Cholesteryl Ester-Rich Lipid Inclusions from Human Aortic Fatty Streak and Fibrous Plaque Lesions of Atherosclerosis*

II. Physical Properties of Swelling, Resiliency and Lyotropy as Liquid Crystal

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The physical properties of the lipids accumulating as spherical inclusions in human atherosclerotic lesions were explored both *in vivo* and *in vitro* conditions. They were mostly anisotropic within the cytoplasm of cells and in an average diameter of $1.96\ \mu$. When suspended in hydrophilic media, say of physiological saline solution, in 4 hours they swelled 2.4 times in volume and changed into isotropic form. They were resilient and deformable in three dimensional configuration, and lyotropic in polar media. When dried they underwent a phase shifting from liquid crystal to true solid crystal. These physical properties of lipid inclusions gained in a new significance for their role in metabolic deterioration of intima cells, rupture and necrosis of foam cells, and destruction of the intimal and medial architectures, which set on mesenchymal reactions leading eventually to thickening and hardening of the arteries.

IN human and animal atherosclerosis, arteries undergo three fundamental changes resulting in proliferation of smooth muscle cells, deposition of intra- and extracellular lipids, and accumulation of interstitial substances like collagen, elastin and proteoglycans¹. Among them, the accretion of lipids is considered to be decisive in the genesis and development of atherosclerosis, because without lipid accumulation no lesions so definitive as atherosclerosis are formed in the arteries².

Previous studies have revealed that bulk lipids accumulate in arterial intima as globular inclu-

sions which possess a number of specific features. (i) They take up lipid stains like Oil-Red O and Sudan III³, (ii) extremely rich in cholesteryl esters^{4,5,6}, (iii) birefringence and interference colors identical with pure liquid crystals of cholesteryl esters⁷, (iv) appearance of dense osmiophilic bodies in transmission electron microscopy⁸, (v) configuration of plump spheres with smooth surface and occasional defective spheres observable under scanning electron microscopy^{9,10} and (vi) propensity to arise from metabolic disturbances in LDL pathway of arterial smooth muscle cells¹¹. Since the lipids in this globular form of inclusions account for over 50% of the total lipids increased in the lesions^{4,12} they must be important for the atherogenesis in man and animals.

Key Words:

Smectic mesophase
Anisotropic inclusions
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Progression of atherosclerosis

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However, the physical properties of the lipid accumulation in the form of spherical cholesteryl ester-rich inclusions and their role in the pathogenesis of atherosclerosis are not precisely understood, though their importance is pointed out.¹³ By previous studies,^{4-7,14} the physical nature of the globular inclusions was defined as lyotropic smectic A mesophase,¹⁵ and their inner make-up as concentric lamellar structure of the constituent cholesteryl ester molecules.

The present paper deals with the results of our investigations, still in progress, of the cholesteryl ester-rich inclusions in human atherosclerotic lesions of fatty streaks and fibrous plaques, and goes to elaborate on their physical properties as liquid crystal by studying behaviors *in vivo* and *in vitro* conditions. In the *in vivo* study the optical properties and size of inclusions in tissue site were investigated, and in the *in vitro* their volume swelling was quantitatively determined. As medium were used such common materials as distilled water, physiological saline, phosphate buffer, 10% formalin, and 1% sucrose solutions, including preservatives of glycerol, propylene glycol and gelatin, and such fixative or embedding materials as glutaraldehyde, osmium tetroxide, cyanocrylate and glycol methacrylate. We have further demonstrated *in vitro* their dependency on water molecules for the formation and maintenance of three dimensional configuration, and finally observed the phase transition from liquid crystal to solid crystal upon drying.

The importance of lipids in atherosclerosis is well known, but the exact mechanism by which they initiate the early lesions in arterial tissue, or the process in which the lesions advance from the initial stage to fibrous plaque or complicated lesions is yet unexplored. In view of this, we have tried to correlate the physical properties of the inclusions with their progressive changes in arterial tissue.

