Virchows Arch. Abt. A Path. Anat. 351, 179—192 (1970)

Studies on the Pathogenesis of Atherosclerosis with Experimental Model Systems

III. An Ultrastructural and Lipid-Histochemical Study on the Effect of Injections of Physiologic Saline into the Lumen of the Doubly Ligated Rabbit Common Carotid Artery

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Received June 18, 1970

Summary. The common carotid artery of the rabbit was doubly ligated and Ringer's solution was injected into the lumen; the changes produced in the vessel were studied by lipid histochemical techniques and electron microscopy from one to seventeen days. A characteristic change was the appearance of intracellular lipid droplets spread diffusely throughout the arterial wall although physiologic saline was present only in the lumen. These droplets of lipid were present in twenty-five percent of the cells one day after ligation, reached a maximum of forty-five percent three to seven days after ligation, then decreased later. Phagocytic or autophagic vacuoles were found, especially within endothelial cells. Intimal thickening comprised predominantly of proliferating smooth muscle and endothelial cells was observed later. The relevance of this study as a control for similar double ligation studies, in which lipid substrates were injected rather than a physiologic saline, is mainly dependant on the pattern of localization and distribution of the intracellular lipid droplets.

Zusammenfassung. Die Doppelligatur der A. carotis communis des Kaninchens mit anschließender Injektion von physiologischer Ringer-Lösung in das Lumen, führte zu einer Reihe von Veränderungen, die nach ein bis 17 Tagen nach Ligatur mit Hilfe lipid-histochemischer und elektronenmikroskopischer Techniken untersucht wurden. Eine charakteristische Veränderung bestand in dem Auftreten intracellulärer Lipid-Tröpfchen, die in der Arterienwand diffus verteilt waren, obwohl nur physiologische Salzlösung in dem Lumen vorhanden war. Einen Tag nach Ligatur fanden sie sich in 25% der Zellen, erreichten ein Maximum von 45% nach 3—7 Tagen und gingen danach wieder zu niedrigeren Werten zurück. Hetero- oder autophagocytotische Vacuolen waren in erster Linie in Endothelzellen zu beobachten. Zu einem späteren Zeitpunkt trat eine Intimaverdickung auf, die vor allem auf eine Proliferation glatter Muskelzellen und Endothelzellen zurückzuführen war.

Die Bedeutung derartiger Untersuchungen als Kontrollversuch zu ähnlichen Doppelligatur-Untersuchungen, bei denen Lipid-Substanzen anstelle von physiologischer Salzlösung injiziert worden waren, besteht darin, daß die Lipid-Tröpfchen eine unterschiedliche Lokalisation und ein anderes Verteilungsmuster zeigten.

Introduction

It has been shown that when an arterial segment is isolated from its oxygen and nutrient supply by the application of two ligatures, the lumen of this segment becomes reduced as the result of intimal cell hyperplasia. This intimal thickening following double-ligation has been documented by Sokoloff (1893), Malyschew (1929), Mehrotra (1953), Williams (1956), and Zollinger (1967) using light microscopy, and by Buck (1961), Hackensellner, David and Uerlings (1965)

and Hoff and Gottlob (1969a, b) using electron microscopy. The latter studies have been particularly useful in identifying the proliferating intimal cells as smooth muscle cells originating from the media. Intimal thickening is also associated with the early stage of atherosclerosis (Wissler, 1968; Parker and Odland, 1966; Knierem, 1968). Further similarities between the disease and this model (double-ligation) have been demonstrated which recommended this system for studies on atherosclerosis (Friedman and Byers, 1965; Hoff and Gottlob, 1969a, b). However, while *in vivo* intimal hyperplasia is initiated by any one of a number of noxious stimuli (hypertension, turbulence etc.) (Dock, 1967; Esterly, Glagov and Ferguson, 1968), in this system it is caused by hypoxia (Hoff and Gottlob, 1969a, b). This model has already been used extensively to investigate the effects of various stimuli on arteries. For example, lipids or lipoproteins have been injected to simulate hyperlipemia (Friedman *et al.*, 1966; Hoff and Gottlob, 1969a, b). In the latter studies injection of physiologic saline (Ringer's) acted as a control.

This present detailed report on the morphologic alterations following such control studies was made for several reasons: First, no illustrations of the saline injected control artery had been included in our previous reports. Second, none of the previous studies by other authors had described the morphologic changes following such injections. Finally, previous studies done in this laboratory on saline injected arteries demonstrated the presence of some intracellular lipid droplets. Since these lipid droplets also represent the most characteristic change following lipid or lipoprotein injections into this experimental system, it was expected that a semiquantitative and morphologic study of the cells containing lipid droplets following saline injections would provide information relevant to the origin of these droplets.

Materials and Methods

Rabbits of mixed strain and of either sex, averaging three kg were anesthetized with pentobarbital. The right common carotid artery was exposed and a double ligature applied as previously described (Hoff and Gottlob, 1969a). A physiologic saline solution (Ringer's) to which 10 mg% carbon black (Günther Wagner, Hannover, West Germany, Batch no. c 11/1431 a) was added, was injected into the lumen of the ligated artery, prior to application of the distal ligature. With this procedure all blood is flushed out of the ligated segment. At periods of one, two, three, seven, eleven and seventeen days following ligation, the ligated segment was excised and prepared for lipid histochemistry and electron micoscopy. The contralateral carotid was used as a control. Blood was permitted to remain in one of the segments studied (eleven days). Each segment was divided into several samples, some of which were immersed in Baker's Ca-formol, frozen sections cut, and stained with oil red 0, OTAN, (to differentiate between neutral and polar lipids) (Adams, 1967), and PAN (to demonstrate cholesterol) (Pearse, 1968). Other segments were fixed 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. $^1/_2$ 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.

