SIMILARITY OF LIPID INCLUSIONS IN EXPERIMENTAL ATHEROSCLEROSIS OF RABBITS TO THOSE OF HUMANS

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A comparative study was made on lipid inclusions of human and rabbits. Atherosclerotic lesions were produced in rabbits fed 0.67% cholesterol for seven months. The loci of the inclusions produced in tissue, three-dimensional configuration, crystallographic characteristics, physical properties and chemical compositions were compared, and found to be similar and even identical between man and rabbit. The lipid inclusions were thus presumed to be formed in arterial tissues by a cellular metabolism common to man and rabbit, and shaped by a physico-chemical effect independent of difference in species. Hence, rabbits are found usable as a model for the study of lipid inclusions in experimentally induced atherosclerosis.

Human atherosclerosis develops in the arterial tissue resulting in a proliferation of smooth muscle cells, deposition of intra- and extracellular lipids, and accumulation of such interstitial substances as collagen, elastin and proteoglycans (28). Lipid accumulates in the arterial intima as globular inclusions which show such characteristics as: (i) take-up of lipid stains like Oil-Red O and Sudan III (6), (ii) extreme richness in cholesteryl esters (8, 14, 19), (iii) birefringence and interference colors identical with pure lipid crystals of cholesteryl esters (11), (iv) configuration of dense osmiophylic bodies in transmission electron microscopy (22), (v) configuration of plump spheres with smooth surface with occasional defective spheres under scanning electron microscopy (15, 16), and (vi) propensity to arise from metabolic disturbance in LDL (Low Density Lipoprotein) pathway of arterial smooth muscle cells (7). These changes in the arteries start at an early age (23), and slowly but insidiously grow into advanced lesions, which are usually undetectable until clinically evident, even fatal, symptoms of complications appear (29).

Although atherosclerosis is known to be a major cause of death, research has been hindered by certain basic drawbacks (21). There is no way of observing directly the inside of arteries of living persons, where atherogenesis and complications occur. Vascular surgery and autopsy are the only means of observation, and the both have supplied us with useful information on early changes as well as advanced

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lesions. However, surgery has limitations on the location and extent of visualization, and autopsy is inevitably accompanied by a post-mortem change in tissue and enzymes, often mixed with secondary alterations of complications.

For these reasons animal experiments are resorted to and rats, rabbits, fowls, dogs, swine, and monkeys are commonly used, but the advantages and disadvantages of these animals as experimental models are in dispute (3, 34, 35). From among them rabbits are singled out as suitable on the grounds that they produce atherosclerotic lesions morphologically resembling those of human (5, 24), chemically similar in lipid composition (26), and metabolically alike in enzymatic activities (17, 18, 36).

The nature of lipids in the form of lipid inclusions in rabbits were, however, not examined to see if they bear the same characteristics as in humans, but their morphological, crystallographic, and physico-chemical observations revealed the same characteristics as in humans, thus the use of rabbits is justified as a model for experimental atherosclerosis.

MATERIALS AND METHODS

Tissue Specimen Eleven male albino rabbits were fed daily 150 g of laboratory chow (Oriental RC-4, Oriental Kobo Industries, Tokyo) with 1 g of cholesterol (Nakarai Chemicals, Ltd., Kyoto) added. Their body weights and serum lipids were checked every three weeks, and after seven months of this feeding regime the rabbits were killed under ether anaesthesia and their aortic atherosclerosis was examined. The pertinent data revealed during this period are given in Table 1. The serum cholesterol was determined by autoanalysis method (33).

