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Studies on the Pathogenesis of Atherosclerosis with Experimental Model Systems

II. An Electron Microscopy Study on the Uptake of Egg Lipoproteins by Endothelial and Smooth Muscle Cells of the Doubly-Ligated Rabbit Carotid Artery

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Studien über die Pathogenese der Atherosklerose mit experimentellen Modellsystemen

II. Eine elektronenmikroskopische Untersuchung zur Aufnahme von Eilipoproteinen durch Endothel- und glatte Muskelzellen der doppelt ligierten Arteria carotis

Zusammenfassung. Aus Eigelb isolierte Lipoproteine wurden in das Lumen der doppelt ligierten Arteria carotis des Kaninchens injiziert, um ihre Aufnahme in die Endothel- und glatten Muskelzellen im Zustand der Hypoxie zu studieren. Man nimmt an, daß die Hypoxie möglicherweise eine wichtige Rolle bei der Pathogenese der Atherosklerose spielt. 2—20 Tage nach der Injektion füllten sich alle Zellen, insbesondere aber die glatten Muskelzellen mit Lipidtropfen. Da die Färbung für Cholesterin negativ und für Phospholide schwach ausfiel, kann man annehmen, daß sie hauptsächlich Triglyceride enthielten. Die Endothelzellen, die mit ihren Microvilli, Lysosomen und freien Ribosomen den Makrophagen ähnelten, enthielten auch Vacuolen mit intakten Lipoproteinpartikeln. Man kann vermuten, daß die Lipoproteine im wesentlichen an der Zelloberfläche zu freien Fettsäuren bzw. Glyceriden abgebaut werden, und daß diese nach Eintritt in die Zelle Triglyceridtropfen bilden. Die freien Fettsäuren müßten zuvor in Triglyceride überführt werden, während die Glyceride direkt Tropfen bilden können.

Summary. Egg lipoproteins were injected into the lumen of doubly-ligated rabbit carotid arteries in order to study the uptake of lipoproteins by endothelial and smooth muscle cells under hypoxic conditions, a state believed to possibly play an important role in the pathogenesis of atherosclerosis. All cells, in particular smooth muscle cells, were filled with lipid droplets from two to twenty days following injection. These inclusions were believed to contain primarily triglycerides, since staining was weak for phospholipids and negative for cholesterol. The endothelium, which resembled macrophages with microvilli, lysosomes and abundant free ribosomes, were also filled with vacuoles containing intact lipoprotein particles. It is suggested that most of the lipoprotein was broken down on the cell surface, possibly to free fatty acids or glyceride esters and resynthetized or coalesced to triglyceride droplets.

Introduction

In a previous study the hypoxic state induced by double ligation of the rabbit carotid artery was studied in order to simulate one or a group of unspecific irritants believed to be important in the pathogenesis of atherosclerosis (Hoff

and Gottlob, 1969). When a triglyceride emulsion, Intralipid, was injected into the ligated segment, both the endothelial and smooth muscle cells of the thickened intima accumulated large amounts of the lipid in form of intracellular droplets, far more electron-dense than the Intralipid particles. Since no clearcut entrance mechanism was observed, it was suggested that the triglycerides might be broken down at the cell surface either to smaller triglyceride droplets, mono- or diglycerides, or free fatty acids, and consequently taken up by some mechanism still obscure. Since triglycerides are not found free in serum, it was deemed necessary to continue such studies but using lipoproteins of high triglyceride composition. Egg lipoproteins which fulfill this requirement were the first of a series of various lipoproteins to be used in combination with the doubly-ligated carotid artery. This investigation therefore deals with the morphological alterations to the rabbit carotid artery as observed light microscopically on lipid-stained frozen sections and electron microscopically, when egg lipoproteins were injected into the doubly-ligated carotid artery. Particular attention was devoted to the form of the intracellular lipid inclusions and the possible mechanism of uptake.

Material and Methods

Lipoprotein Isolation

Lipoproteins were isolated from hen egg yolks by the following procedure: two yolks carefully washed free from egg white were mixed with 100 ml Ringer solution, density 1.007 and the suspension first centrifuged at 2,000 g and the supernatent then ultracentrifuged at 110,000 g for 18 hours. The egg lipoprotein fraction floated to the top of the centrifuge tube forming a gel which could easily be brought into solution with Ringer. This isolated fraction of egg lipoprotein belonged to the $S_{F20,\,1.06}$ 16—126 class and chemically consisted of 11% protein and 89% lipid, of which 66.3% was glyceride ester, 28% phospholipid, 5.2% free cholesterol and 0.5% cholesterol ester (Nichols *et al.*, 1969). Concentrations of the macromolecule in Ringer were measured with a Zeiss Abbé refractometer using a specific refractive increment of 1.58×10^{-3} n/g/100 ml (Lindgren *et al.*, 1954).