An average of five sections ($^1/_2$ micron thick toluidine blue-stained Araldite sections) from different planes of each ligated segment were used for a semiquantitative determination of percentage cells containing lipid droplets using light microscopy. Approximately 1000 cells were counted per section. All cells containing at least one lipid droplet (seen as yellow-green colored vacuoles) were tabulated.

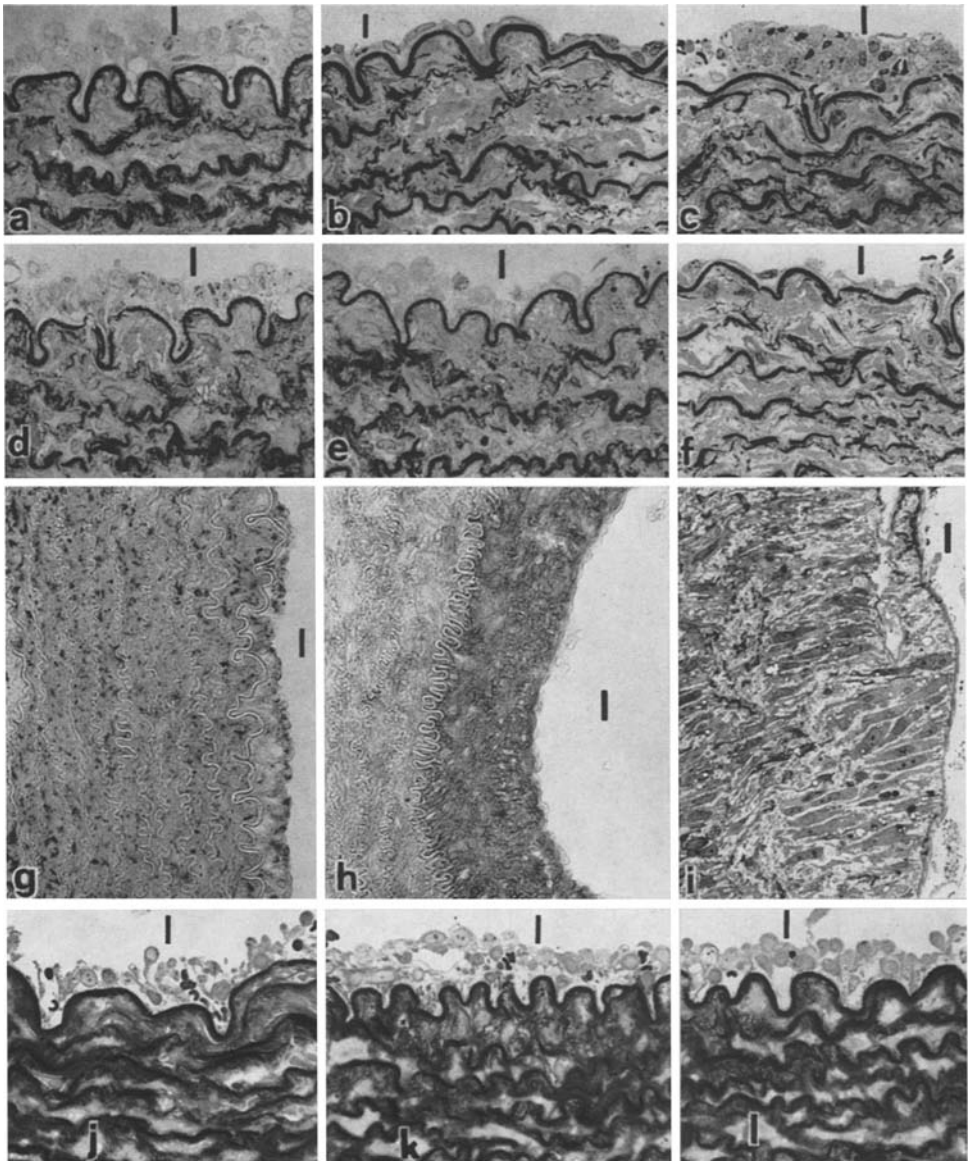

Fig. 1 a–l. Light micrographs of sections of the doubly ligated rabbit carotid artery. a–f Toluidine blue-stained $^1/_2$ micron-thick Araldite sections three days after ligation. Note the small dots in the endothelial lining (a and b) and the cells in the subendothelial space (c). ×320. g OTAN-stained frozen section of artery seven days after double ligation. Note the fairly numerous dark granules indicating droplets containing neutral lipid. ×200. h OTAN stained frozen section of artery seventeen days after double ligation. Note the complete lack of stained lipid in the thickened intima or the media. ×200. i–l Toluidine blue-stained $^1/_2$ micron-thick Araldite sections. i Seven days after ligation. Note intracellular lipid droplets seen as clear vacuoles. ×320. j, k and l Artery filled with blood eleven days after ligation. Note blood cells in subendothelial space. ×320. *l* lumen