Intimal Surface Studies and Histology For macroscopic evaluation the aorta was taken out from the arch to the bifurcation, the adventitial adipose tissue was removed, and the tubular portion of the arch was preserved in 10% buffered formalin

TABLE 1. Duration of cholesterol feeding, body weight, serum cholesterol levels and involved surface area of experimental rabbits

No.	Rabbit #	Cholesterol Feeding (days)	Body Weight at Terminus (g)	Serum Cholesterol (mg%)		Involved Surface Area (%)
				Beginning	Terminus	
1	11	230	3550	56	384	12.8
2	18	204	3130	74	950	25.5
3	49	197	2340	44	2490	27.4
4	51	198	2550	22	534	5.3
5	84	259	2900	52	2000	44.9
6	92	230	2710	72	1840	36.1
7	94	231	2770	80	1500	34.3
8	120	215	3260	172	1098	42.4
9	122	215	4150	108	2340	42.2
10	123	212	3060	48	1164	39.0
11	148	188	3500	—	1416	26.6
Mean±SD		216±20 (n=11)	3084±517 (n=11)	73±42 (n=10)	1429±690 (n=11)	30.6±12.7 (n=11)

were used. The aorta was cut open longitudinally along the anterior margin, laid flat in a transparent polypropylene bag, and xeroxed for the study of the intimal surface. All lesions, flat and elevated, were copied to mark their contrast to the normal tissues. The outlines of lesions were transferred by tracing onto graph paper, and the number of 1 mm squares within the lesion's contour and aortic surface was counted. The intimal area with atherosclerotic lesions was expressed in percentage to the total surface area of the aorta (13).

Crystallographic Study To show the lipid inclusions accumulated in the cells of the lesion site, a small portion of a fresh aorta was teased in 80% glycerol and made as thin as possible by cutting with a fine needle under a dissecting microscope, and observed under a polarizing light microscope with polarizer and analyser crossed at 90°. For the crystallographic examination, the specimen aorta, after its wet weight was determined, was minced with fine scissors into cubes of about 1 mm³, and homogenized in a tissue grinder, fitted with a Teflon pestle with a wall clearance of 0.10 mm to 0.15 mm, which was made to rotate at 1,200 rpm for two minutes. Homogenization at room temperature served to release all inclusions from the tissue without damaging the optical properties, sphericity and diameter of the inclusions. Transferred to 15 ml round-bottomed centrifuge tubes filled with 10 ml of distilled water, the homogenate was centrifuged in a swinging bucket head in a refrigerated centrifuge (Model RC-5 with Type HB-4 head, Ivan Sorvall, Inc., Newtown, Connecticut, USA) at 3,800 G for 45 minutes at 4°C. After centrifugation the homogenate was separated into three fractions: on top, the pale yellow fragile thin layer of lipid inclusions; in the middle, the translucent supernatant; at the bottom, the residue sediment.

The top layer mingled with 0.1 ml to 0.2 ml of supernatant was drawn off into a 1 ml glass syringe through a blunt 22 gauge needle. One or two drops of the harvested inclusions were mixed with 80% glycerol on a microscope slide for examination (11). From prior studies the inclusions were known to be stabilized in 80% glycerol. With a tungsten lamp as light source, the extinction angles and the optic axis were observed on crossed polarizers under orthoscopic lights. The interference figures were observed with crossed polarizers and Bertland lens under conoscopic lights. Elongation sign, optic sign and interference colors were observed with a tint plate of $R=530\text{ m}\mu$ under orthoscopic and conoscopic lights. The order of interference colors was determined by a quartz wedge plate inserted in the microscope's optical path (4).

The isotropic inclusions were observed with the polarizer and the analyser crossed at 80°, slightly off 90°. This angle gave the microscopic field enough brightness to visualize the isotropic forms without distorting the cross image of the anisotropic forms.

