Virchows Arch. Abt. A Path. Anat. 347, 1—15 (1969)

Studies on the Pathogenesis of Atherosclerosis with Experimental Model Systems

I. An Electron Microscopy Study of the Effect of Artificial Fat-Emulsion Injections into the Lumen of Doubly-Ligated Rabbit Carotid Arteries

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Received November 4, 1968

Studien über die Pathogenese der Atherosklerose mit experimentellen Modellsystemen

I. Eine elektronenmikroskopische Studie über die Wirkung von Injektionen artifizieller Fettemulsionen in das Lumen der doppelt ligierten Carotis des Kaninchens

Zusammenfassung. Eine experimentelle Atherosklerose wurde am Kaninchen durch Injektionen der künstlichen Fettemulsion Intralipid in das Lumen der doppelt ligierten Carotis des Kaninchens nachzuahmen versucht. Es wurden Schnitte dieser Arterie licht- und elektronenmikroskopisch in der Zeit von 3—20 Tagen nach Ligation studiert. Beim Vergleich zur normalen zeigt die unterbundene Arterie nach 7 Tagen vor allem eine Verdickung der Intima durch einsprossende und proliferierende glatte Muskelzellen und Fibroblasten. Als weiteres Kontrollpräparat diente eine doppelt ligierte Carotis, in die Ringerlösung injiziert wurde. Im Vergleich zu dieser zeigte die Testarterie als hauptsächliche Veränderung Ansammlungen von Fetttröpfchen in allen Zellen der Arterienwand. Diese intracellulären Fetttröpfchen waren elektronendichter als die ursprünglichen Intralipidpartikel, wahrscheinlich infolge der Umwandlung der Fettsäuren innerhalb der Trigylceride in eine ungesättigtere Form. Die Intralipidtröpfchen an der lumennahen Seite des Endothels waren oft viel kleiner als die ursprünglichen, was auf ihren Abbau durch eine Lipase hinweist. Die aus den Lipiden entstandenen freien Fettsäuren dürften von den Endothelzellen aufgenommen worden sein, z. B. durch Vesikel, die rosettenförmige Strukturen aufweisen. Zahlreiche ultrastrukturelle Veränderungen wurden nach Doppelligatur, sowohl mit als auch ohne Fettinjektion beobachtet und mit ähnlichen morphologischen Veränderungen bei spontaner, experimenteller Atherosklerose und anderen Modellen verglichen, die zu Arterienschädigung und Hyperlipämie führen. Unsere Ergebnisse und die Befunde von anderen Arbeitskreisen lassen vermuten, daß die Schädigung der Arterienwand die eigentliche Ursache der Arteriosklerose ist, hingegen scheint die Fettansammlung, wenn sie auch die Morphologie beeinflußt, sekundärer Natur zu sein.

Summary. Experimental atherosclerosis in rabbits was simulated by injecting the artificial fat emulsion Intralipid into the lumen of doubly-ligated rabbit carotid arteries. Sections of these arterial segments were studied with light and electron microscopy at periods from three to twenty days following ligation. When comparing the test vessel with the normal artery, the main alteration was an intimal thickening comprised of proliferating smooth muselc cells and fibroblasts. This hyperplasia was not prominent until the seventh day. The main change when comparing the test artery with a control, doubly-ligated one injected with Ringer solution was the accumulation of lipid droplets within all cells of the arterial wall. At earlier time-intervals these intracellular droplets were more electron-dense than the original Intralipid trigliceride particles, suggesting a metabolic transformation of the fatty acid moiety in the lipid to a more unsaturated form. The Intralipid particles close to the luminal

surface of the endothelia were often much smaller than the original ones, suggesting their breakdown by a lipase; lipid might then be taken up by these cells in the form of free fatty acid, perhaps by vesicles forming rosette-shaped structures. Numerous ultrastructural changes following double-ligation, both with and without lipid, were demonstrated and compared to similar morphological changes in spontaneous and experimental atherosclerosis and in other model systems which combined arterial injury and hyperlipemia. We concluded that our results together with the numerous studies by other investigators suggest that injury to the arterial wall is the primary cause of arteriosclerosis and that lipid accumulation, although influencing the resulting morphology, is only secondary.

In the past years a wealth of information has accumulated demonstrating that the reaction of the arterial wall to injury of various types is not only qualitatively similar to one another (Lorenzen, 1963; Waters, 1954), but resembles to a remarkable degree the non-lipid form of early spontaneous atherosclerosis (Schenk et al., 1966). The similarity between early lipid-containing atheroma and this injury-reaction is further enhanced when hyperlipemia is also induced concurrently with injury (Courtice and Schmidt-Diedrichs, 1962; Constantinides, 1968; Friedman and Byers, 1965; Still and Dennison, 1967; Kirkpatrick, 1967).

Studies on doubly-ligated arterial segments, in particular, have been used by a number of investigators (Buck, 1961; Hackensellner et al., 1965; Friedman et al., 1966) as a model for studies on experimental atherosclerosis. Recently Friedman et al. (1966) combined the hypoxic and mechanical irritation of double ligation of carotid artery segments in rabbits with hyperlipemia. The latter was simulated by injecting suspensions of rat thoracic lymph chylomicrons into the lumen of the doubly-ligated segment. Light microscopic observations of the vessel wall were then made. Friedman et al. (1966) were mainly concerned with the genesis of foam cells which they suggested were derived from endothelial cells. If this model system were to be combined with an electron microscopy study, not only could more information be possibly obtained on foam cell genesis, but ultrastructural similarities with spontaneous and diet-induced atherosclerosis could also be ascertained, as could some details on lipid uptake by arterial cells. The right common carotid artery of rabbits was therefore doubly-ligated and the lumen filled with an artificial fat emulsion. The segments were then studied at various periods of up to twenty days following ligation both with the light and electron microscope.