Results

Intracellular lipid droplets were found in both endothelial and smooth muscle cells at all time intervals studied following ligation of the saline-containing carotid artery. They were uniformly distributed throughout the media, but more focally in the intima. On the average only about two lipid droplets were found per cell. As can be seen in the graph (Fig 8), by one day after ligation, twenty-five percent of all cells present in the intima and media contained lipid droplets (appearing as clear vacuoles in Fig. 1i). The percentage of lipid droplet-containing cells reached a maximum between three and seven days after ligation and then declined thereafter. The number of lipid droplets in the nonligated artery was negligible. The fact that mainly non-polar lipid was stained using the OTAN method (black granules in Fig. 1g) while the PAN staining for free or esterified cholesterol was negative, implies that these lipid droplets were comprised mainly of triglycerides. At later time intervals (seventeen days) hardly any lipid droplets were found (Fig. 1h), especially in the thickened intima. Ultrastructurally, lipid droplets were seen as grey-amorphous masses usually surrounded by a darker rim which was sometimes crenated (Fig. 5). Occasionally myelin-type figures were found within such droplets suggesting the presence of some phospholipid (Fig. 3b). Lipid droplets in endothelial and smooth muscle cells occasionally appeared to have a localization within the cisternae of the rough-surfaced endoplasmic reticulum (Fig. 3b), while those in smooth muscle cells usually had a perinuclear localization free in the cytoplasm and grouped together with other organelles (Fig. 5).

During the earlier time intervals studied, the non-necrotic part of the endothelial lining contained dark organelles (Fig. 1a and d) shown ultrastructurally to be vacuoles incorporating lumen-injected carbon black particles (Figs. 2c, 3b). Such carbon particles were also seen extracellularly in the edematous subendothelial space (Fig. 2c). The endothelial cells were full of membrane-bound organelles containing material of various electron densities (Figs. 2a and b, 3a), perhaps autophagic or phagocytic vacuoles. Lysosome-like bodies were also found in both endothelial and smooth muscle cells (Fig. 4b and c). Endothelial cells often has a rounded appearance (Figs. 1a and d, 4) and overlapped one another (Fig. 1a). Occasionally groups of cells resembling monocytes were seen in the subendothelial space (Fig. 1c) as in experimental hypertension (Still, 1967), most notably, when blood was permitted to remain in the lumen (Figs. 1j–l, 4). These cells were often rich in lipid droplets and resembled foam cells.

In these series of experiments concentric intimal thickening was found seventeen days following ligation. The cells on the lumen side were particularly

Fig. 2a–c. Electron micrographs of sections of the doubly ligated rabbit carotid artery three days after ligation. a and b Endothelial lining contains large membrane-bound bodies (small arrows) presumably either phagocytotic or autophagic. c Lumen-derived particles of carbon black can be seen in an intraendothelial vacuole and in the subendothelial space *ss* (large arrowhead). Note that no carbon particles are seen within the membrane-bound structure (*P*) which is assumed to be a section through a pseudopod. a ×20000, b ×17000, c 28000. *l* lumen