Surgical Procedure

The right common carotid artery of ether-anesthetized rabbits (male and female, yellow-silver strain, about four months old, averaging 1.5 kg) was exposed, a ligature applied on the caudal end of the exposed area and a plastic catheter inserted into the lumen in the cranial direction. A suspension of egg lipoprotein (approximately 20% in Ringer solution) was then injected via the catheter into the carotid artery until the lumen was completely filled with the suspension. A second ligature was then applied two centimeters cranial to the first ligature, a third one just cranial to the hole created by the catheter, the artery re-inserted, and the wound sutured. At periods of one, two, three, five, seven, eight, ten and twenty days following ligation the isolated segments were removed and prepared for microscopy. Segments of the doubly-ligated right carotid artery but injected with Ringer solution and the untreated left carotid artery were also studied and used as controls.

Microscopy

In some experiments segments of the artery were fixed in Baker's calcium formol, frozen sections cut and stained with Sudan black and oil red 0 for neutral lipids, OTAN to distinguish between neutral and polar lipids, and PAN for cholesterol (Pearse, 1968). Segments from experiment were also fixed in 5% glutaraldehyde in 0.1 m cacodylate buffer pH 7.4 followed by fixation in 1% osmium tetroxide-0.23 m sucrose in 0.1 m veronal-acetate buffer pH 7.4 for two hours at 4° C. These segments were then dehydrated in graded ethanols and embedded

in Araldite. Sections were cut on a Reichert ultramicrotome. One micron-thick sections were stained with alkaline toluidine blue and viewed with the light microscope for survey purposes. Ultrathin sections stained with 1% lead citrate were viewed with a Siemens Elmiskop 1 or a Zeiss EM 9 electron microscope.

Results

Light microscopically both endothelial and smooth muscle cells were filled with vacuoles (Fig. 1a) which presumably contained lipid since they were sudan (Fig. 1b) and oil red 0 positive. OTAN-stained sections also demonstrated the localization of some, if very little phospholipid within these droplets. Cholesterol staining was negative. Subendothelial edema could be seen (Fig. 1c) and the endothelial cells often overlapped one another (Fig. 1a and c). From two days on, at which time the first lipid appeared in cells, there was a general increase in the number of cells containing lipid with time, although after five days these cells often appeared quite necrotic. A number of experiments were complicated by the appearance of thrombi particularly at later times. They became organized quite rapidly and contained capillary sprouts within twenty days (Fig. 1d). The borders of the thrombi were often coated by an endothelial lining (Fig. 1e and f).

Ultrastructurally, lipid droplets were observed in all cells of the thickened intima and media (Fig. 2a). These inclusions were not very electron-dense, but the lipid did not appear to be extracted from these droplets (although this possibility was not studied further). The morphology of the smooth muscle cells had been altered to one resembling fibroblasts with abundant rough-surfaced endoplasmic reticulum. Often such cells were oval rather than spindle-shaped, and when filled with lipid droplets resembled the typical foam cell (Fig. 4c). Some of these cells often with a necrotic appearance also contained giant vacuoles full of large lipid spheres (Fig. 4b).

The morphology of the endothelial cell also changed after five days of hypoxic environment and presence of high lipoprotein concentrations (Fig. 2b). These cells were filled with numerous lysosomes and vacuoles possibly phagocytotic and frequently containing intact lipoprotein particles, identified as rings as seen in the lumen and in these vacuoles (Fig. 2b). Mitochondria were often swollen, clusters of ribosomes were found free in the cytoplasm and the cell surface often extended microvilli into the lumen. Some unidentifiable electron-dense material was found in the subendothelial space (Fig. 2b). The abnormal overlapping of endothelial cells was at times so extreme that three cells sometimes separated the lumen from the subendothelial space (Fig. 3a). Not only microvilli but large protrusions were also seen extending from such cells (Fig. 3b). The lipid droplets seen in endothelial cells (Figs. 2b and 3b) were identical to those seen in smooth muscle cells. They did not appear to be surrounded by a membrane, but were often in close association with ribosomes (Figs. 2b, 3a, c) and the endoplasmic reticulum (Fig. 5) but extremely rarely in mitochondria or the Golgi zone. The dense but diffuse specks on most lipid droplets represent lead precipitate following lead staining. Although the egg lipoprotein particles were often seen in direct contact with the lumen side of the endothelial lining (Fig. 4d), no particles were seen in the cytoplasm except in vacuoles (Figs. 2a, 4a).

