against 60%).

Although there was no difference in total TG content of HSV-infected cells and cLDL-treated cells (Fig. 1), the fatty acid profile of cellular TG are not identical (Fig. 2). The principal fatty acid in TG in both HSV-infected and cLDL-treated cells is oleic acid. In addition, there were equal percentages of monounsaturated fatty acids in both treatment groups (40% in HSV-infected cells as against 46% in cLDL-treated cells). However, HSV-infected cells contained nearly double the percentage of saturated fatty acids in TG as compared with cLDL-treated cells (41 as against 23%), but only half the percentage of PUFA in TG as compared with cLDL-treated cells (18 as against 40%) (Fig. 2).

D.s.c. studies of smooth-muscle-cell derived foam cells

D.s.c. experiments were conducted with intact cells containing CE-rich inclusions and also with control cells that had not been previously loaded with CE. The curves in Fig. 3(a) were generated with control cells. The continuous line in this Figure illustrates the heat-capacity curve obtained during the initial scan. The broad isotherm centred near 60 °C is probably due to denaturation of cellular protein and nucleic acids. [It is unlikely that enthalpy absorbed during the initial scan of control cells is due to lipid transitions, because of the insignificant amounts of CE and the large percentage of membrane cholesterol, which inhibits phospholipid gel-to-liquid-crystalline transitions (Ginsburg *et al.*, 1984).] The curve represented by the dotted line in Fig. 3(a) was obtained by cooling the sample to –20 °C before rescanning. The absence of any peak in this scan demonstrates

Fig. 2. Fatty acid analysis of neutral-lipid content: effect of HSV infection (a) and cLDL treatment (b)

Confluent cultures of arterial smooth-muscle cells grown in T-25 cm² flasks were exposed to either HSV infection (MOI = 1.0, for 72 h) or cLDL (100 µg of protein/ml for 1 week with one media change). Cell lipids were extracted into hexane/propan-2-ol (3:2, v/v). After addition of cholesteryl heptadecanoate (30 µg), the fatty acid profile of TG (■) and CE (▒) was determined by g.l.c. after separation of these cell lipid classes by t.l.c. and derivatization to their corresponding methyl esters using BF₃/methanol (60 µg of cholesteryl heptadecanoate was added directly to the TG spot to quantify these fatty acids). The protein content was assayed in cells digested in 0.2 M-NaOH. Results are expressed as µg/mg of cell protein unless otherwise specified (means $\pm$ S.E.M. for triplicate samples).

that the denaturation of protein and nucleic acid observed during the initial scan was irreversible. Similar results were obtained when the cells were cooled to 5–10 °C for 1 h of rescanning (results not shown).

The continuous line in Fig. 3(b) shows the heat-capacity curve generated during the initial scan of a dilute suspension of intact HSV-infected cells (*n* = 2). A relatively large amount of enthalpy was absorbed, with one endotherm centred near 63 °C, and a larger, unresolved, endotherm at higher temperatures. Although the cellular transitions due to protein and nucleic acids are thermally irreversible (cf. Fig. 3a, dotted line), phase changes due to cellular CE-rich inclusions are known to be reversible for both J774 macrophages (Glick *et al.*, 1987) and Fu5AH rat hepatoma cells (Glick *et al.*, 1983). Thus any peak observed upon cooling then re-heating the cells should be due to such reversible lipid transitions. The curve represented by the broken line in Fig. 3(b) was obtained after cooling the cells to less than 10 °C for 1 h before rescanning; however, there is no evidence for any co-operative transition in this second scan, indicating that the lipid had not yet crystallized before rescanning them. Polarized optical microscopy performed with HSV-treated cells revealed that the lipid was isotropic, or disordered, in agreement with the d.s.c. results. Evidently, the lipid must be cooled well below 10 °C to

Fig. 3. Excess-heat-capacity curves for control smooth-muscle cells

(a) —, Initial scan; ·····, second scan obtained after refrigerating cells at -20°C for 48 h. (b) Typical excess-heat-capacity curve for HSV-infected cells ($n = 2$): —, initial scan; ----, second scan obtained after cooling and maintaining cells at $5\text{--}10^{\circ}\text{C}$ for 1 h. (c) Heat-capacity curve for cLDL-treated smooth-muscle cells ($n = 2$): —, initial scan; ----, second scan obtained after cooling and maintaining cells at $5\text{--}10^{\circ}\text{C}$ for 1 h; ·····, third scan obtained after removing cells from the calorimeter, then refrigerating them at -20°C for 48 h [note 1 mcal = 4.184 mJ (SI unit)].

induce crystallization. [CE can typically exist in long-lived, metastable, crystalline states at temperatures for which the crystalline phase is thermodynamically more stable (Ginsburg *et al.*, 1984).]