Scanning Electron Microscopy To demonstrate the lipid inclusions in tissues, a portion of aortic tissue 5 mm $\times$ 5 mm was fixed with 2.5% buffered glutaraldehyde for 30 minutes, washed with buffer solution, post-fixed for 16 hours with 1% osmium tetroxide in phosphate buffer at pH 7.4, and finally washed with distilled water. The interior of the lesion tissue was exposed by lifting up the overlying tissue with dissecting needles. The preparations were air-dried and coated uniformly *in vacuo* with gold about 200 Å thick. To view the isolated lipid inclusions, about 0.1 ml of the suspension with inclusions was fixed for 16 hours with 1% buffered osmium

for histological examination in which Oil-Red O and hematoxylyn-eosin stain tetroxide, and washed with distilled water. The osmium fixation was found better than other fixations such as 2.5% glutaraldehyde, phosphotungstic acid, acrolein or a double fixation with osmium and glutaraldehyde. A few drops of the suspension of fixed and washed inclusions were placed on a sample stub and dried by evaporation in a gentle air current. In 18 hours the dried sample was gold-coated and immediately examined with a scanning electron microscope (Model, JSM-S1, JEOL Ltd., Tokyo).

Chemical Analysis Lipids were extracted from the inclusions, the supernatant and the residue with 20 volumes of a 2:1 chloroform:methanol mixture (2). The extracts were dried by evaporation under nitrogen stream at 40°C. Cholesteryl esters, triglycerides, free cholesterol, total phospholipids, and individual phospholipids were separately determined by quantitative thin layer chromatography on silicagel HR (E. Merck AG., Darmstadt, GFR). The plates for lipid determination were developed first with acetone-benzene 20:80, secondly with iso-octane-ethyl-acetate 100:1, and lastly with chloroform-methanol-water 65:25:4. They were dried in a vacuum oven (Model RV-3, Hirano Mfg. Co., Koshigaya) under -760 mmHg for 30 minutes at 50°C. The lipid bands were made visible with iodine vapor and scraped off from the plates. The scrapings were digested with acid dichromate reagent and the reduction in optical density was measured at 350 m μ .

For the gas-liquid chromatography (GLC) of the cholesteryl ester fatty acids, the remainder of each extract, when available, was spotted in a wide band on a thin layer chromatography plate to isolate the cholesteryl esters. They were interesterified with super-dry methanol containing 5% HCl, and the fatty acid methyl esters were extracted with hexane. A gas-liquid chromatography of the methyl esters of lauric through arachidonic acid was made with a GLC machine (Model 4B-PF, Shimizu Seisakusho Ltd., Kyoto) using a column packed with 15% ethyleneglycol succinate and an ion flame detector (20). The fatty acid compositions were measured on a chart by the triangle method and expressed in percentage.

RESULTS

Intimal Surface Studies and Histology The aortas of the rabbits fed cholesterol for 7 months had an intimal surface presenting slightly elevated pale yellowish lesions varying in size from small, flat and yellowish spots around the orifices of intercostal arteries to yellow, opaque, confluent and elevated lesions covering the whole lumen of the arch. The percentage of the surface area of the aorta covered with the lesions was 30.6% on an average, as seen in Table 1.

Figure 1 shows an histological specimen of the arch from one of the rabbits fed cholesterol for 7 months, whose intima covered with lesions stained deep red with Oil-Red O in 60% isopropanol. The lipids accumulated in clusters mostly in the thickened intima and partially in the medial layers of the tissue.

FIG. 1. Histology specimen showing lipids accumulating mostly in clusters in the intimal layer of the aortic arch of a rabbit fed 0.67% cholesterol for 7 months. $\times 4$

FIG. 2. Birefringent lipid inclusions with a black cross image filling the interior of spindle-shaped intimal cells in atherosclerotic lesion of rabbit viewed under a polarizing light microscope. $\times 40$

Fig. 1.

Fig. 2.

FIG. 3. Isolated lipid inclusions secured from the atherosclerotic lesion of rabbit and viewed under a polarizing light microscope. $\times 40$

Crystallographic Study Figure 2 and 3 represent the anisotropic inclusions clustered *in situ* in the fresh lesion of atherosclerosis and the isolated lipid inclusions suspended in 80% glycerol under a polarizing light microscope. The inclusions clustered mostly in anisotropic form with a black cross image, whose arm directions coincided with those of the microscope's polarizer and analyser. They appear to fill uniformly the interior of the intimal cells demarcated in spindle shape.