The thickened intima frequently contained capillaries twenty days following ligation and injections of lipoproteins. The cells of the thickened intima were

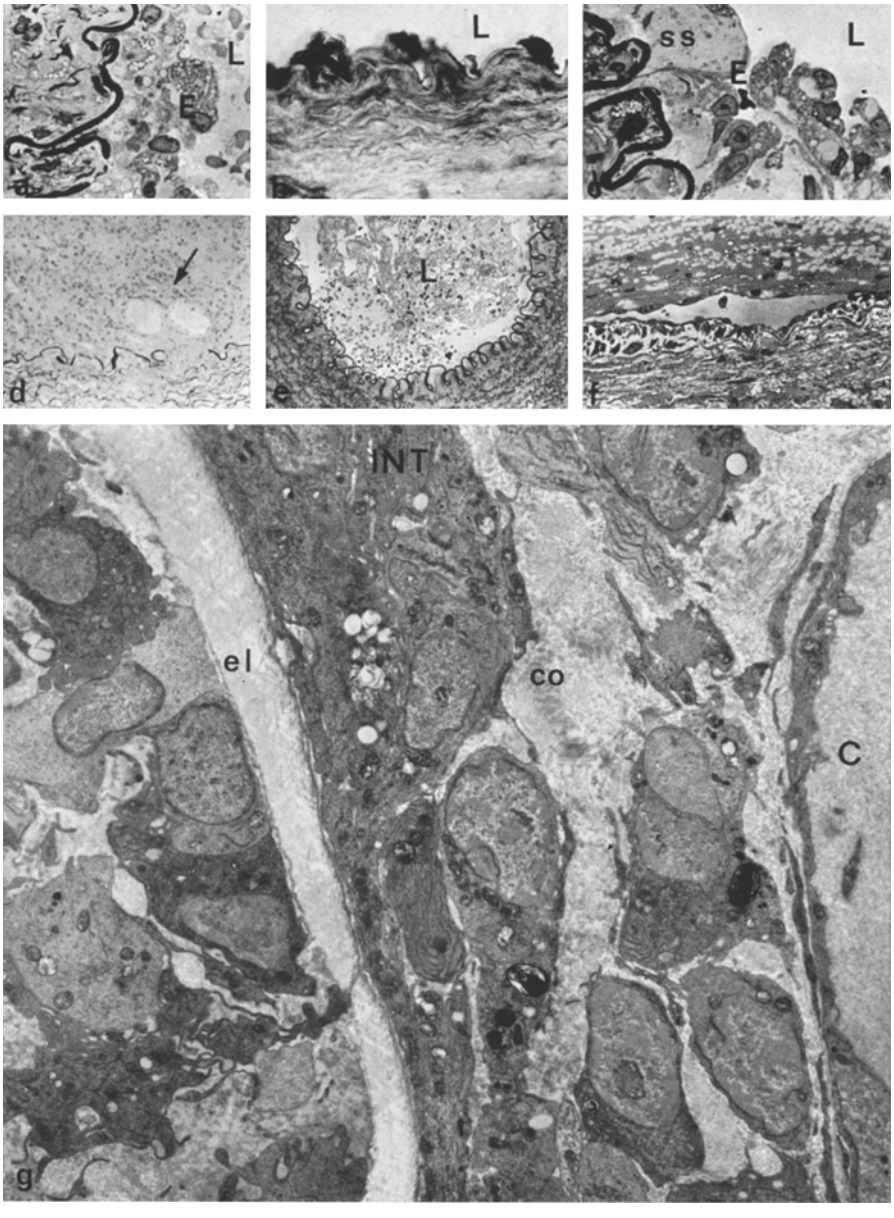

Fig. 1. a Toluidine blue-stained section of artery five days after ligation and lipoprotein injection. Note the numerous vacuoles in the endothelial cells (*E*) which overlap each other. ×800. b Sudan black stained frozen section demonstrating lipid in the intima and parts of the media of an artery after three days, ×200. c Toluidine blue-stained section of artery after seven days. Note the edema in the subendothelial space, ×800. d, e and f Toluidine blue-stained section of artery. d After twenty days, note the two capillaries (arrow) in the thickened intima and the gap in the elastic membrane which appears to be a focus of cell outgrowth into the lumen, ×120. e After twenty days, note the endothelial sheet around the thrombus of red cells and lipoproteins in the center of the lumen (*L*), ×60. f After twenty days, the lumen is almost completely filled with a similar thrombus as in e which is also endothelialized, ×120. g Electron micrograph of an artery twenty days after ligation and injection. Note the fibroblast-like cells in the thickened intima (*INT*) and a capillary (*C*). Numerous collagen fibers (*CO*) can be seen between these fibroblasts or modified smooth muscle cells, ×4000