Fig. 3(c) shows the results of a typical d.s.c. experiment with cells exposed to cLDL ($n = 2$). During the initial scan (continuous line), a large amount of enthalpy was absorbed, as was observed for HSV-infected cells. A single peak occurs near 40°C , and a broad, unresolved, endotherm occurs at higher temperatures. The curve represented by the broken line in Fig. 3(c) shows that cooling the cells to less than 10°C for 1 h before rescanning was not sufficient to induce crystallization of the cellular CE. The sample was then removed from the calorimeter and then refrigerated at -20°C for 48 h. The curve represented by the dotted line in Fig. 3(c) shows a co-operative transition near 34°C

[$H_{\text{fus}} = 3.6 \text{ J (0.86 cal)/g}$ of cellular CE]. In this case, maintaining the cells at -20°C for 48 h was sufficient to induce a transition to a more ordered phase. Polarizing optical microscopy performed with cells exposed to cLDL revealed that the lipid was isotropic, in agreement with the d.s.c. results.

DISCUSSION

A reduction in cellular CE hydrolysis may be one of the principal mechanisms favouring CE accumulation and foam-cell development. Small and his colleagues have hypothesized that the physical state of the intracellular CE may strongly influence CE hydrolysis (Small & Shipley, 1974; Katz *et al.*, 1976; Small, 1977a,b, 1988). Recent data from this laboratory suggests that viral infection induces host-protein shut-off at the post-transcriptional level, causing partial reduction of CE hydrolase in the cell (Hajjar *et al.*, 1989); furthermore, lipid loading of smooth-muscle cells by viral infection or exposure to cLDL causes reduced lysosomal and cytoplasmic CE-hydrolytic activities (Hajjar *et al.*, 1985, 1987; Pomerantz & Hajjar, 1990). However, these reductions in CE hydrolase in both the HSV-infected and cLDL-treated group were not sufficient to explain the large increases of CE accretion in these cells. In the present study we have tested the hypothesis that the physical state of the neutral lipids of CE-enriched foam cells, as assessed by d.s.c., may be such that they are poor substrates of CE-hydrolytic enzymes, thus promoting CE accumulation.

The biochemical elements which confer a particular physical state (anisotropy as against isotropy) to neutral lipids include the relative amounts of TG to CE as well as the amount and degree of unsaturated fatty acids comprising the neutral-lipid compartment. As Fig. 1 shows, HSV infection and cLDL exposure produces a lipid profile in rabbit arterial smooth-muscle cells which resemble the human arteriopathy (Small, 1977a,b, 1988). We have demonstrated that HSV infection and treatment of arterial smooth-muscle cells with cLDL does not alter the total TG mass, but dramatically increases the mass of CE. Although there are fatty acid compositional differences in TG between HSV-infected cells and cLDL-treated cells, the effect of these differences may be rather subtle when compared with the significant differences in the CE mass, as well as the large differences in the fatty acid composition of these CE. Our data demonstrate that the cLDL-treated cells have a greater CE/TG ratio than HSV-infected cells; in addition, the CE fatty acid profile reveals that cLDL-treated cells have a higher PUFA/SFA ratio than have HSV-infected cells, with an equal percentage of mono-unsaturated fatty acids. Therefore these parameters predict that the CE of cLDL-treated cells would be more disordered than that of HSV-infected cells.

The d.s.c. results allowed us to describe the physical state of the cellular CE for both HSV-infected and cLDL-treated SMC. Knowledge of the physical state of this lipid is important, because clearance of cellular CE is known to depend on its physical state. Specifically, clearance of CE from cells containing anisotropic (ordered) CE-rich inclusions occurs more slowly than from cells containing isotropic (fluid) inclusions (Minor *et al.*, 1989; Glick *et al.*, 1983; Adelman *et al.*, 1984). Intact cells were used for these experiments because the thermotropic transitions observed for isolated inclusions are typically irreversible, whereas they are reversible when intact cells are used which contain the inclusions (Snow *et al.*, 1988). During the initial scan of intact cLDL-treated cells, more enthalpy (H) was absorbed at lower temperatures (between 30 and 45°C) than was absorbed by HSV-infected cells (Figs. 3b and 3c). This is consistent with the observation that the PUFA/SFA ratio of CE was greater in cLDL-treated cells than it was for HSV-infected

cells (Fig. 2), since these lipids melt at lower temperatures (Ginsburg *et al.*, 1984).