MATERIALS

Tissue samples were aortic fatty streak lesions secured from ten subjects with an average age of 37.6 ± 11.6 , and fibrous plaque lesions from two subjects with an average age of 61.0 ± 1.4 , all died a sudden and unexpected death. The fatty streak lesions were defined as yellowish, slightly raised areas of opaque spots or streaks covered by a delicate translucent membrane, whose underlying internal elastic lamina and media appeared normal in color, texture and consistency. The fibrous plaque lesions were recognized

as pearly whitish raised areas of opaque firm tissue covering a pale yellow core of lipids in the intima which often extended into the media. Fatty streak and fibrous plaque lesions were removed from the aorta by semi-microdissection using ophthalmologic surgery instruments and a dissecting microscope (Model-SMZ, Nikon Inc., Garden City, N.Y.) at 8 to 20 x magnification.

As reagents were used sucrose, 99.5% glycerol (Matheson Coleman & Bell, Norwood, Ohio), diethylene glycol, propylene glycol, gelatin, isopropanol, liquid paraffin, cyanocrylate, glycol methacrylate (Polysciences, Inc., Warrington, Pa.), glutaraldehyde (Fisher Scientific Co., Fair Lawn, N.J.), and osmium tetroxide (Polysciences, Inc., Warrington, Pa.) which were obtained, unless otherwise stated, on the market and were of reagent grade.

METHODS

Examination of Size and Optical Characteristics of Inclusions in Situ To determine the size and the optical characteristics of lipid inclusions nesting in the cytoplasm of the foam cells, 14 fatty streak lesions were removed from 6 aortas and each piece of lesion tissue teased in 80% glycerol with fine needles to make it thin enough to make visible the individual foam cells under a polarizing light microscope (Model S-Ke, Nikon Inc., Garden City, N. Y.) in which the polarizer and the analyzer were crossed at 90°. As medium was used 80% glycerol, a concentration in which the inclusions were proved to be best stabilized. The inclusions were judged to be localized within the cell when clustered in a spindle form delineating the shape of a foam cell.

Diameters of the inclusions were measured with an image shearing eyepiece (I.S.E. Model 123A-1, W. Watson and Sons Ltd., Barnet, England), calibrated with a stage micrometer graded in 0.01 mm divisions (American Optical Co., Buffalo, N. Y.). Only the inclusions locating near the peripheries of the clusters had to be measured, as any overlapping of the inclusion's image interfered with accurate shearing of the images. The measurements of diameters and characterization of the inclusions were continued until at least fifty inclusions were examined *in situ* in one specimen.

Observations on Swelling in Size and Loss of Anisotropy of Isolated Inclusions Swelling in size and change in optical properties were ob-

Fig.1. Lipid inclusions *in situ* in thin layer preparation of unfixed fatty streak lesion from a 48-year-old male examined under a polarizing light microscope. ($\times 450$)

served on isolated inclusions in various suspension media. They were isolated from tissue by mincing with fine scissors in about 0.5 ml to 1 ml of the medium to be tested. As soon as a turbid suspension was produced, a few drops were drawn with a Pasteur pipet, mounted on a hole slide glass, and observed under a polarizing light microscope fitted with the image shearing eyepiece. The media examined were (a) distilled water, (b) 0.9% sodium chloride solution, (c) 1/15 molar phosphate buffer solution (pH 7.4), (d) 1% sucrose solution, (e) 10% acetate buffered formalin solution, (f) 80% glycerol, (g) 99.5% glycerol, (h) diethylene glycerol, (i) propylene glycol, (j) gelatin, (k) isopropanol, (l) 2.5% glutaraldehyde, (m) 1% osmium tetroxide, (n) cyanocrylate, and (o) glycol methacrylate.

To check short-term changes in size and anisotropy after immersion, several inclusions

were chosen for continuous observation on their diameters and optical characteristics at 2, 5, 10, 15, 30, and 60 minutes at room temperature if they did not deteriorate instantly in the medium.

For long-term changes one hundred inclusions were examined at 1, 3, 6, 12, and 24 hours, and subsequently at 24-hour intervals for a total of 168 hours. To prevent the evaporation of the suspension in the course of observation the cover glass was sealed at the edges with liquid paraffin.