For crystallography, isolated inclusions were used as they were free of the surrounding tissue substances which would interfere with optical observations. The inclusions appeared as small optically clear and colorless spheres of various sizes, which were resolved with polarizing attachments into mixtures of isotropic and anisotropic forms. They showed smooth circular images at all points of the focus, irrespective of their orientation when they were tumbled and rotated through the field of view. Each anisotropic inclusion appeared as a bright gray sphere divided into equal quadrants by a black cross image, whose arms spread out centrifugally

with concave margins, and the arm directions coincided with those of the polarizer and analyser north-south, east-west, i.e., a Formée cross. As the arms of the cross fanned out with concave and not straight margins, the image should be referred to as a Formée cross, and not as a Maltése whose arms broaden with straight margins. Under polarized lights, each quadrant gave an interference color of first order whitish gray, whereas under a tinted plate of $R=530\text{ m}\mu$ inserted in the light path the whitish gray of all quadrants changed into yellow in the upper left and lower right quadrants, and blue in the upper right and lower left. The order of the interference colors was confirmed to be first order gray, first order yellow and second order blue by means of a quartz wedge plate inserted in the optical path. The location, form and tint of the interference colors underwent no change when the inclusions were rotated by turning the microscope stage through 360° or when the inclusions were tumbled

FIG. 4. Three-dimensional view of lipid inclusions nesting in the atherosclerotic lesions of rabbit.
 $\times 1,800$

through the suspension. This indicated that the inclusions had neither a particular extinction position or optic axis, nor sign of elongation, which is characteristic of a true crystal. The anisotropic inclusions were found to have formed no isogyres under conoscopic lights as in pure crystals. The absence of isogyres indicates that the positive and negative optic signs were not applicable to them, though their cross image and interference colors were similar to those of the crystals of positive optic sign.

Some more physical characteristics of the anisotropic inclusions were observed under a polarizing light microscope. The first was resiliency in deformation when

FIG. 5. Three dimensional configuration of lipid inclusions isolated from atherosclerotic lesions of rabbit. $\times 3,600$

they were squeezed between a cover slip and a microscope slide. They could be changed into an ellipsoid, and if not pressed too hard through a narrow channel they would flow transiently through a dumbbell shape, but they would recover their spherical form after the pressure was released. During this transient and moderate deformation the Formée cross image was distorted, but its characteristic shape was eventually recovered.

Secondly, when dried the anisotropic inclusions irrevocably lost their spherical shape, and the Formée cross image and the interference colors disappeared. They disintegrated into whitish brilliant birefringent bodies. Water was thus essential for the maintenance of three-dimensional integrity, hence they were lyotropic.

Thirdly, when exposed to distilled water, physiological saline solution, or 1/15 molar phosphate buffer or 1% sucrose solution, the anisotropic inclusions gradually swelled 15% in diameter in 4 hours and developed isotropy, starting in the center and progressing centrifugally until they were completely isotropic. Swelling was minimum in 80% glycerol among others.

The isotropic inclusions appeared as homogeneous gray spheres and remained unchanged when the microscope stage was rotated or the tint plate was inserted. They tended to possess a larger diameter than the anisotropic.

Scanning Electron Microscopy Figure 4 represents the lipid inclusions nesting in the tissue site. As shown in Figure 1, where the lipids stained with Oil-Red O scattered in the disrupted intimal tissue, the spherical inclusions were deposited in clusters in the destroyed architecture of the tissue. Individual inclusions appeared spherical in basic shape and smooth in the surface texture, but a few showed defects of various sizes on the surface.