Material and Methods

Surgical Procedure

The right common carotid artery of ether-anesthetized rabbits (male and female, yellow-silver strain, about four months old, averaging three kgm) was exposed and a two centimeter segment tied off from the remaining circulation. The proximal ligature was applied first so that the doubly-ligated segment would not be filled with blood. The segment was then pierced with a fine hypodermic needle close to one of the ligatures and approximately 0.2 to 0.3 ml of a 20% Intralipid suspension (soybean oil suspension comprised of triglicerides with some phospholipid as an emulsifier, Vitrum, Stockholm) injected into the lumen. In controls the segments were filled with Ringer solution. Another ligature was then applied to prevent leakage through the hole created through the injection. At periods of three, five, seven, ten and twenty days following application of the ligatures, the segments were excised from the anesthetized animals and prepared for electron microscopy. At least two rabbits were used for the test experiment and one for its control in each time sequence studied. The left common carotid artery was investigated in a number of the experiments as a control of a normal unligated artery.

Microscopy

Each segment was cut into several discs and immersed in 5% glutaraldehyde in 0.1 m cacodylate buffer, followed by post fixation in 1% osmium tetroxide — 7.86% 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 I electron microscope with a 50 μ objective aperture.

Results

Light Microscopy

Subendothelial edema (Fig. 1a) and an increase in the extracellular space between medial smooth muscle cells were seen from three to five days following double ligation of the right common carotid artery. One could observe intracellular vacuoles (Fig. 1a), focal necrosis of endothelial cells, and some elastica fragmentation. Cells resembling smooth muscle cells were seen in the intima. All these changes were also found in the ligated arteries injected with Ringer solution. ZOLLINGER (1967) and HACKENSELLNER et al. (1965) also described subendothelial edema in their studies on doubly-ligated arteries. Elastica fragmentation and focal endothelial necrosis were also described by HACKENSELLNER et al. (1965) and FRIEDMAN et al. (1966).

At seven days and later, more extensive intimal thickening could be oberved (Fig. 1b and d) in the test experiments than in the controls. Twenty days after double ligation, the lumen was completely occluded in one artery (Fig. 1c and e). Here, proliferating cells formed around the lipid particles and remaining erythrocytes in the lumen. The intimal thickening was sometimes concentric (Fig. 1d) and sometimes focal (Fig. 1b). One obtained the impression that the thickening occurred around areas of revascularization from vasa vasorum. Occasionally, sections were observed in which rows or spindle-shaped cells were seen as if penetrating a large gap in the elastic membrane and forming a hyperplastic intimal thickening with proliferating smooth muscle cells. It was interesting to observe that most of the endothelia covering those areas away from the thickened intima were either necrotic or completely denuded. This would strengthen the speculation that revascularization keeps the cells in a small vicinity viable and also permits hyperplastic intimal thickening, in an area which is otherwise anoxic. Some new elastica formation was found close to the elastic membrane in very thick intimas (Fig. 1d) in agreement with the ligation experiments of BUCK (1961) and HACKENSELLNER et al. (1965), but in contrast to that of ZOLLINGER (1967). In concentric intima-thickening the same orientation of smooth muscle cells was observed by BUCK (1961) on rats, namely an inner concentric orientation and outer longitudinal one (Fig. 1d). Non-necrotic endothelial cells showed metachromatic staining with alkaline toluidine blue in the form of intracellular lipid droplets presumably triglycerides. At later times almost all cells in the thickened intima contained these droplets. This finding was the most striking difference relative to the controls in which hardly any cells of the thickened intima contained fat droplets.

The left carotid artery never showed any intimal thickening nor lipid accumulation in these studies.

Electron Microscopy

At three and five days following double ligation and before intimal thickening had commenced, the endothelial lining showed patches of necrotic or completely denuded areas. Morphologically normal cells were filled with lipid droplets far