Reheating both cell types after cooling and maintaining them at approx. 5–10 °C for 1 h produced evidence for neither liquid → crystalline nor crystalline → lipid transitions, which indicates that the CE must have remained as an isotropic fluid at these temperatures. It is not surprising that the d.s.c. results offered no evidence for liquid → crystalline transitions, owing to the presence of relatively large amounts of TG (Fig. 1). The presence of TG in small amounts (about 5%, w/w) is known to abolish the smectic liquid-crystalline phase and eventually the cholesteric liquid-crystalline phase (about 10%, w/w) in CE mixtures (Croll *et al.*, 1985). When the cLDL-treated cells were subsequently refrigerated at –20 °C for 48 h, a broad phase transition occurred near 34 °C (Fig. 3c, dotted line). Evidently, the fluid CE-rich inclusions in cLDL-treated cells undergo partial crystallization between 5 and –20 °C, and the CE-rich inclusions in HSV-infected cells must have crystallized below 5 °C as well (cf. Fig. 3b, broken line).

The phase behaviour of model dispersions of binary CE mixtures can be explained by classical nucleation theory (Snow & Phillips, 1990). The results of these model system studies enabled us to predict that the CE would not crystallize after 24 h at 37 °C in either HSV-infected cells or cLDL-treated cells, on the basis of the observed thermal history of their phase behaviour. This is an important observation, since cellular CE in living cells is in a dynamic state, continually undergoing a cycle of hydrolysis and re-esterification (Brown *et al.*, 1980). Since the half-time for hydrolysis and re-esterification of cellular CE is on the order of 24 h, this is apparently the minimum time period for which the metastability of fluid, undercooled, CE must be maintained if the inclusions are to be fluid under steady-state conditions. These results, then, provide strong evidence that the cellular CE in both HSV-infected and cLDL-treated cells were present as isotropic liquid under steady-state conditions at 37 °C. It is noteworthy that the CE-rich inclusions present in cLDL-treated cells melt below 37 °C, so they would be fluid at physiological temperature regardless of their thermal history. [Since the CE-rich inclusion of cLDL-treated cells melt below 37 °C, the isotropic fluid phase would be thermodynamically stable at 37 °C and should therefore remain fluid indefinitely, provided their composition does not change. The isotropic phase for these inclusion would then be metastable at 37 °C for melting points above this temperature, and would eventually crystallize at physiological temperatures (Ginsburg *et al.*, 1984).]

The biochemical data reported here demonstrate increased cholesteryl linoleate in HSV-infected and cLDL-treated cells. This is consistent with a decrease in lysosomal clearance of lipid. Recent electron-microscopic and biochemical criteria support this concept (Pomerantz & Hajjar, 1989). These data also support the viewpoint that decreased lysosomal function is characterized by cholesteryl linoleate enrichment, and yields phase-transition isotherms which parallel lysosomal dysfunction (Small, 1988). The liquid or 'random' state of the CE in these two groups of foam cells may allow for partial CE hydrolysis, but obviously is insufficient to fully catabolize CE (Hajjar *et al.*, 1987). In fact, it has been shown that exposure of J774 macrophages to acetylated LDL caused CE inclusions to develop, which could explain the reduction in the hydrolytic rate of these esterified lipids (Snow *et al.*, 1988). Furthermore, mononuclear cells from patients with primary Type II hypercholesterolaemia exhibit reduced CE hydrolase activity, concomitant with increased CE, especially cholesteryl linoleate (Hagemenas *et al.*, 1984). Recently, it has

been demonstrated that isotropic TG/CE droplets are cleared at a different rate from anisotropic lipid droplets composed principally of cholesterol oleate (Minor *et al.*, 1989).

In conclusion, results of this combined biochemical and structural analysis of arterial CE support the contention that the activities of CE hydrolases are critically dependent upon both the magnitude and physical state of deposited intracellular CE. However, it remains to be fully defined whether alterations to CE hydrolase activities are consequential to, or a contributory factor towards, the physical state of accumulated neutral lipid.

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