Figure 5 is a scanning electron micrograph of the isolated lipid inclusions. All inclusions appeared spherical with a diameter ranging from $0.3\text{ }\mu\text{m}$ to $3.0\text{ }\mu\text{m}$, with a mean and standard deviation of $1.0\text{ }\mu\text{m} \pm 0.5\text{ }\mu\text{m}$ ($n=419$). They had predominantly smooth surfaces with occasional low profile ridges, but a few showed defects of

TABLE 2. *Chemical compositions of lipid inclusions, supernatants, and residue in experimental atherosclerosis of rabbits*

Lipids	Inclusions (n=11)	Supernatants (n=10)	Residue (n=11)	Statistical Significance	
				between Incl & Sup	between Incl & Res
Total Lipids (mg/wet g)	11.6 ± 7.7	6.4 ± 1.7	5.7 ± 4.0	—	—
Percent Distribution (%)	(49.0)	(27.1)	(23.9)	—	—
Cholesteryl Esters (%)	$52.6 \pm 13.9^*$	31.2 ± 7.7	39.1 ± 6.3	$p < 0.01$	NS
Triglycerides	13.4 ± 13.3	7.2 ± 6.4	10.6 ± 7.5	NS	NS
Free Cholesterol	15.5 ± 3.7	31.2 ± 2.1	29.5 ± 7.7	$p < 0.01$	$p < 0.01$
Phospholipids	18.1 ± 6.1	30.5 ± 6.9	20.7 ± 7.5	$p < 0.01$	NS
Cephalin (%)	17.3 ± 12.9	—	12.6 ± 9.7	—	NS
Lecithin	20.9 ± 9.6	—	21.0 ± 11.3	—	NS
Sphingomyelin	37.4 ± 15.4	—	38.1 ± 14.0	—	NS
Lysolecithin	24.4 ± 6.7	—	28.9 ± 8.9	—	NS

* Mean $\pm$ SD

Incl: Inclusions; Sup: Supernatant; Res: Residue

TABLE 3. *Proportions of various fatty acids in cholesteryl esters of inclusion and residue in experimental atherosclerosis of rabbits*

Fatty acid		Percent of total fatty acids by tissue preparation	
Common name	Short hand	Inclusions	Residue
Myristic	14 : 0	3.3 ± 3.0*	4.3 ± 2.7
Palmitic	16 : 0	27.3 ± 10.0	31.7 ± 12.9
Palmitoleic	16 : 1	6.2 ± 3.3	3.5 ± 2.7
Stearic	18 : 0	6.1 ± 2.3	6.6 ± 2.8
Oleic	18 : 1	41.9 ± 15.5	37.5 ± 11.4
Linoleic	18 : 2	11.2 ± 5.9	13.0 ± 6.4
Eicosatrienoic	20 : 3	3.3 ± 1.6	3.7 ± 1.2
Arachidonic	20 : 4	2.6 ± 0.6	—

* Mean ± SD (%)

various sizes for lack of substances. Occasional small globular structures were found adhered to the surface of inclusions.

Chemical Analysis In Table 2 are given the chemical compositions of the lipid inclusions, the supernatant and the residues. In 11 experimental rabbits whose aortas developed atherosclerotic lesions in 30.6% of the surface, 49% of the lipids accumulated in the tissue were in the form of lipid inclusions, 27% in a solubilized form of lipids in the supernatant, and 24% in the form of structurally bound lipids in the residue. The lipid compositions differed appreciably from preparation to preparation, but were remarkably uniform within each preparation. The inclusions were constituted largely of cholesteryl esters, with small proportions of triglycerides, free cholesterol and phospholipids. The supernatant and the residue fractions consisted largely of equal portions of free cholesterol and phospholipids with a smaller amount of cholesteryl esters as compared with the inclusions ($p < 0.01$). Sphingomyelin was the major phospholipid both in the inclusions and the residues.

The fatty acid composition of the cholesteryl ester fraction is presented in Table 3. Eight fatty acids were commonly found: lauric, myristic, palmitic, palmitoleic, stearic, oleic, linoleic, and arachidonic acids. The inclusions contained more palmitoleic and oleic acids, while the residue contained more palmitic and linoleic acids.