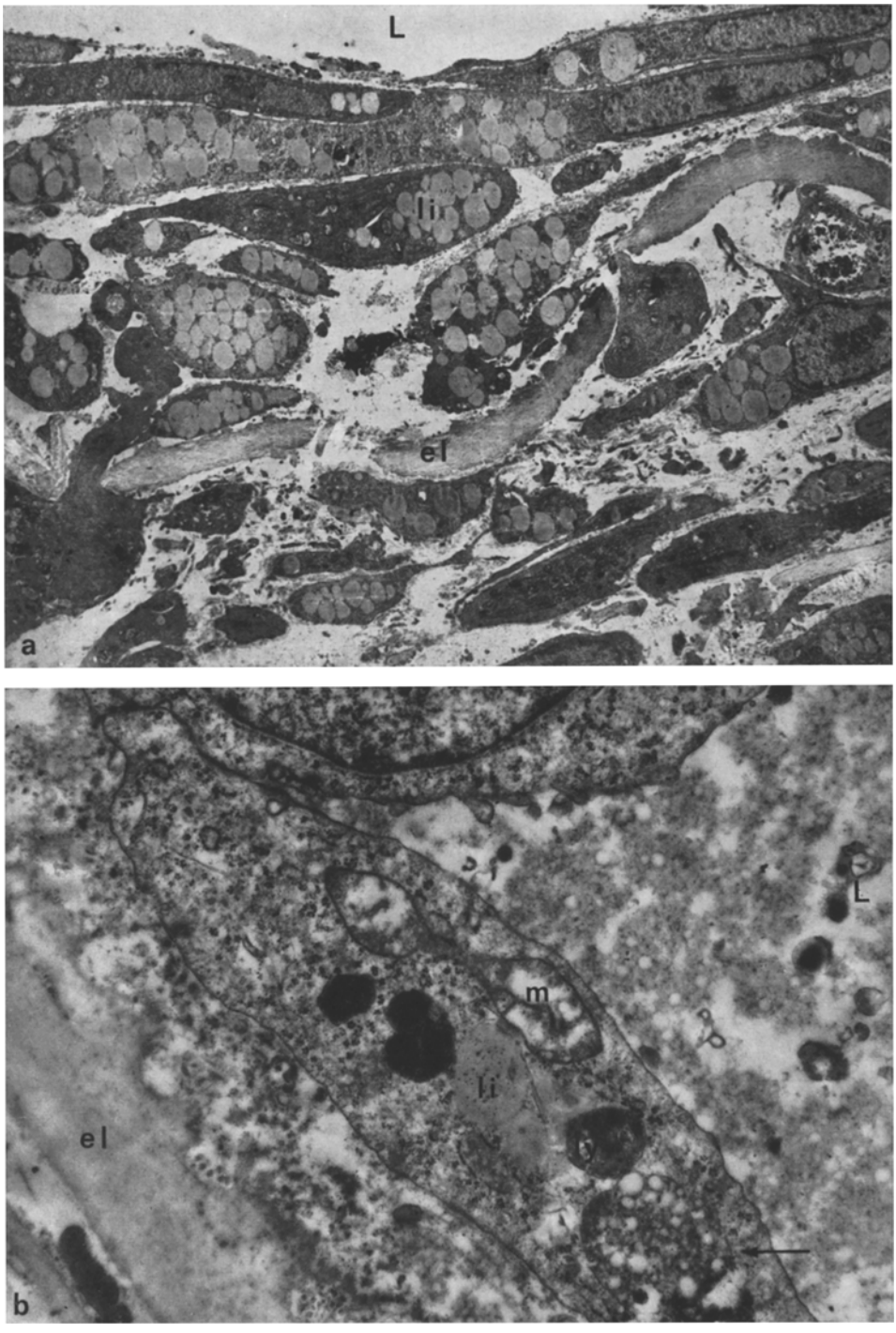

Fig. 2a and b. Electron micrograph of the thickened intima (a) and the endothelial lining (b) after five days following ligation and injection. Note in a the numerous lipid inclusions in the modified smooth muscle cells within the thickened intima and media. The stripping of the endothelial layer in this micrograph is a procedural artifact, ×5,000. Note in b the swollen mitochondria (*m*), the lysosomes (*ly*), amorphous lipid droplet (*li*) and the vacuole containing presumably intact lipoprotein particles (arrow) resembling the particles in the lumen (*L*), ×13,000. *el* elastica