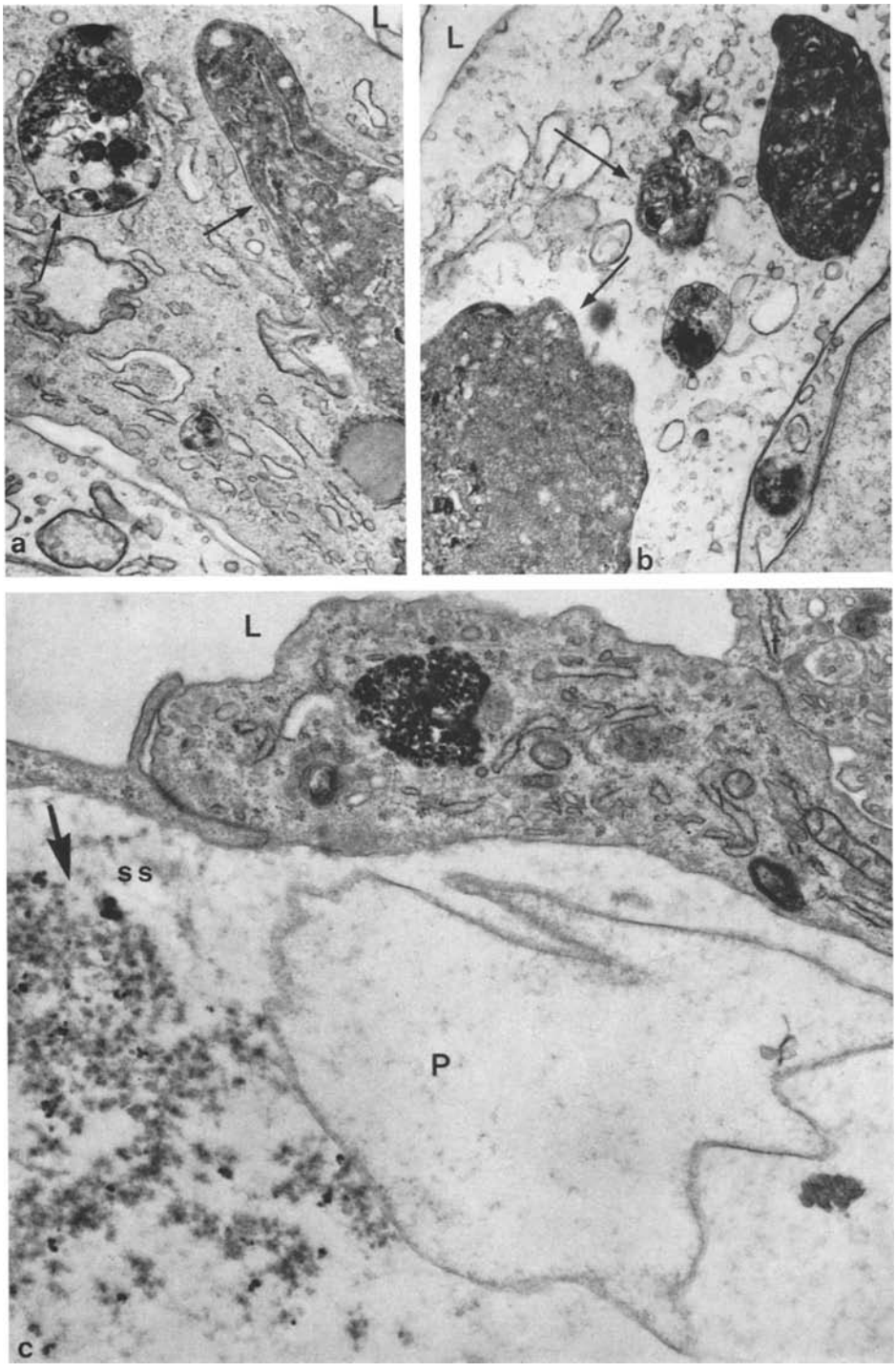

Fig. 2 a–c

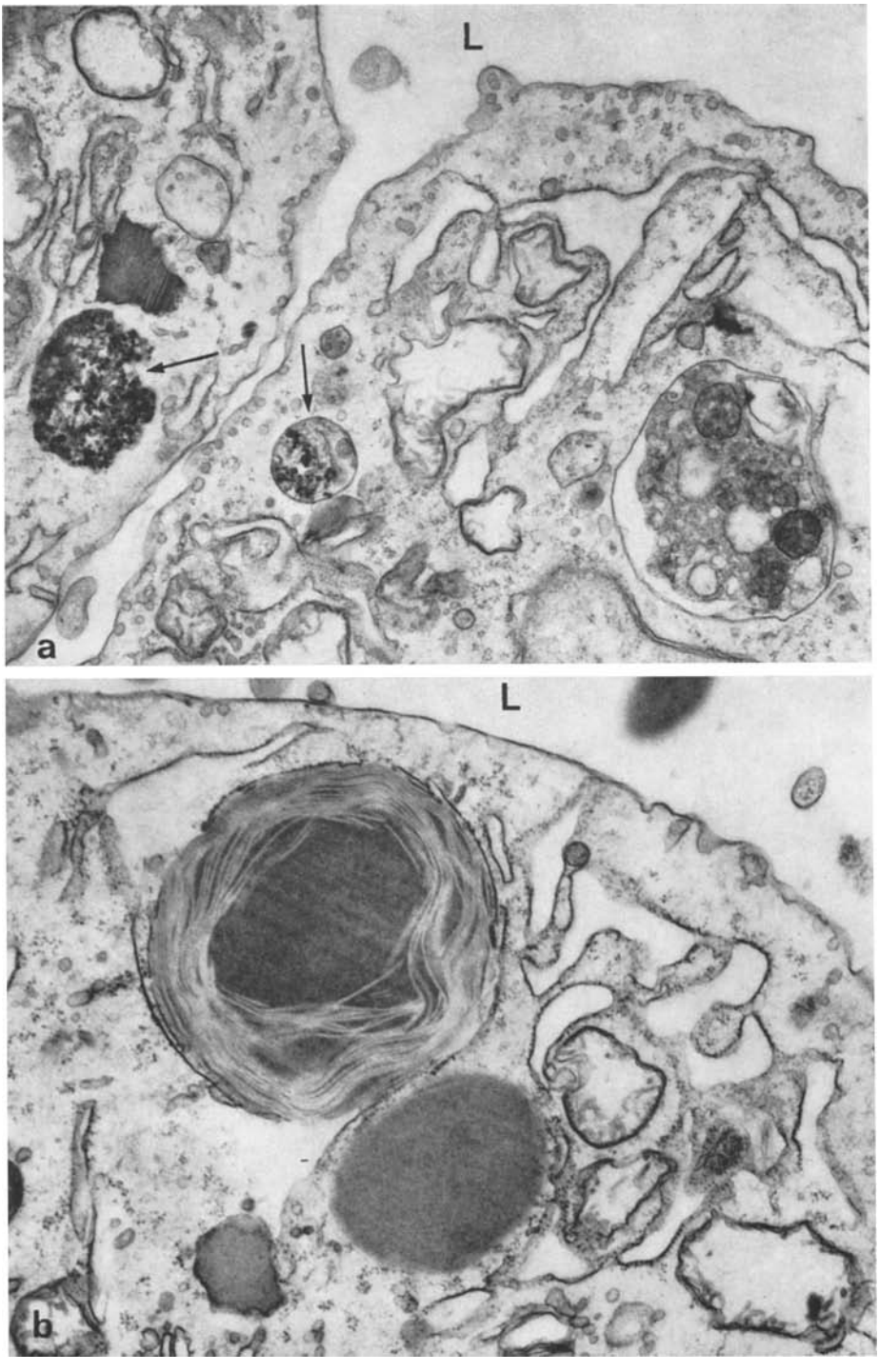

Fig. 3 a and b

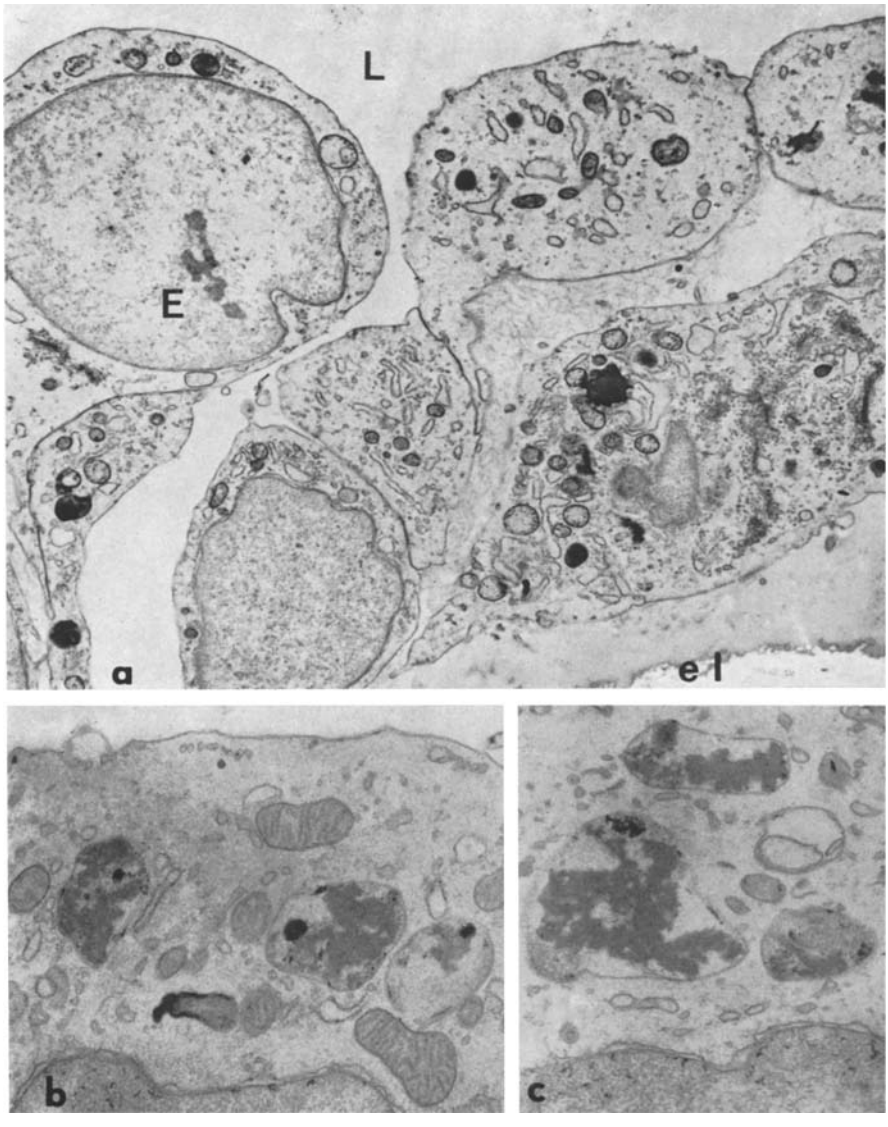

Fig. 4a-c. Electron micrographs of sections of the doubly-ligated rabbit artery filled with blood eleven days after ligation. a Intima comprising of rounded endothelial cells (*E*) and subendothelial cells of unknown origin. ×8000. b an endothelial cell and c a smooth muscle cell containing lysosomal-like bodies. b 16000, c 20000

Fig. 3a and b. Same as Fig. 1. a Note vacuoles in endothelial cells containing lumen-derived carbon particles (arrows). ×28000. b Note the lipid droplets within an endothelial cell. The large droplet also contains myelin-type structures resembling negative staining. The droplet appears to be within the rough-surfaced endoplasmic reticulum. ×28000. *l* lumen