DISCUSSION

In order to compare the lipids in experimental atherosclerosis of rabbits with those of human, we have produced atherosclerotic lesions in rabbits by feeding them with 0.67% cholesterol for seven months. The lesions have involved on the average 31% of the intimal surface of the aorta, and the lipid contents are increased four-fold. They are isolated from the tissue and separated into three fractions: lipid inclusions, supernatant and residue. The lipid inclusions are bulk lipids accumulated in particulate form, and float as the top layer after isolation by centrifugation, while the supernatant fraction contains lipids in solubilized form, like lipoproteins released from intimal cells and interstitial space. In the residue

the lipids are bound to the structural components of tissue like cell membrane matrix or interstitial substances. As the fraction of the lipid inclusions has so increased in content as to attain half of the four-fold increase of lipids, a form never to be found in normal tissue, it can be safely assumed that they represent atherosclerotic lipids in the arterial tissue. Thus we have compared them to those in man.

To make the comparison valid we have used exactly the same methods as used in the study of the inclusions in man (11, 15, 16, 19), and found their loci and three dimensional configuration similar to those of human, their crystallographic characteristics and physical properties identical to and chemical compositions very close to those of the human counterpart.

The lipid inclusions have revealed such unique characteristics as, (i) localization in intimal tissue in a particulate form clustering within the cells and subsequently in extracellular space, (ii) invariable spherical form with diameters ranging from 0.3 μm to 3.0 μm on an average of 1.0 μm , whose three dimensional appearance remains unchanged while nesting in tissue or after isolation from it, (iii) a Formee cross image dividing the sphere into quadrants, whose interference color is first order gray under polarized lights, (iv) interference colors resembling the pattern of an optic sign positive material, second order blue in the upper right and lower left quadrants and first order yellow in the upper left and lower right quadrants, (v) constancy of optical appearance irrespective of the inclusion's orientation, (vi) failure of anisotropic inclusions to demonstrate extinction positions, optic axis, sign of elongation and true optic sign, (vii) resiliency to modulate deformation in shape, (viii) swelling in volume and loss of anisotropy when exposed to various solutions, (ix) lyotropy requiring water as an essential constituent of the inclusion's integrity, (x) mostly smooth surface of the inclusions with only occasional defects, and (xi) unique chemical composition high in cholesteryl esters, particularly in cholesteryl oleate.

As shown by histological studies on the localization of the bulk lipids in the form of inclusions, they accumulate at an early stage within the cell of the thickened intima like the foam cells of human fatty streak lesions, but after seven months of cholesterol feeding they precipitate in the form of extracellular clusters of various sizes aggregating in the deeper layers of media as shown in Figure 1. Scanning electron microscopy has confirmed this observation by showing the spherical lipid inclusion nexus scattered in the destroyed architecture of the tissue. The loci of lipids are thus similar to human lesions, though different in their topography. In rabbits, atherosclerotic lesions start at the arch, rather than at the abdominal aorta as in the human (27), and develop from there to peripheries; reasons for this difference are still unknown.

The spherical form of lipid inclusions may hint at a thermodynamically stable state where molecules above a critical concentration minimize their free-energy to form a structure with less freedom of movement within certain regions. In the inclusions, the longer axis of the constituent molecules of cholesteryl esters is orientated everywhere parallel to its radii, e.g., in radial symmetry. This symmetry obtains throughout the inclusion only by forming a concentric lamellar structure, in which the constituent molecules are uniformly aligned parallel to the radius, and with decreasing radius in the more central positions the decreased circumference is accommodated by reducing the number of molecules per layer (10, 11).

On the basis of this structural concept, the crystallographic characteristics

of the inclusions are explained: the black cross image of the anisotropic inclusions is an interception of the orthoscopic light vibrating perpendicular to the privileged directions of polarizer and analyser, and no isogyres being formed under conoscopic light. The brilliance of the whitish gray color in the inclusion's quadrants is due to the interference of slower and faster lights whose retardation in wave velocity is caused by the optically active cholesteryl ester molecules. By inserting the tint plate which has the privileged axis in the direction of upper right to lower left quadrants, retardation in wave velocity is incremented in the same quadrants where the interference color changes from first order whitish gray to second order blue, while in the upper left and lower right quadrants where the privileged direction of the tint plate becomes perpendicular to that of cholesteryl ester molecules, the interference color changes from first order whitish gray to first order yellow. The molecular alignment in radial symmetry does not give any extinction position or optic axis, because they have a symmetry axis only in the center of the sphere.