Demonstration of Resiliency Deformability of the anisotropic lipid inclusions was observed both *in vivo* and *in vitro*. For *in vivo* observation, foam cells visualized by thin section of fatty streak lesions were used. They were squeezed between a cover slip and a microscope slide under a polarizing light microscope, and

Fig.2. Isolated lipid inclusions from unfixed fatty streak lesion from a 43-year-old male suspended in 80% glycerol. ($\times 500$)

Fig.3. Swollen lipid inclusions in 0.9% saline solution. ($\times 500$)

forced to move through a narrow intercellular space. For *in vitro* observation, isolated lipid inclusions were suspended in 80% glycerol, and poured forth through a capillary of about 0.5μ in inner diameter (Capillary Electrode Puller,

Narishige, Tokyo), and their deformation observed under a polarizing microscope with an objective lens of a long working distance.

Verification of Lyotropy The inclusions were isolated by mincing the tissue of fatty streak and

fibrous plaque lesions, and suspended in distilled water after washing twice by repeated suspension and centrifugation at 3,800 G for 10 minutes at 4°C. Then they were mounted on a hole slide glass, and left to stand till they were dry at room temperature. The dried specimens were checked by a polarizing light microscope. Use of distilled water must have served to prevent the contamination of the specimens with other crystalline substances.

RESULTS

Lipid Inclusions in Situ Figure 1 presents foam cells containing anisotropic lipid inclusions in cytoplasm. They were spherical in shape and showed an interference color of whitish gray divided into equal quadrants by a black cross image, whose arm directions coincided with those of the polarizer and analyzer, i.e., east-west and north-south directions. When a tint plate of $R = 530 \mu$ was inserted between the polarizer and the analyzer, the interference color of whitish gray changed into yellow in the upper left and lower right quadrants, and blue in the upper right and lower left quadrants. The order of interference color was confirmed by inserting a quartz-wedge plate into the microscope's optical path as first order whitish gray, first order yellow and second order blue, respectively. The diameters were measured in total of 800 primarily anisotropic inclusions within the outline of cell shape. They ranged from 1.2 to 2.7μ in diameter with an average of $1.96 \pm 0.10 \mu$.

Swelling in Size and Loss of Anisotropy of Isolated Inclusions in Various Media When the isolated inclusions were suspended in diethylene glycol, propylene glycol, isopropanol, osmium tetroxide, cyanocrylate, and methacrylate, they instantly swelled in size, and deteriorated by losing their anisotropy into swollen isotropic forms.

However, in other solutions of 0.9% sodium chloride, 1% sucrose, 1/15 molar phosphate buffer, 10% formalin, 2.5% glutaraldehyde, and in 80% and 99% glycerol and distilled water, the swelling and transformation took place in two steps: the initial rapid swelling and the secondary gradual alterations.

The initial rapid swelling occurred by 7 to 15% in diameter within 5 to 15 minutes after extrusion of the inclusions into the above mentioned media except in 80% glycerol, where the swelling averaged only 5% in diameters. Since

Fig.4. Swelling in size and loss of anisotropy of lipid inclusions in various media. Increase in average diameters of 100 inclusions and decrease in number of anisotropic inclusions are presented in percentage of the initial value.

the initial swelling was not controllable with other solutions, 80% glycerol turned out as the most adequate stabilizing medium, as far as the size and optical properties were concerned (Fig. 2 & 3).

The secondary alterations in size and optical properties took a slow and gradual course, though the rate of swelling and loss of anisotropy differed from medium to medium as seen in Figure 4 demonstrating the secondary alterations of inclusions until 24 hours. For instance in 3 hours of suspension in physiological saline solutions, the size swelled 19% and anisotropy lost 15%, in phosphate buffer 24% and 20%, in 1%

Fig.5. A schematic presentation of anisotropic lipid inclusion deformed in a narrow channel of capillary of about 0.5μ in inner diameter. After released in suspension medium of 80% glycerol, it restored the original round shape (above). If the channel is narrower, the anisotropic figure of inclusion does not recover when released (below).

Fig.6. A schematic presentation of possible roles of lipid inclusions in the genesis and development of atherosclerosis.

sucrose 26% and 29%, in 2.5% glutaraldehyde 13% and 15%, in 10% formalin 20% and 19%, in 99% glycerol 6% and 2% and in 80% glycerol 3% and 1% respectively. After a total of 168 hours of suspension they severely deteriorated in any kind of medium. Thus, the secondary swelling and deterioration of the inclusions were again minimum by 80% glycerol.