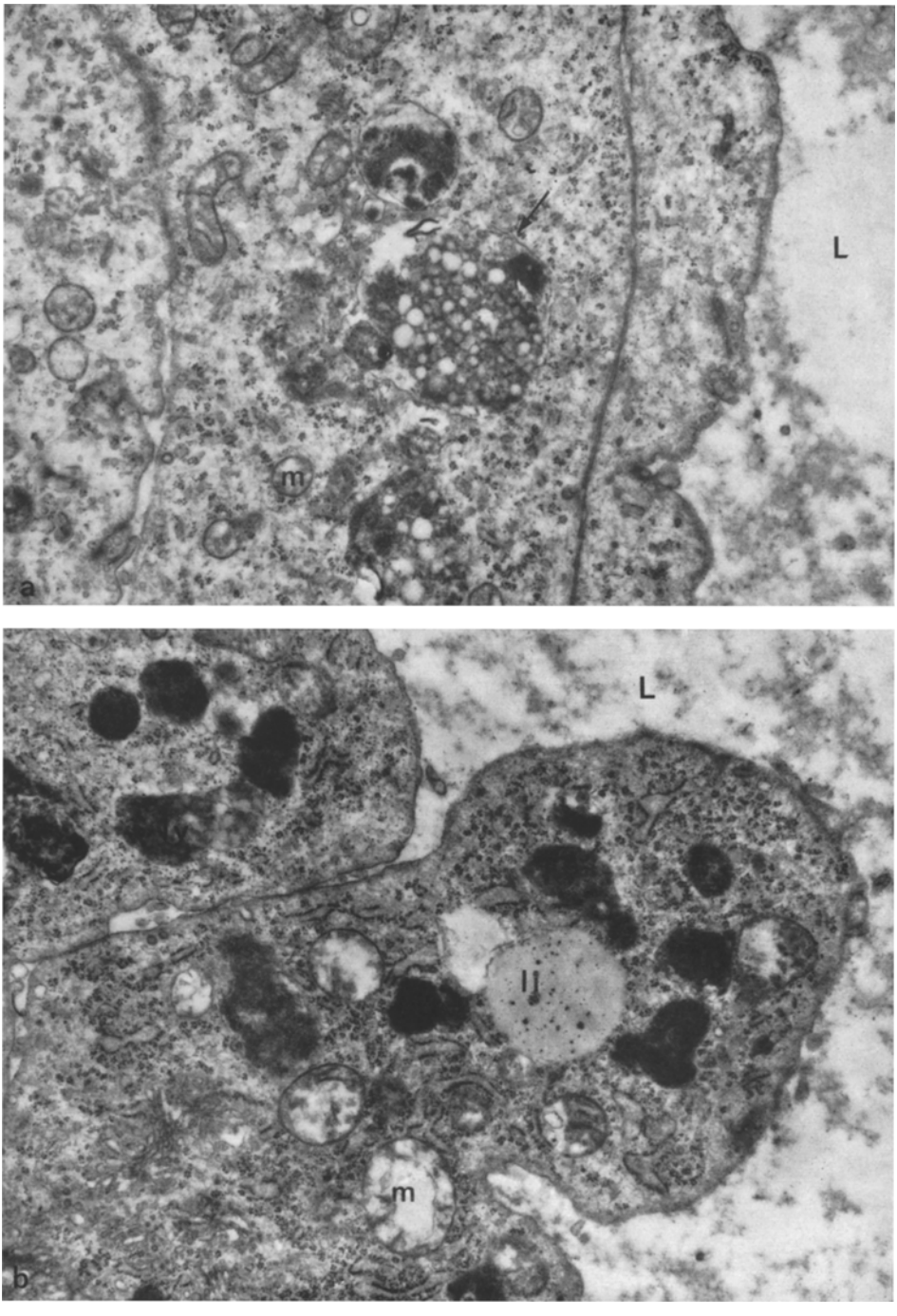

Fig. 3 a and b. Endothelial lining of an artery five days following ligation and lipoprotein injection. In a one can see an abnormal overlapping of cells which contain abundant free ribosomes and phagocytotic vacuoles possibly containing intact lipoprotein particles, ×14,000. In b one can observe large protrusions extending into the lumen (*L*). Note also the swollen mitochondria (*m*), the lipid droplets (*li*) and the numerous lysosomes (*ly*), ×15,000