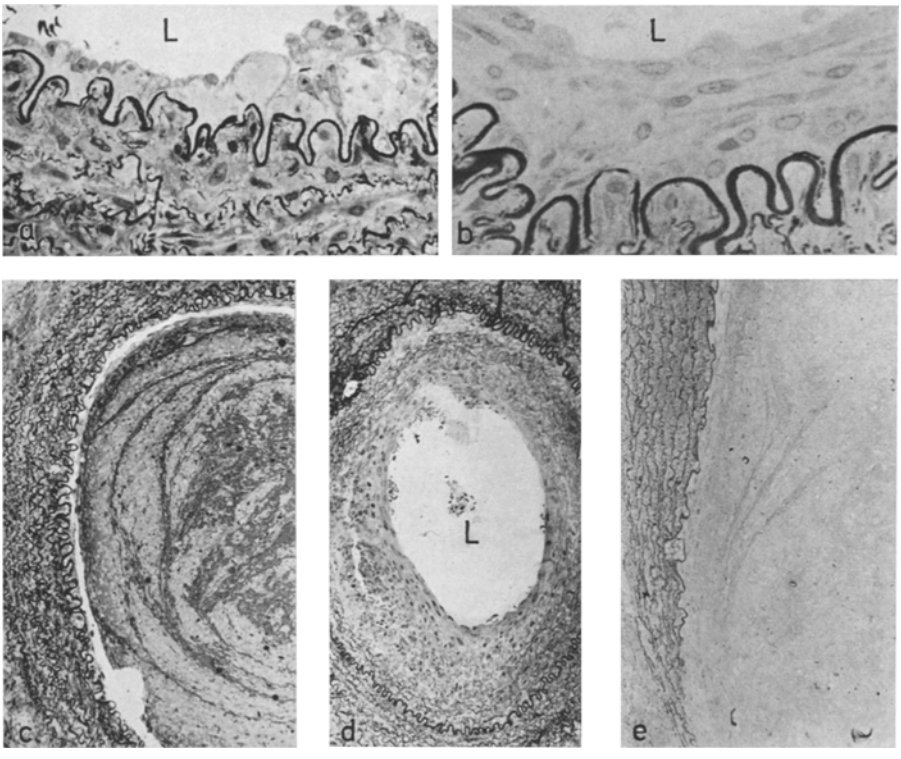

Fig. 1a—e. Light micrographs of the doubly-ligated rabbit carotid artery injected with Intralipid (alkaline toluidine blue). a Artery after 5 days. Note the subendothelial edema, the overlapping of endothelial cells, and the appearance of intracellular lipid droplets. × 200. b Artery after 10 days. Note the thickened intima which was focal in this area. The endothelial lining is intact and the intima is filled with spindle-shaped cells. × 800. c Artery after 20 days. Note the complete occlusion of the lumen which is now filled with proliferating cells that have mingled with the Intralipid fat particles and a few erythrocytes. The small gap between elastica and the occluded lumen is a procedural artifact. × 100. d Artery after 10 days. Note the relatively concentric intimal thickening with an intact endothelial layer, an inner concentric array of spindle-shaped cells and an outer longitudinal array of these cells; and new formation of elastica on the outer edges of the thickened intima. × 100. e Artery after 20 days. Micrograph shows a longitudinal section of an organized thrombus of fat particles and erythrocytes occluding the lumen. × 100

more electron-dense than the original Intralipid particles (Figs. 2b and 3a). These droplets had an even darker rim which was fairly round. In the controls hardly any endothelial cells had such droplets within their cytoplasm. These viable endothelial cells also showed signs of phagocytosis such as autophagic vacuoles (Fig. 2a). The Intralipid particles were often in close apposition to the endothelial cell membrane in scallop-shaped indentations (Fig. 3b), yet never so close as to give the impression of "melting" into the membrane as observed by Schoefl and French (1968) in capillaries of the rat mammary gland. Often the Intralipid emulsions were in the form of smaller particles when close to the endothelial layer (Fig. 2a and b). No lipid could be detected in any organelles such as the membrane invaginations or pinocytotic vesicles which might have lead to

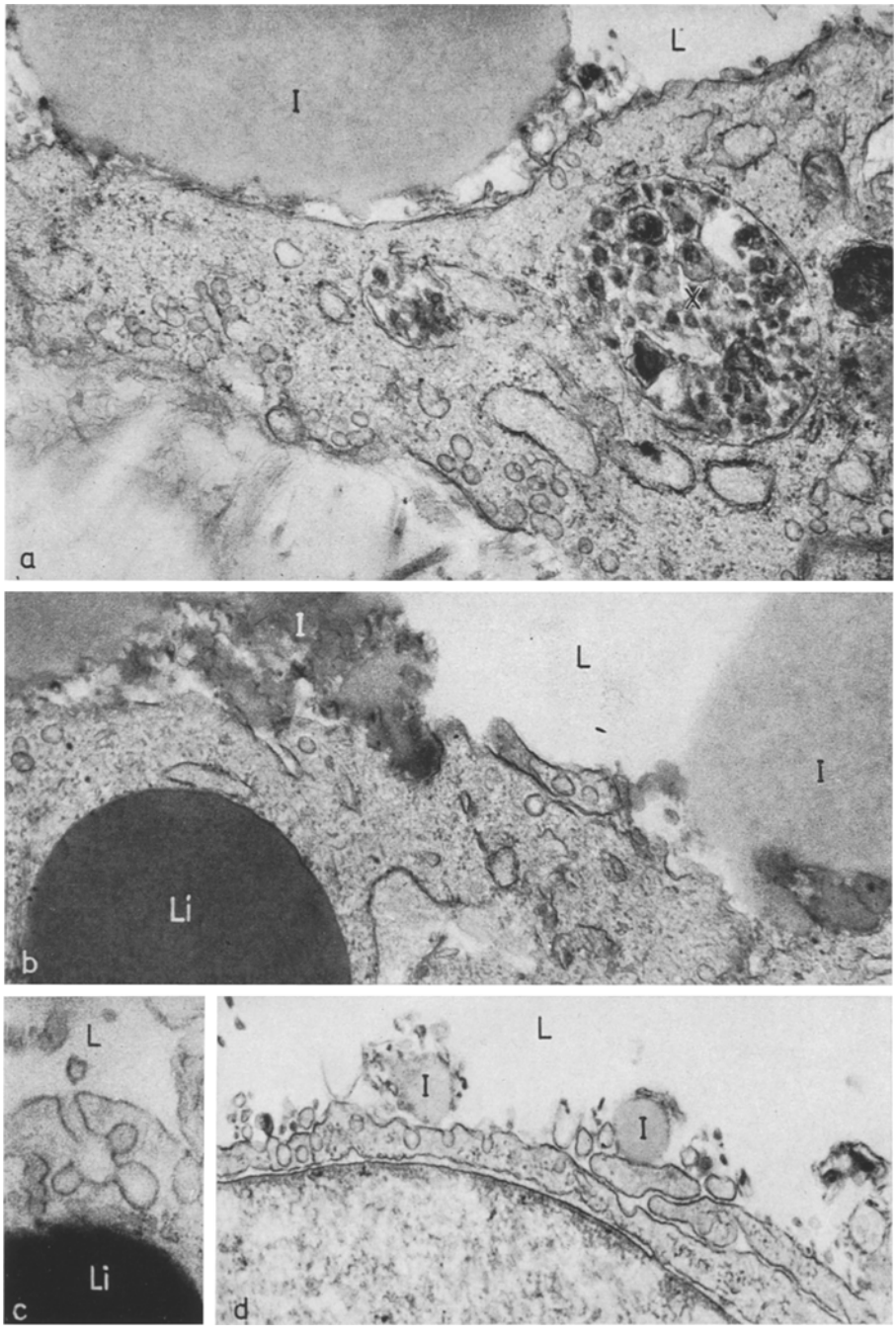

Fig. 2a—d. Electron micrographs of the doubly-ligated carotid artery injected with Intralipid three days after application. Pb stained. a Note the autophagic vacuoles (*X*) within the endothelial cell and the Intralipid particle (*I*) in the lumen (*L*), × 40,000. b A lipid droplet (*Li*) is seen in the endothelial cell. Note that the Intralipid droplets have been broken down when in close contact with the cells' surface. × 30,000. c A rosetta-shaped vesicle formation on the endothelial surface close to an intracellular lipid droplet (*Li*). × 60,000. d Two Intralipid particles can be seen on the endothelial surface which appears ruffled, perhaps forming microvilli. × 30,000