Demonstration of Resiliency When the inclusions were squeezed *in vivo* between a cover slip and a microscope slide to move through a narrow channel of intercellular space, or forced to run through a narrow capillary *in vitro*, they could be made to change into an ellipsoid or to flow transiently through a dumbbell shape as in Figure 5, and then to resume their spherical form upon release of the pressure. When the transient deformation was moderate, the black cross image was distorted, but in time its characteristic shape was restored. However when the deformation into a dumbbell shape was severe, the black cross image was often permanently disrupted, and within the reformed shape, partly anisotropic and partly isotropic portions appeared.

Verification of Lyotropy When the anisotropic inclusions were dried, the black cross image and the first order whitish gray interference color were lost and changed into smaller amorphous whitish brilliant double refractive bodies. Their interference color was of first order gray.

DISCUSSION

The lipid inclusions from human aortic fatty streaks and fibrous plaque lesions are examined for such physical properties as their volume swelling in various suspension media, resiliency

in response to deformation of the spherical form, lyotropy in hydrophilic media, and transition to solid crystal upon drying. Thus these physical properties gain in a new significance in atherosclerosis.

On the basis of crystallographic^{7,14} and chemical studies^{4,5,6} a structural model has been proposed for the inner structure of lipid inclusions^{7,14} in which the longer axis of constituent molecules of cholesteryl esters are aligned throughout the inclusion in parallel to the inclusion's radii, namely, in radial symmetry, and the cholesteryl ester molecules form concentric lamellae. As the radii of the lamellae decrease toward the central positions, the diminishing circumferences are accommodated by reducing the number of molecules per layer, while the interlayer spaces are filled with water molecules.

This structural model for the inner make-up of the anisotropic lipid inclusions is found valid by the present study of their physical properties; that is, (i) the swelling of the inclusions in polar media indicates the presence of water between the layers, and when the water is increased, the inner molecular alignment is distorted by the incorporated water molecules, and the anisotropic inclusions alter into isotropic forms, (ii) the resiliency is accountable by the fact that in contrast to the solid crystal where the constituent molecules are oriented in three dimensions and packed tight, the inclusion's molecules are aligned only in one direction of the radius and free to rotate around the radial axis and translate along the circumference,^{15,16} as long as they remain radially oriented, which

makes the molecular packing less tight and enables it to deform and restore the anisotropic spherical configuration under a moderate pressure, provided the molecular orientation remains undestroyed, (iii) the inclusion's lyotropy is related to the fact that it is formed in a hydrophilic media^{15,16} at a given critical concentration of the cholesteryl ester molecules, and that water molecules interact with cholesteryl ester molecules to maintain both the inclusion's concentric lamellar structure and their three dimensional spherical form, and finally (iv) the transformation of the inclusions to amorphous birefringent pieces upon drying involves a phase transition from liquid crystal to solid crystal with changes in orientation from one to three dimensions. This phase transition can not be reversed from solid crystal to liquid crystal by simply adding water to the solid unless it is heated beyond the limits of physiological conditions and the melt shaken vigorously in a hydrophilic medium, thus the phase transition can be defined an enantiotropic.¹⁷

All these physical properties of the lipid inclusions not only warrant the structural concept of the inclusion's inner structure as lyotropic smectic A mesophase,^{7,14,15} but also point to new significance of the inclusions in two aspects for the study of atherosclerosis: one is the vulnerability of the isolated inclusions when manipulated under *in vitro* conditions, and the other is the relation of their physical properties to the development of atherosclerosis.

The inclusions isolated from tissue are very susceptible to distortion by solvents, temperature, mechanical stress or dryness. They are dissolved in an organic solvent of isopropanol, deteriorated in such fixatives as formalin, glutaraldehyde and osmium tetroxide, and swollen in size and deformed in anisotropic figure in such common media as distilled water, physiological saline, phosphate buffer, sucrose solutions, and in the long run deteriorate into swollen isotropic forms.