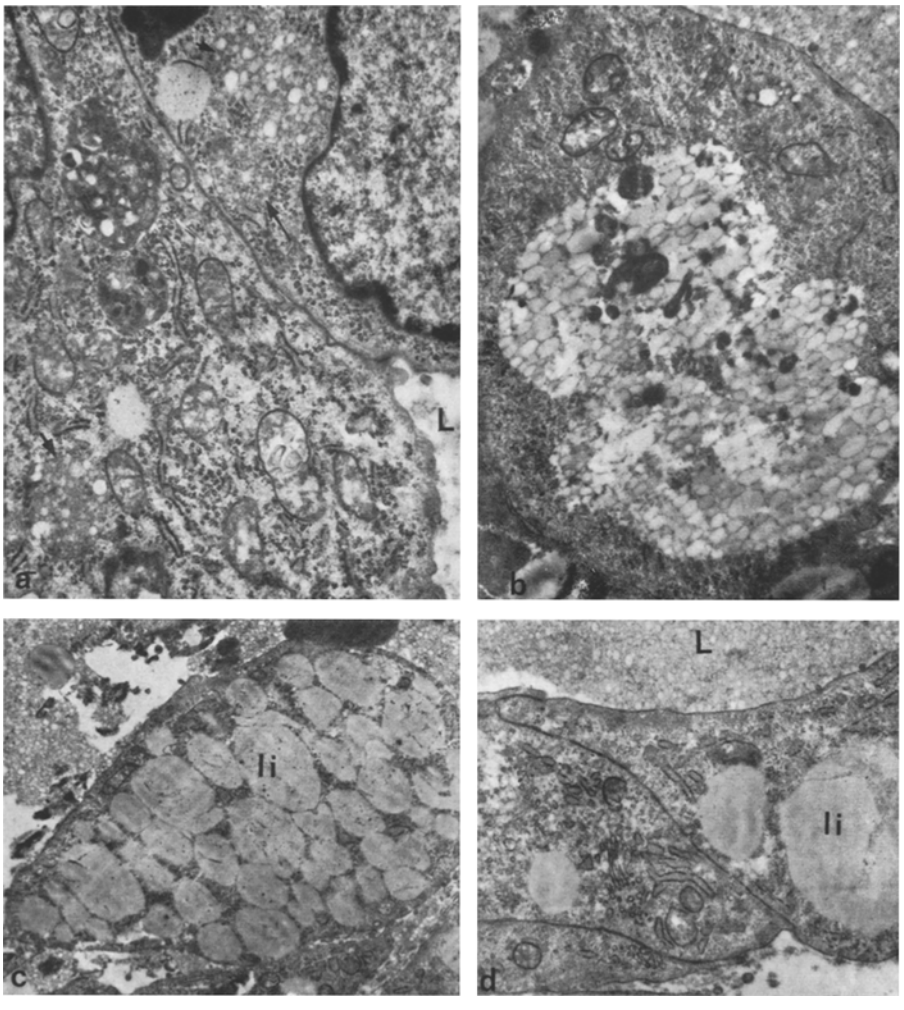

Fig. 4. a Endothelial lining after five days containing phagocytotic vacuoles filled with intact lipoprotein particles, ×12,000. b A necrotic modified smooth muscle cell with a large lipid inclusion consisting of numerous rings in cross-section after five days. Note the egg lipoproteins in the extracellular space on the upper right, ×11,000. c An oval-shaped cell full of lipid droplets resembling a foam cell after seven days, ×8,000. d The endothelial lining after seven days showing the lumen (*L*) filled with lipoprotein particles, ×10,000

often multinucleated and had extensive endoplasmic reticulum (Fig. 1g). Some areas had surprisingly little intracellular lipid. Collagen bundles filled the extracellular space.

Discussion

Although we initially used the hypoxic condition produced by double-ligation merely as a model for the unspecific irritating stimuli believed to occur in atherosclerosis, there is strong evidence that hypoxia may play an important role in