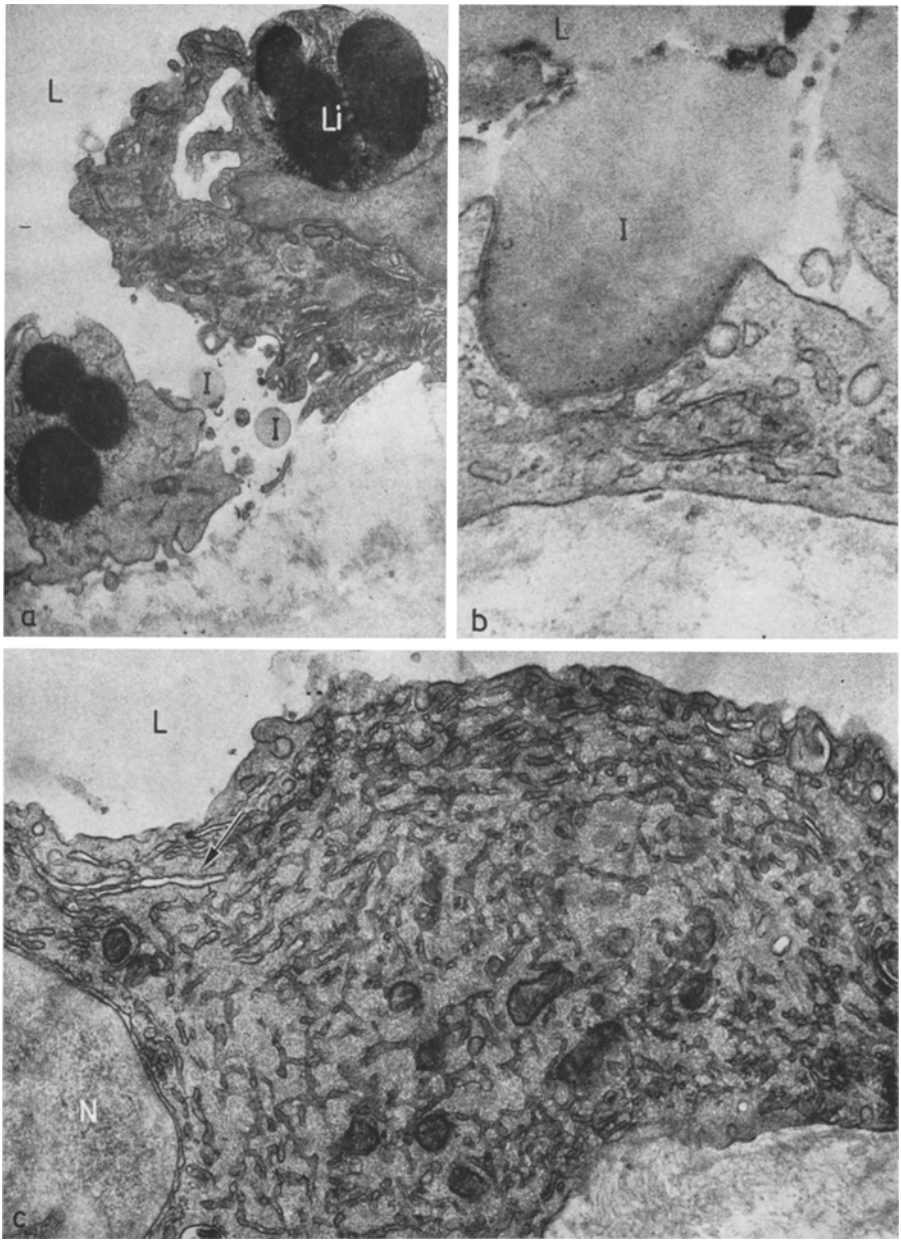

Fig. 3a—c. Same as in Fig. 2. a Note the lipid droplets (*Li*) in the two endothelial cells. These droplets are electron-denser than the Intralipid particles (*I*) seen here in a gap between the endothelial cells. × 15,000. b An Intralipid particle (*I*) in the lumen (*L*) is seen within a crescent-shaped pocket of the endothelial cell. × 65,000. c An endothelial cell is filled with vesicles presumably belonging to the Golgi zone (arrow). The cells cytoplasm is also filled with fibrils. (*N* nucleus). × 28,000

some prediction on the mode of lipid uptake. A number of rosetta formations of vesicles could be seen (Fig. 2c) forming a possible entrance route. The surface of the lining occasionally appeared rough (Fig. 2d) presumably by the formation