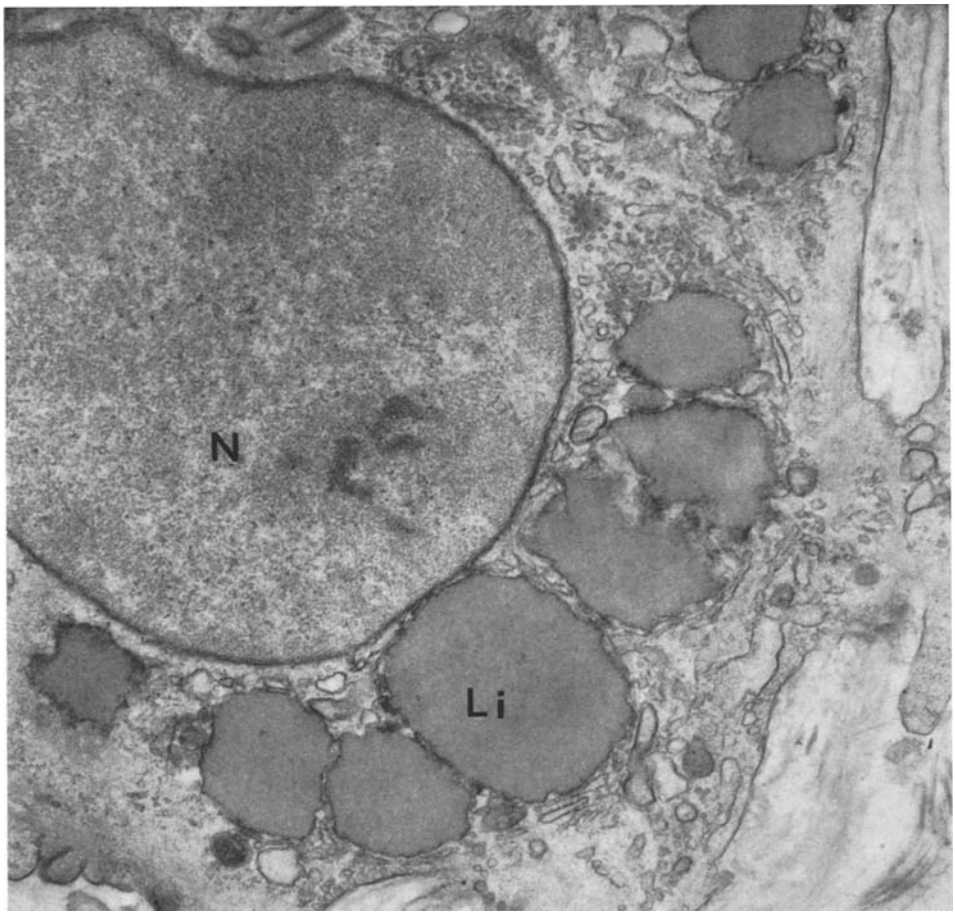

Fig. 5. Same as Fig. 4. A typical intimal smooth muscle cell containing numerous perinuclear-localized lipid droplets (*Li*). ×16000

swollen and were closely apposed basally to cells with the same general appearance (Figs. 6, 7). Their cell membranes had very few invaginations (Fig. 7). The surface cells had very long intercellular gaps and pseudopods were often seen extending from these cells into the lumen (Fig. 6b). Some lysosome-like bodies were present in the surface cells, although not in the abundance observed at earlier time intervals in the endothelial lining. Smooth muscle cells were seen to have various orientations between the elastica interna and the several-layer-thick-aggregate of swollen surface cells; namely radially close to the elastica and concentrically in the middle intima (Fig. 6c). Between the middle intima and the swollen cells was a group of spindle-shaped cells of unknown origin and foam cells (Fig. 6d) which were also seen in the edematous areas close to the elastica (Fig. 6c). Only these sparsely localized foam cells contained lipid in the thickened intima. Cells resembling fibroblasts were often seen in the intima of arteries in