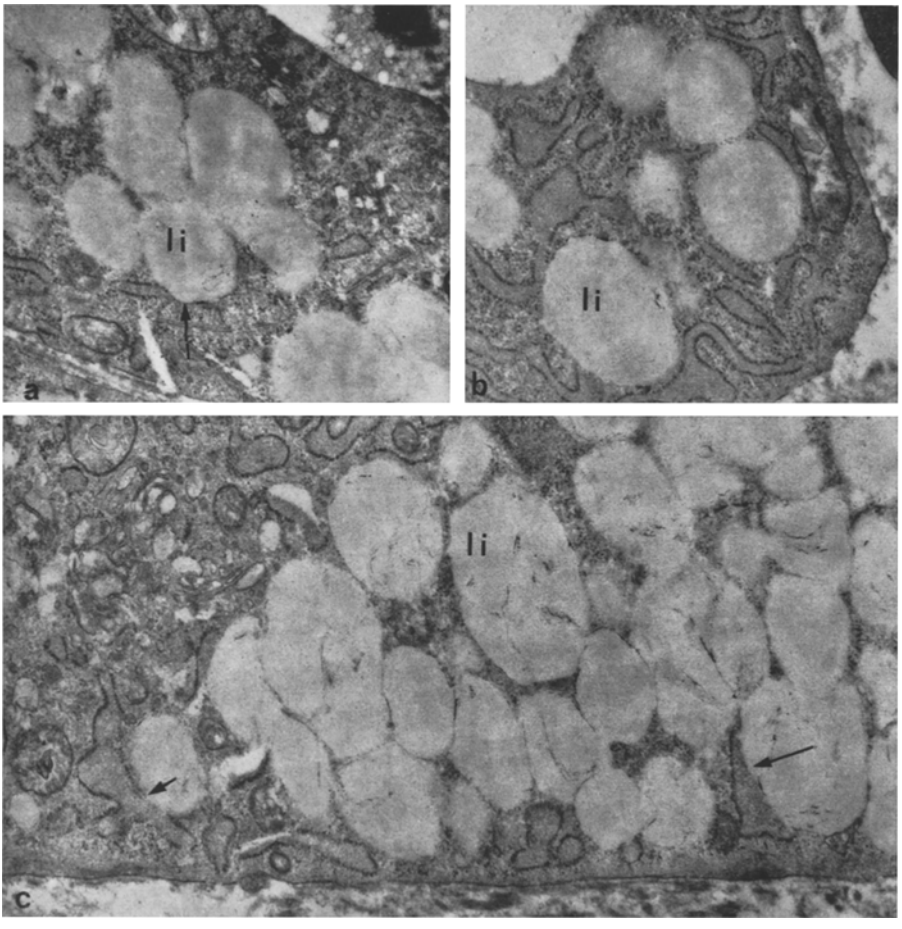

Fig. 5a—c. Lipid inclusions in intimal modified smooth muscle cells of arteries after seven days. Note the close association of these lipid inclusions (*li*) with the rough surfaced endoplasmic reticulum (arrows), ×18,000

the pathogenesis of atherosclerosis. Helin and Lorenzen (1969) have succeeded in inducing arteriosclerosis through systemic hypoxia, while Kjeldsen (1965) showed that arterial cells accumulated lipid in greater amounts under hypoxic conditions than normally, as did Robertson (1961) in tissue cultures of aortic cells. In both hypoxia and early atherosclerosis there is a general reduction in enzyme activity of the Krebs cycle. Most oxidative enzyme activity is reduced as is ATPase (Adams, 1967). Glycolysis, in particular the anaerobic form, plays an important role in arterial metabolism (Kirk, 1959). This appers to be the reason why the arterial cells survive an environment with such low oxygen tension. Many cells appear morphologically viable even though their mitochondria are swollen. Assuming an initial breakdown of the lipoprotein lipid to free fatty acids, which is only one of many possibilities, it seems that enough energy is derived from glycolysis for the consequent synthesis of triglycerides and possibly also phos-

pholipids, using exogenous free fatty acids, although such synthesis requires a great deal of energy. If the catabolism of fatty acids is blocked, as may be the case in cells whose mitochondria are disrupted; the free fatty acid pool, could then be used entirely for synthesis of lipids. Perhaps a dynamic equilibrium occurs which is shifted in the direction of lipid synthesis, hence leading to the extreme case when practically the entire cell consists of fat droplets.

The altered morphology of the endothelium found in this study and resembling to some extent macrophages, has also been described by BUCK (1961) in his double-ligation studies. The phagocytotic or autophagic vacuoles and lysosomes seen in this study have also been reported by DAOUD *et al.* (1968) and WELLER *et al.* (1968). The latter author also localized a phospholipid to cholesterol fatty acid acyltransferase (ADAMS, 1967) within such bodies. WELLER *et al.* (1968) suggested that the whorl bodies in atherosclerotic vessels contained phospholipid and cholesterol and then became transferred to amorphous droplets when the cholesterol became esterified. The appearance of intact lipoproteins within vacuoles separate from the lipid inclusions, resembles the separate transport mechanism for particulate matter and lipid by endothelial cells as described by STILL and PROSSER (1964). This phenomenon may also represent a phagocytotic mechanism of endothelium toward the foreign antigen egg lipoprotein by these cells. CONSTANTINIDES (1968) discusses the phagocytosis of egg lipoproteins by arterial cells following ergocalciferol injury. However, this mode of lipid uptake in the form of intact particles may represent the only mode. Assuming the vacuoles to contain lysosomal enzymes, the lipid could then be broken down and the vacuoles coalesce with others eventually leading to the creation of a lipid droplet. The aggregates of egg lipoproteins in the subendothelial space resemble the aggregates of lipoproteins between endothelium and pericites in experimental xanthoma as demonstrated by PARKER and ODLAND (1969).

Although some egg lipoproteins were observed intact within cytoplasmic vacuoles of endothelial cells, a large fraction also appears to be broken down possibly by a lipase on the endothelial surface. This initial breakdown of the lipoprotein is in line with the experiments of HASHIMOTO and DAYTON (1966) and NEWMAN and ZILVERSMIT (1966). These authors suggested that cholesterol influx into the aorta is independant of lipoprotein transfer. Similarly the protein moiety and the lipid components of lipoproteins appear to have different entrance rates and localization according to the autoradiographic studies of ADAMS *et al.* (1968) and ROBERTSON (1967).