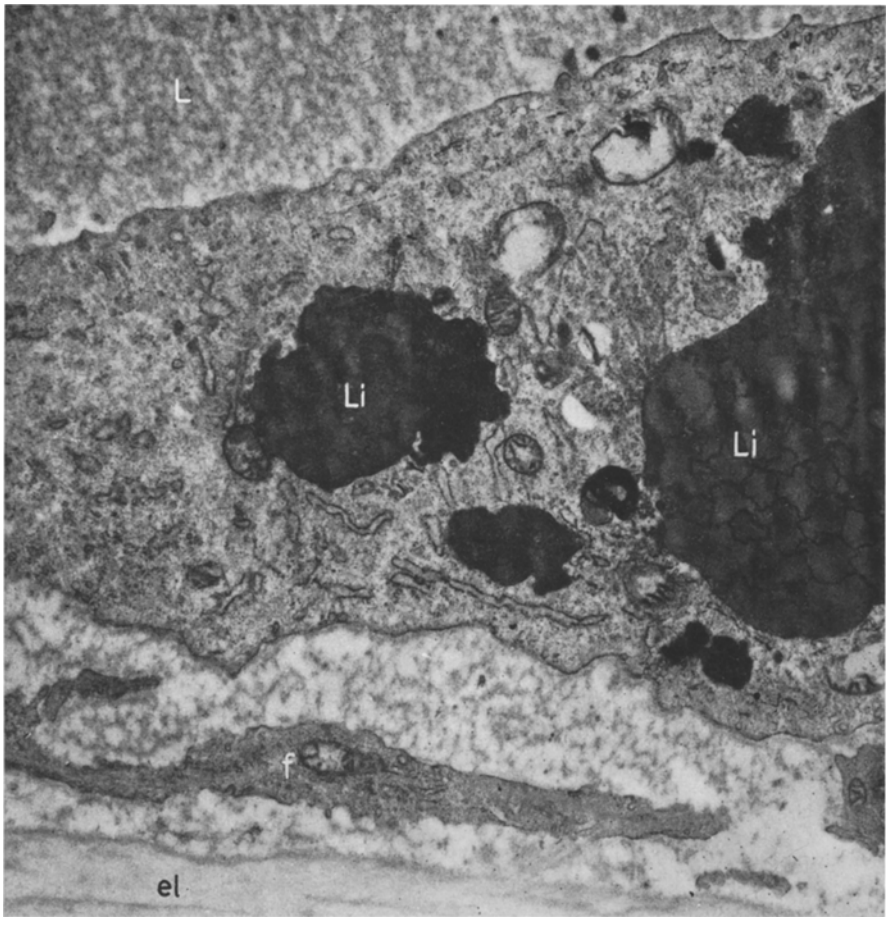

Fig. 4. A swollen endothelial cell 10 days following double-ligation and injection of Intralipid. Note the ruffled shape of the lipid inclusions and the swollen mitochondria. An arm of a fibroblast is seen in the subendothelial space. This micrograph was taken from an area not showing any thickened intima. × 12,000

of microvilli, which could also play a role in lipid uptake. Occasionally in areas of necrotic endothelial cells, large gaps could be seen in which smaller Intralipid particles could penetrate into the arterial wall (Fig. 3a). The few myointimal cells now present in the intima contained lipid also as round electron-dense droplets, as did some medial smooth muscle cells. Occasionally an endothelial cell was filled with vesicles presumably belonging to the Golgi zone (Fig. 3c).

At seven days and later, lipid droplets were seen in almost all cells comprising the thickened intima (Fig. 5). These cells sometimes resembled smooth muscle cells with their myofilaments and basement membrane, and sometimes fibroblasts with their numerous ergastoplasm (Fig. 5). The controls also showed cells without lipid droplets in the thickened intima looking like smooth muscle cells and to greater extent fibroblasts. At later time sequences the endothelial cells resembled phagocytic cells (Fig. 4). They were often squamous-shaped and swollen,