The inclusion is resilient, because the molecules are orientated only in one direction of the inclusion's radii, consequently each molecule has the freedom to rotate along its radial axis and to translate along the circumference of the inclusion's sphere as long as it keeps the axis parallel to the radii. Aside from this uni-directional molecular orientation, water molecules between the concentric layers of cholesteryl ester molecules make molecular packing of the inclusion less tight than that of the true solid crystals. This uni-directional molecular orientation and the interlayer water molecules make the inclusion less rigid to moderate deformation.

Furthermore, water plays an essential role in maintaining the inclusion's three-dimensional configuration. Once the water molecules are lost upon drying the inclusions, the inclusions lose not only the optical characteristics but also disintegrate into fine amorphous pieces of birefringence. When excess water enters the interlamellar spaces of the structure in various kinds of media, the inclusions swell in volume, and eventually lose anisotropy and are distorted in their inner structure when excess water is incorporated.

The chemical composition of the inclusions in rabbits high in cholesteryl esters is similar to that of the human. Cholesteryl esters, particularly oleate, account for about one-third of the total increased lipids, as the cholesteryl esters amount to about 53% of the total lipids and the oleate about 42% of the cholesteryl ester fatty acids. This high concentration of oleate in the cholesteryl ester is unmatched to any kind of lipids except the adrenal lipids in animal bodies (25) and only the cholesteryl oleate can form lyotropic mesophase of birefringent globular form at temperatures including the body and the room temperatures (30).

In the meantime, there are some arguments for and against the use of rabbits as experimental models for the study of atherosclerosis (34, 35); for example, (i) the rabbits are said to be different from the human in lipid and cholesterol metabolism, (ii) their topography of lesions is different, (iii) they seldom develop lesions corresponding to the human fibrous plaque or complicated lesions, (iv) their lesions are often complicated by cholesterol storage in macrophages and reticuloendothelial cells, and (v) their lesions are very resistant to regression (1).

We have used, however, rabbits for our experiments, as they are very susceptible to hypercholesterolemia and quick to develop atherosclerotic lesions, and also they are already well documented, easy to handle in daily care and experiment, and

their low cost is suitable for mass studies (35). Moreover when we consider that the rabbit inclusions bear good resemblance to the human counterpart in the localization in tissue, three-dimensional configuration, crystallographic characteristics, physical properties and chemical compositions, we come to the conclusion that the rabbit is a suitable model for animal experimentation of atherosclerosis in so far as the nature of accumulated lipids, particularly in the form of inclusion, is concerned.

These similarities may stem from the fact that the lipid inclusions are formed in arterial tissue by a cellular metabolism common to both (7, 9), and shaped by a physicochemical mechanism like hydrophobic effect of lipid molecules (31, 32) independent of difference in animal species. Only tissue reactions against lipid inclusions may vary from animal to animal and bring about different kinds of atherosclerotic lesions. Our preliminary observations of the lipid inclusions from atherosclerosis in dogs and rats show that they are the same in nature as those of the human. These facts lead us to a further insight that the cholesteryl ester-rich inclusions may be the causative entity of atherogenesis common to the mammal, while the tissue reactions vary from artery to artery and species to species. Although the exact mechanism for formation and resorption of the lipid inclusions is not yet fully known, the elucidation of the cellular and physico-chemical processes which produce such inclusions as uniquely rich in cholesteryl esters is not only important for the validation of animals as experimental model, but also crucial to the understanding of the role of lipids in atherogenesis in mammals including man. The identification of the difference in tissue reactions to the inclusions may give a clue to the explanation for differences in atherosclerosis in different arteries of different animal species.

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