It is curious that in this study lipoprotein particles consisted of ringshaped structures in section with presumably extracted centers, whereas the intracellular lipid droplets, although not extremely dark, were greyish, amorphous, and apparently not extracted. We recall that in a previous study injecting Intralipid into the douply-ligated carotid artery (HOFF and GOTTLOB, 1969), intracellular lipid droplets were far electron-denser than the original Intralipid particles. With the knowledge that lipid containing more unsaturated fatty acids are electron-denser and less prone to extraction by procedures in electron microscopy, it is tempting to speculate that under such hypoxic conditions, there is a general shift of the fatty acid in the cell's metabolic pool to a more unsaturated form. This could occur by unsaturation of the original exogenously derived fatty acid,

or synthesis of new fatty acid. The latter has been shown to occur in atherosclerotic vessels by Whereat and Orishimo (1968). However, these changes in density and structure may be procedural artifacts, that is, the result of lipid extraction during dehydration and embedding.

One of the essential questions in the pathogenesis of atherosclerosis is the mode of entrance of lipoproteins, the physiologic vehicle for lipids, into the arterial wall. A filtration through the interendothelial gaps has been suggested by Duncan *et al.* (1965), Kramsch *et al.* (1967) and Watts (1968) in their tracer, autoradiographic, and immunohistochemical studies. However, since these gaps are no wider than 20 mμ, that is, smaller than the diameter of most β-lipoproteins, one should not expect too much penetration except if the gaps are widened by hypertension. Indeed, Olsen (1968) demonstrated with fluorescent-labelled proteins that penetration occurred primarily in dilated areas of rat arterioles following acute angiotensin-hypertension. Only under special conditions have lipoproteins or lipids been observed within the intercellular gaps, namely in the lymphatics by Casley-Smith (1962) and in capillaries of newly born rats by Suter and Majno (1965). Massive penetration of lipoproteins into the arterial wall at focal points of endothelial necrosis or denudation, as seen in this report, is also possible. Once having passed the endothelial barrier the lipoproteins could travel via the subendothelial space in an axial direction as suggested by Doerr (1963), until they reach areas of focal intimal thickening containing modified smooth muscle cells. These presumably dedifferentiated cells perhaps take up the lipid from the lipoproteins at a faster rate than neighboring cells do, or conversely cannot remove the ingested lipid, thus resulting in an atheromatous plaque. Any one of a number of noxious stimuli could induce such focal endothelial injury such as hypercholesterolemia or vasoactive agents, just to mention two.

Since staining for cholesterol was negative and weak for phospholipids it must be assumed that the amorphous lipid droplets in this study represent primarily triglycerides. The facts that egg lipoprotein contains predominantly triglyceride and that endogenous lipid is synthetized in arteries to phospholipid, particularly in atherosclerotic arteries, (Parker, 1966; Stein and Stein, 1962) suggest that the intracellular lipid droplets are exogenously derived. Furthermore, doubly-ligated arteries injected with Ringer rarely had cells containing lipid droplets. These lipid inclusions in this study appear, if not within, at least closely associated with the cisternae of the endoplasmic reticulum. Geer (1965) suggested that lipid inclusions in smooth muscle cells from canine atherosclerotic arteries, form by the coalescence of smaller cisternae of lipid-containing agranular endoplasmic reticulum. Stein and Stein (1967) using autoradiographic techniques with the electron microscope, observed radioactive free fatty acids within the cisternae of the endoplasmic reticulum in mammary gland alviolar cells a few minutes following intravenous injections in mice, thereby suggesting some relationship between this organelle and lipid uptake. Pinocytotic vesicles are occasionally in contact with cisternae of the endoplasmic reticulum, thus representing a possible entrance route for free fatty acids (Williamson, 1964). Still (1964), however, suggested that the lipid was enclosed in the Golgi apparatus, although these present studies do not confirm this mode of entry. Although we rarely observed lipid within mitochondria, it has been demonstrated in athero-

sclerotic arteries by SMITH *et al.* (1966) in cells cultured from the aortic intima of atherosclerotic-susceptible White Carneau pigeons, and by MURRAY *et al.* (1968) in smooth muscle cells of human and experimental rabbit atherosclerosis. These authors suggested that lipid accumulation in atheroma was a result of injury at the level of the mitochondrion, causing a metabolic blockade that resulted in accumulation of lipid as in fatty degeneration of the liver.

In conclusion it has been shown that when egg lipoproteins are injected into doubly-ligated carotid arteries, the dedifferentiated endothelial and smooth muscle cells accumulate lipid in the form of lipid droplets and as intact lipoprotein particles within phagocytotic vacuoles. Further studies on doubly-ligated arteries following injections of lipoproteins containing higher concentrations of cholesterol would be of interest, particularly in light of the atherogenic effect of this lipid moiety on arteries. Also lipid analysis of the intracellular droplets and a comparison with the composition of the original lipid injected, both with respect to type of lipid and fatty acid ratio of each type, would be of interest. The latter approach might give some insight on the mechanism of uptake and on whether any metabolic shifts have occurred. Such morphological and biochemical studies are presently in progress.

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