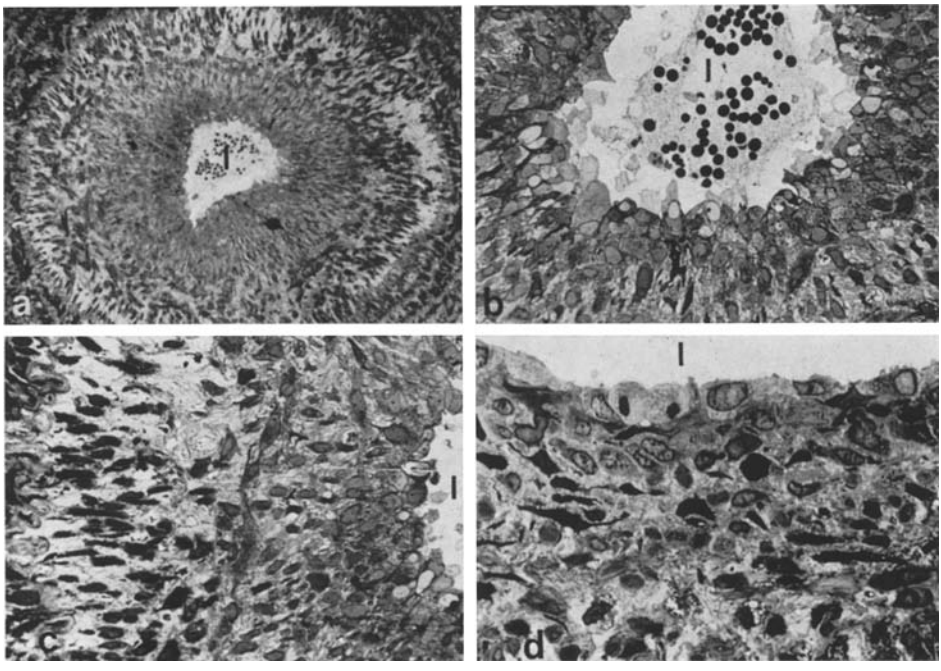

Fig. 6a–d. Light micrographs of toluidine blue-stained $^1/_2$ micron-thick Araldite sections of the rabbit carotid artery seventeen days after ligation. a Concentric intimal thickening of media-derived cells almost obliterates the lumen. ×80. b Note that the cells close to lumen are all rounded, closely apposed, and many appear to be sending pseudopods into the lumen. A thrombus can be seen in the center of the lumen. ×320. c Note the various orientations of cells in the thickened intima. On the left near the internal elastic membrane are radially-oriented cells; then come a group of concentrically-oriented cells, and finally near the lumen come a group of rounded cells several layers thick, resembling the endothelial cells. ×320. d Note the rounded cells of the endothelial lining closely apposed by cells several layers thick. A few lipophages can be seen in the basal layers. Very little lipid is found at this time sequence. ×410. *l* lumen

which the intimal thickening was less oriented, especially in organized thrombi containing capillary sprouts. In general, very little elastogenesis occured in the thickened intima.

Discussion

The double-ligation of arteries injected with lipids has been proposed as a model for studies on atherosclerosis (Friedman and Byers, 1965; Hoff and Gottlob, 1969a, b) and the effect attributed to the lipid injections was distinguished from that of ligation alone (Hoff and Gottlob, 1969a). The present report gives a detailed description of saline-injected arteries, also previously used as controls. Intracellular lipid droplets, noted to be in abundance in earlier studies following lipid injections, surprisingly appeared also following saline injections (Hoff and Gottlob, 1969a, b). The semiquantitative determination of intracellular lipid droplet formation in this

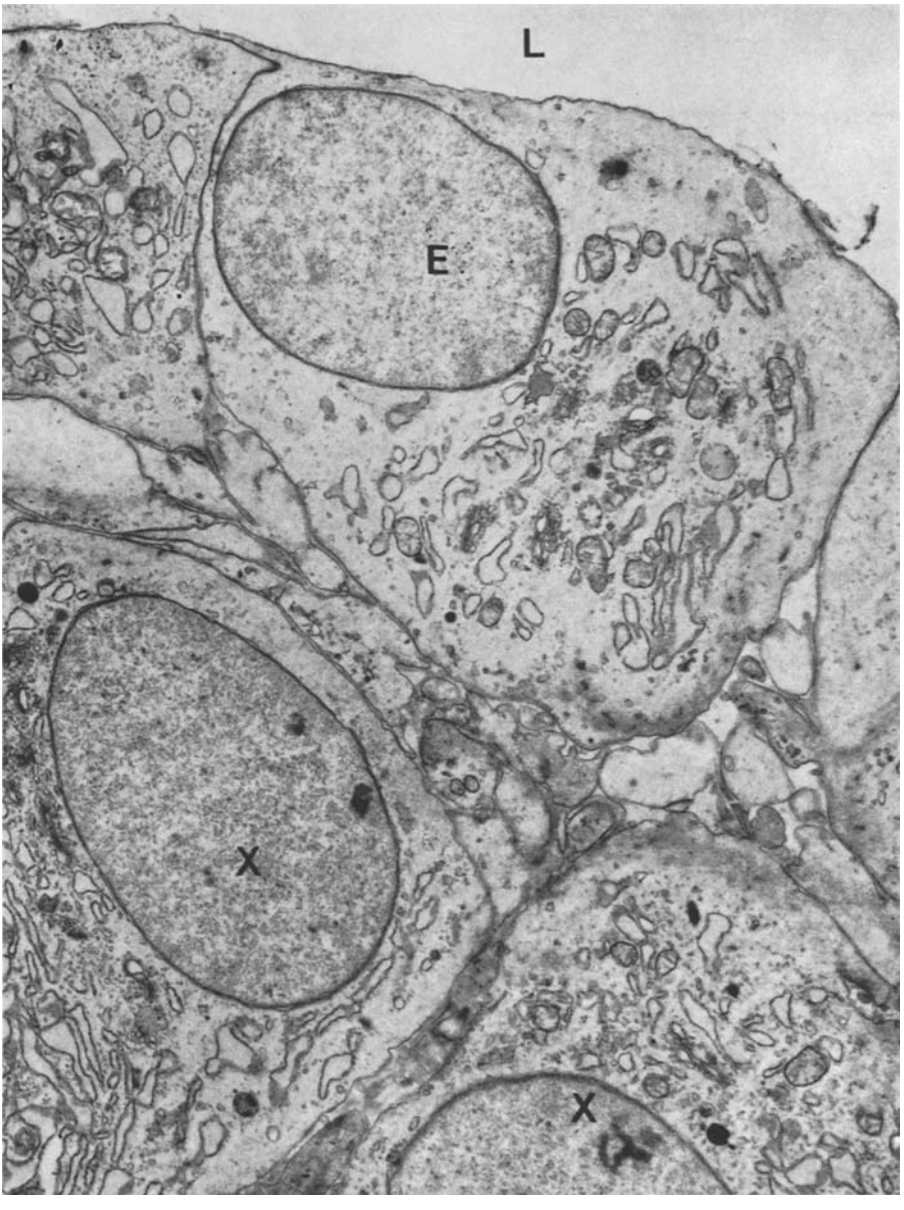

Fig. 7. Electron micrograph of same artery as in Fig. 6, demonstrating the surface lining seen light-microscopically in Fig. 6d. Note the swollen appearance of the endothelial (*E*) and subendothelial cells (*X*), and their close apposition to one another. Both cell types are morphologically very similar. ×8000. *l* lumen

investigation showed that by one day following ligation 25% of the cells were involved, reaching a maximum of 45% three to seven days after ligation and declining at later time intervals. In addition, the same amorphous-grey ultra-