The swelling and deterioration occur in two steps: the initial acute swelling and the gradual chronic deterioration. The initial swelling of about 5% in diameter which occurs within a few minutes of release from tissue into 80% glycerol solution is uncontrollable, but the isolated specimen can be stabilized in it, and the secondary gradual swelling prevented throughout the time of observation. Unless care is taken of the inclusion's vulnerability, the inclusions will be affect-

ed by various kinds of artifact induced in morphological and physico-chemical studies. This is seen in the photographs of lipid inclusions published in the literature in which some deteriorations in the anisotropic form are detectable,¹⁸⁻²⁰ and their diameter differs in range from author to author, say 0.5-5.0 μ by Stewart,¹⁹ 1.0-3.0 μ by Weller,²¹ and 0.3-2.5 μ by Geer.²² The osmolarity or the ionic strength of the medium seems to be an important factor for these variations in the appearance of the inclusions under *in vitro* conditions.

The inclusion's physical properties have an important bearing on the role of lipids in the development of atherosclerotic lesions. In atherosclerosis the lipids are known to accumulate in arterial tissue, but the exact mechanism of the lipid accumulation and formation of foam cells in intimal tissue are little known, though the importance of the physical nature of cholesteryl ester molecules for the formation of inclusions has been pointed out.^{23,24} How the early lesion of fatty streaks develops into an advanced lesion of fibrous plaques or complicated lesions is another unsolved question. Ischemic necrosis,²⁵ intimal hemorrhage,²⁶ mechanical stress,²⁵ mesenchymal reactions,²⁷ and calcium deposit²⁸ have been postulated as causes of progression of atherosclerosis, but the role of lipid inclusions has not been accorded due attention. Studies on the inclusion's physical properties ought to throw some lights on the unexplored field.

The lyotropy of the inclusions seems to be closely related to the formative process of globular anisotropic inclusions in the cytoplasm of the intimal cells. When cholesteryl ester molecules are concentrated to a certain critical point in a hydrophilic medium of cytoplasm, they may start forming anisotropic globules which have a macromolecular organization of the constituent molecules in radial symmetry. This could be due to the hydrophobic effect of cholesteryl ester molecules at high concentration which reduces their free-energy in a certain region of the environment by forming spherical macromolecular assemblage as lyotropic anisotropic inclusions.²⁹

Once inclusions are formed, they remain unchanged for a long period of time in a homeostatic medium of cytoplasm, because of the stable ordered inner structure, and the difficulty for enzymes like cholesteryl ester hydrolase to catabolize it. The formation of inclusions is space-taking in cytoplasm and may hinder cel-

lular metabolism. This *circlus vitiosus* of inclusion's formation and space-filling induces the intimal cells to successive build-up of lipid inclusions, and eventual clustering of foam cells in the intima.

The swelling of the inclusions can be a cause of the rupture and necrosis of foam cells. Although the exact process is unknown, changes either in the osmolarity or ionic strength of the cytoplasm in consequence of some disturbances in cellular membrane transport or permeability in endothelial lining causes swelling of the inclusion's volume as seen in common media. Increment in diameter by 15% by swelling equals 24% expansion in volume for each inclusion. If this dilation occurs all at once in every inclusion, some of the foam cells are unable to accommodate the expansion and will rupture the cell membranes. Emancipation of lipid inclusions and intracellular organelles from cytoplasm into the intercellular space may stimulate the reactive proliferation of interstitial tissues in the intima and the media.³⁰

The inclusions, being resilient and deformable, can be made to pass through a narrow channel of intercellular space not only to form mass of inclusions by drawing lipids from infiltrating lipoproteins in the intimal and medial layers, but also they obtain various surface textures. Mostly they are of plump sphere, but sometimes when two or more inclusions are pressed together, they form a concave surface as observed in the electron micrographs.

The transformation from spherical liquid crystal to solid crystal will give rise to further destruction of the tissue architectures. The precipitation and growth of solid crystals involves an enlargement of their dimensions which will further tear the regular structure of the arterial tissues. This tissue laceration, together with crystallization, will stimulate further mesenchymal as well as interstitial reactions, resulting in hardening and thickening of the tissues.³⁰

The hypothetic relations of these physical properties of the inclusions to the genesis and development of atherosclerosis are schematically represented in Figure 6. We wish to emphasize again the importance of the inclusion's physical properties in the development of atherosclerosis. Their elucidation will give us an important clue to the understanding of atherosclerosis and the prevention or even the regression of it.

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