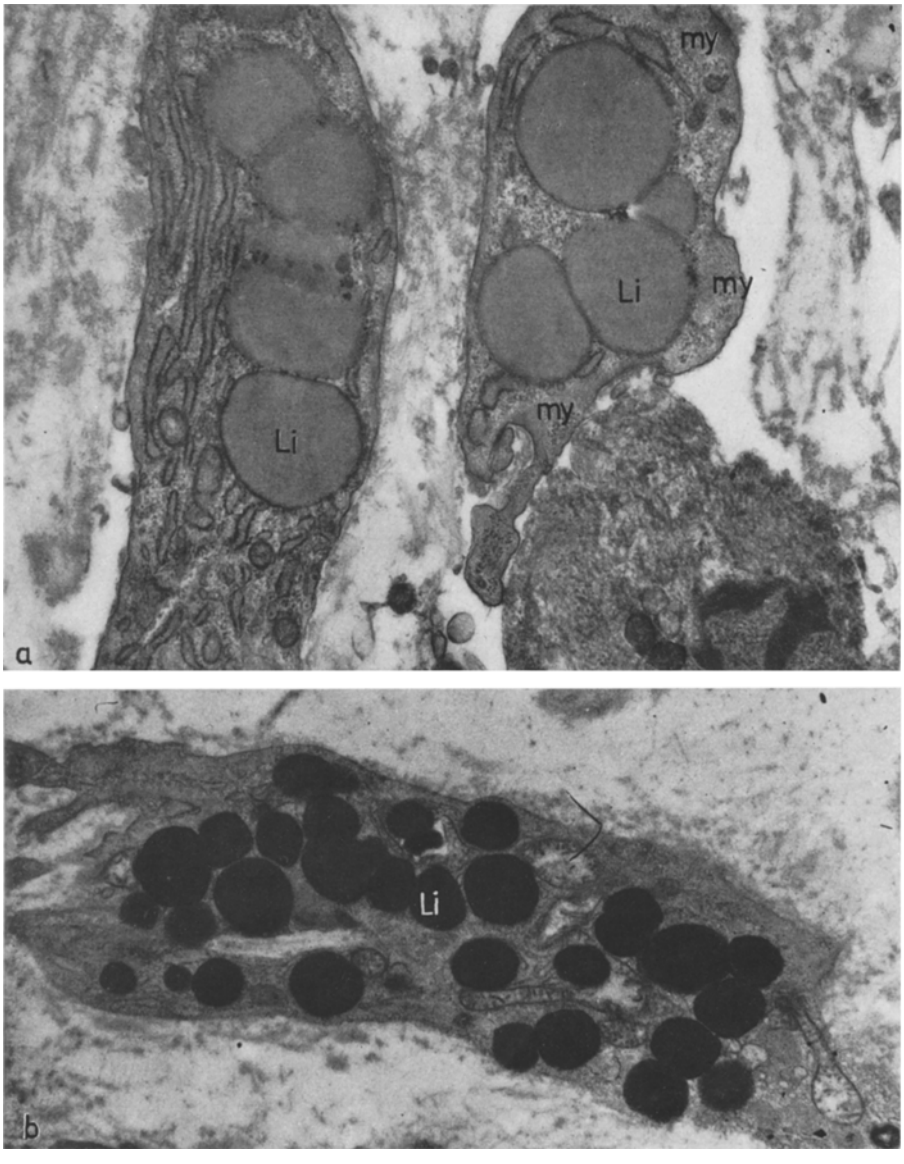

Fig. 5a and b. Cells within the thickened intima 10 days following ligation. a Cells containing moderately dense lipid inclusions (*Li*) with a dark rim. The cell on the left resembles a fibroblast with its abundant rough-surfaced endoplasmic reticulum. The cell on the right still has some remnants of a smooth muscle cell, namely the few myofibrils on its surface (*my*). Note the fragmented elastica in the intracellular space. × 24,000. b A spindle-shaped foam cell full of electron-dense lipid droplets. × 12,000

similar to the cells described by Buck (1961) as monocytes in his ligation study. They contained numerous cell organelles, such as Golgi zones, ergastoplasm and autophagic vacuoles, both in our experimental and control studies. The main difference was the lipid accumulations in the test experiments. These inclusions were, however, scallop-shaped rather than round and diffuse (Fig. 4) and often had

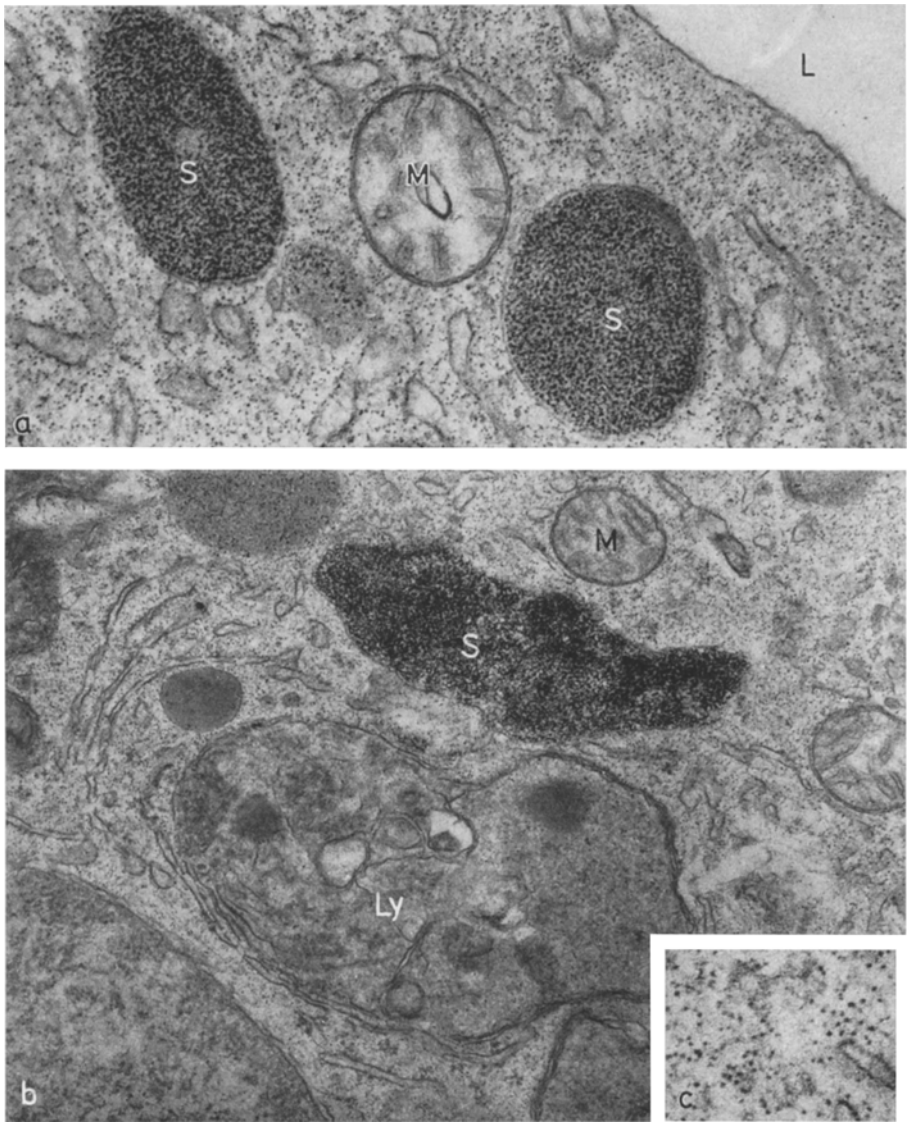

Fig. 6a—c. Artery 20 days after ligature. Ferritin particles are seen in an endothelial cell (a) × 60,000 and a modified smooth cell (b) × 38,000, both free in the cytoplasm and particularly abundant in organelles, presumably siderosomes (*s*). The large autophagic vacuole or lysosome in (b) has even less ferritin than the free cytoplasm. Note the ferritin particles at higher magnification × 100,000 (*M* mitochondria)

light areas which possibly contained saturated lipid extracted during the preparatory procedure. No round foam cells were seen in this study, only spindle-shaped cells full of lipid droplets (Fig. 5b) resembling fibroblasts and modified smooth muscle cells (Fig. 5a). In the extracellular space one could find patches of elastica and dense areas possibly lipid and mucopolysaccharides as was observed by ZOLLINGER (1967) in his ligation study. In one experiment twenty days follow-

ing ligation, ferritin particles could be seen free in the cytoplasm of endothelial and other intimal cells (Fig. 6). The ferritin was particularly abundant in certain organelles probably siderosomes. Buck (1961) described the accumulation of red blood cell degradation products in dense bodies of cells in doubly-ligated arteries. The morphology of ferritin within these cells resembles that of ferritin in glia cells during marginal siderosis of the central nervous system in rabbits following repeated injections of iron-dextran into the cisternae magna (Blinzinger, 1968).

At later times, cells resembling endothelia were found just basal to the endothelial lining, which also contained electron-translucent cells with widened cisternae of the endoplasmic reticulum.

Discussion

In this report an attempt has been made to demonstrate that such a model experiment as double ligation of the carotid artery and injection of an artificial emulsion into the vessel's lumen is a valid system for studies on early atherosclerosis. This is substantiated by the remarkable similarity in morphological alterations (to be described in detail) found in this study, in other studies on injury to arteries, and in experimental and spontaneous atherosclerosis.

The thickening of the arterial intima with proliferating smooth muscle cells is a hallmark of early atherosclerosis both in the experimental variety (Parker and Odland, 1966; Haust et al., 1962) and spontaneous (Schenk, 1966) in rabbits. Various experimental studies producing injury to arteries have resulted in intimal thickening, even without the presence of lipid. This was also found in non-lipid atheroma of rabbits (Schenk et al., 1966). The non-specificity of this injury is illustrated by the wide spectrum of injurous stimuli able to cause this change, such as injections of fibrin thrombi intra-arterially (Rabin et al., 1957), systemic hypoxia (Lorenzen and Helin, 1967), injections of adrenaline and thyroxine (Lorenzen, 1963), arterial stenosis (Kunz et al., 1966), injury through freezing (Gage et al., 1967), experimental hypertension in pulmonary arteries (Esterly et al., 1968), stretching of the artery with a balloon catheter (Baumgartner and Studer, 1966) and following double ligation (Buck, 1961, Hackensellner et al., 1965, Friedman et al., 1966, and Zollinger, 1967). Waters (1954) and Lorenzen (1963) have often commented on the uniformity of the injury pattern in arteries.

A number of investigators have obtained morphological changes similar to early atherosclerosis, in particular intimal thickening, resulting from a combination of arterial injury and hyperlipemia. The forms of injury varied from mechanical (Still and Dennison, 1967, Friedman and Byers, 1965), chemical (Packham et al., 1967), pharmacological (Constantinides, 1968), heat (Courtice and Schmidt-Diedrichs, 1962), to irradiation (Kirkpatrick, 1967). Both this report using the electron microscope and the double ligation experiment of Friedman et al. (1966) further illustrate, that by combining an unspecific injury to the artery with hyperlipemia or hypercholesterolemia, one can create a morphological picture in many ways identical to experimental and early human atherosclerosis. Ssolowjew (1930), already over thirty years ago, suggested that injury together with hyperlipemia brought about atherosclerosis.

A number of specific changes found in this present study resemble the pattern found in atherosclerosis and in other experimental models. The intracellular lipid inclusions in this report looked like those found in atherosclerosis (GEER, 1965; THOMAS et al., 1963; STILL and MARRIOTT, 1964). For instance the intraendothelial lipid droplets as seen by GEER (1965) in canine atherosclerosis resembled those lipid inclusions seen in the endothelia after ten days in this study. Endothelial phagocytosis has been demonstrated following injury plus lipid infusions (CONSTANTINIDES, 1968) and in atherosclerosis (SINAPIUS, 1957; POOLE and FLOREY, 1958). The appearance of ferritin at a later time sequence in this study may be a manifestation of phagocytosis of blood cell degradation products, as was observed in atherosclerotic vessels (SINAPIUS, 1957), with the consequent metabolic conversion to ferritin. Subendothelial edema is another alteration observed here and also following arterial injury to the rabbit femoral artery through ergocalciferol (CONSTANTINIDES, 1968) and in atherosclerosis (DOERR, 1963). Also a greater array of Golgi zones in endothelial cells was seen as in the rat aorta after lipidosis (HESS and STAUBLI, 1963), in porcine atherosclerosis (IMAI and THOMAS, 1968) and in human atherosclerosis (GHIDONI and O'NEAL, 1967). Microvilli on the luminal surface of the endothelia as seen in this present study have also been reported in short- and long-term studies of mechanical and chemical irritation of the rabbit aorta (HOFF and GOTTLOB, 1967, 1968a) and on atherosclerotic arteries (POOLE and FLOREY, 1958; IMAI and THOMAS, 1968). Other changes seen in this study were possible accumulations of ground substance, which were also observed in doubly-ligated arteries (ZOLLINGER, 1967), in experimentally induced arteriosclerosis with injections of adrenaline and thyroxine (LORENZEN, 1963), and in bovine atherosclerosis (KNIERIEM, 1967). Extracellular accumulations of lipid have been reported in canine atherosclerosis (GEER, 1965), in human atherosclerosis (GHIDONI and O'NEAL, 1967), and after combining mechanical irritation with hyperlipemia (BAUMGARTNER and STUDER, 1966). We have observed focal overlapping of endothelial cells and electron-translucent endothelial cells with widened cisternae of the endoplasmic reticulum in this study. These observations confirm those seen by HOFF and GOTTLOB (1968a) after mechanical and by CONSTANTINIDES (1968) after ergocalciferol-induced injury, and by IMAI and THOMAS (1968) in atherosclerosis. This overlapping suggests endothelial mitosis resulting from hypoxic and mechanical injury induced by double ligation. This was substantiated by the autoradiographic studies using tritium labelled thymidine on stenosed arteries (KUNZ et al., 1967) and on mechanically injured arteries (SPAET and LEJNIEKS, 1967). Endothelial mitosis has also been reported by DAOUD et al. (1968) on porcine atherosclerotic arteries. The same configuration of smooth muscle cells in concentric intimal thickenings seen in this investigations was observed in the studies of IMAI and THOMAS (1968

Aside from the many similarities in morphological alterations in this study and in experimental and spontaneous atherosclerosis, two other interesting aspects have been touched on. The first involves the etiology of foam cells. We have failed to observe the round foam cells found so often in experimental atherosclerosis of rabbits and believed to be derived from blood monocytes (STILL, 1963). STILL (1967) was able to observe the diapedesis of monocytes into the subendothelial space of the rabbit aorta in experimentally induced hypertension. In this

report these cells ladened with lipid, appeared to be modified smooth muscle cells or fibroblasts. Parker and Odland (1966) suggested that foam cells were derived from such smooth muscle cells. Friedman and Byers (1965) and Friedman et al. (1966) postulated that foam cells were derived from endothelial cells, and strengthened their theory by demonstrating such foam cells in doubly-ligated segments, in which no monocytes could have entered, except via capillaries formed during revascularization. Furthermore they showed that when the endothelium was denuded, no foam cells appeared. We feel that the discrepancy between the various groups lies first in the definition of the morphology for foam cells, that is, whether round or spindle-shaped foam cells are meant, and secondly in the inability of the light microscope to make a valid identification of these cells. Our studies also demonstrated endothelial cells full of lipid droplets but which could be differentiated from the round foam cells described by Imai et al. (1966) in rabbit atheroma. We feel that the spindle-shaped foam cells in this report are derived from proliferating smooth muscle cells which have dedifferentiated into fibroblasts (Wissler, 1968, Murray et al., 1966, Zollinger, 1967).

The other topic to which this report might have contributed, is in the general problem of lipid uptake by cells, and more specifically by the endothelial lining of blood vessels. As was briefly described in a preliminary report (Hoff and Gottlob, 1968b) the intracellular accumulations of lipid in the form of droplets are more electron-dense than the original Intralipid particles. It had been suggested that this result implied a metabolic shift of the lipid by the cells of the artery to a more unsaturated form. Osmium tetroxide reacts with the double bonds in the fatty acid moiety to give a darker-staining fat (Bahr, 1954). Schoefl (1968) observed that when Intralipid or chylomicrons are fixed and stained with osmium tetroxide *in vitro*, a darker rim is formed around the particles. This, however, was not the case following injections of these lipids *in vivo* Schoefl and French, 1968). Our present observations confirm these results. We had suggested (Hoff and Gottlob, 1968b) that perhaps the endothelial cells preferentially take up phospholipids (stated by Schoefl to form the outer surface of these fat particles), thereby explaining the disappearance of the dark rim in our experiments. This, however, seemed unlikely since the phospholipids would most likely form myelin figures in the aqueous medium of the cell's interior. Furthermore, the diffuse texture of the droplets, at least at earlier time sequences, resembles pure triglycerides.

The mode of lipid uptake by endothelial and smooth muscle cells could not be clearly elucidated in this report. The presence of smaller Intralipid particles close to the endothelial surface suggets that a lipase on the surface of these cells attacks the lipid. It is curious that the lipid was often in close contact with the cell surface within crescent-shaped indentations. Yet, we failed to find the "melting"of the lipid into the cell membrane as was observed by Schoefl and French (1968). Microvilli formation on the endothelial surface might enhance lipid uptake by increasing the surface area. Free fatty acids possibly released by the action of lipase on the fat emulsion, but not visible with the electron microscope, could then enter the cell by some mechanism still unknown. Williamson (1964) suggested from his studies on lipid transport between the circulation and adipose tissue, that the free fatty acids, released on the endothelial surface, are taken up by the fat cells via pinocytotic vesicles which form rosetta-formed structures, also

seen in this report. In this way the non-esterified fatty acid could be transferred into the cell interior by passing from one vesicle to another. In support of an energy-linked transport by these vessels is the finding of an ATPase, localized within these organelles of rabbit aortic endothelial and smooth muscle cells (HOFF and GRAF, 1966; HOFF, 1968). Very small lipid droplets or lipoproteins have been observed in pinocytotic vesicles of endothelial cells in lymphathics (CASLEY-SMITH, 1962). Chyle has been seen within invaginations of endothelial cell membranes in capillaries of newly born rats (SUTER and MAJNO, 1965). 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. A mode of lipid uptake could be postulated if a link were found between the rosetta-shaped vesicle formations and these cisternae. Yet this entire mechanism is still highly speculative, since one cannot clearly establish whether the free fatty acids are in these vesicles.

In conclusion it has been shown that a model system combining the techniques of experimental pathology and electron microscopy can be used to obtain valuable information on such questions as lipid uptake by cells and on the pathogenesis of atherosclerosis. On this latter point the results of these experiments, together with numerous studies of other observers, suggest that the primary cause of atherosclerosis is an irritation of the arterial wall, as suggested over a century ago by VIRCHOW (1856). Since the intima and subintima are close to the limiting diffusion distance for oxygen (WHEREAT, 1967), they are particularly sensitive to any changes in their enviroment which might reduce their oxygen uptake, such as microthrombi formation, thereby leading to focal necrosis. The necrotic or injured areas are apparently more permeable to macromolecules than normally (PACKHAM et al., 1967) as is the case in atherosclerotic arteries (DE BRUJN and SCHORNAGEL, 1967). The cells also engulf lipid in greater amounts. Clearly, neither the combination of hypoxia and mechanical injury used in this study to simulate this unspecific irritation occurring *in vivo*, nor the simulation of hyperlipemia with an artifical fat emulsion, is physiological. Yet, the fact that the results obtained are so similar to atherosclerosis makes one wonder what noxious stimuli really occur *in vivo*. IMAI et al. (1966) made the interesting suggestion that hypercholesterolemia in itself injures the arterial endothelial lining, leading to the secondary permeation and uptake of this lipid moiety. Other possible irritants are hypertension, turbulent blood flow, direct or indirect nervous stimulation and hormonal changes.

Acknowledgments. The authors wish to thank Prof. DAHME and Dr. MENAHAN for their helpful suggestions in the preparation of this manuscript, and Dr. BLÜMEL, Miss SCHELLHAAS and Miss LÖBBECKE for technical assistance. Dr. HOFF is a recipient of an Advanced Research Fellowship of the American Heart Association.

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