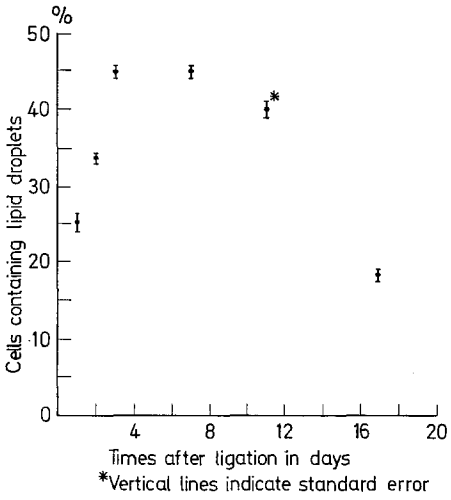

Fig. 8. Percentage of arterial cells containing lipid droplets at various times following double ligation

structure for intracellular lipid droplets was observed, following both injections of egg lipoproteins (Hoff and Gottlob, 1969b) and of physiologic saline. This implies that not all the intracellular lipid seen after injecting lipids or lipoproteins was exogenously derived. While the function of the lipid droplets is unknown, it appears likely that these droplets, consisting mainly of triglycerides were derived from fatty degeneration, as was similarly interpreted following injury to arterial smooth muscle cells (Murray, Schrodt and Berg, 1966). Triglyceride has been claimed to be the most easily removable lipid moiety by arterial tissue, which would explain why only very low levels of triglycerides are found in atherosclerotic arteries (Adams, 1967). The reduction in relative number of cells containing lipid droplets after seven day's ligation could then imply either a reduction in fatty degeneration, an increased ability of the artery to remove intracellular lipid accumulations (Adams, 1967), or a combination of these. As fatty degeneration is known to occur following hypoxia (Büchner, 1957), the reduction of lipid droplets may well be correlated with the reoxygenation of the artery, as is also suggested by the return of oxidative and ATPase activities to these areas (Hoff, 1970). The presence of blood rather than physiologic saline in the ligated artery appeared to have little effect on the distribution or intensity of the intracellular lipid droplets or the ultrastructural changes.

In addition to the unexpected presence of lipid droplets found after double ligation and saline injections, a series of morphologic alterations, previously described in similar studies (Hoff and Gottlob, 1969a), were observed. These changes appeared to be the direct consequence of double ligation, and were again focussed around the endothelial cells at early time intervals and on the various components of the thickened intima at later time sequences. Particularly striking was the presence of viable endothelial cells in an anoxic environment for several days after double-ligation (Malyschew, Buck, 1961; Hackensellner, David and

Uerlings, 1965; Hoff and Gottlob, 1969a, b) and the abundance of lysosomal-like bodies which were either phagocytic or autophagic. That some are phagocytic is suggested by the presence of lumen-injected carbon black within such organelles, as was also seen previously with thorium dioxide (Buck, 1961). However, the tracer may have entered via cell membrane invaginations. The lysosomal-like bodies may be the ultrastructural counterpart of the acid phosphatase-containing granules seen in the intima in a parallel enzyme histochemical study of these ligated arteries (Hoff, 1970). Intimal hyperplasia, encountered in numerous systems involving arterial injury (La Taillade, Gutstein and Lazzarini-Robertson, 1964; Friedman and Byers, 1965; Gage, Fazekas and Riley, 1967; Kunz *et al.*, 1967; Esterly, Glagov and Ferguson, 1965), has been shown by immunohistochemical staining for actomyosin to consist of smooth muscle cells (Knierem, Kao and Wissler, 1968) which are rapidly undergoing mitosis (Spaet and Lejnieks, 1967; Florentin *et al.*, 1969). The cells sometimes resemble smooth muscle cells ultrastructurally (Buck, 1961), and other times fibroblasts (Hackensellner, David and Uerlings, 1965; Hoff and Gottlob, 1969b). Their orientation in the thickened intima is usually concentric inwards and longitudinal outwards (Buck, 1961; Imai and Thomas, 1968; Hoff and Gottlob, 1969a), but on occasions a radial array is found, as seen here and following double stenosis of the rat aorta (T'sao and Spaet, 1967). Elastogenesis of the thickened intima was prominent in some ligation studies (Buck, 1961) but weak or non-existent in others (Zollinger, 1968; Hoff and Gottlob, 1969a), while it is quite extensive in the thickened intima induced by mechanical injury (Björkerud, 1969). The swollen surface cells consisting of several layers in the hyperplastic intima, are presumably proliferating endothelial cells (Kunz, Kranz and Keim, 1967; Florentin *et al.*, 1969). The pseudopods extending from these cells have also been seen frequently in the past (Esterly, Glagov and Ferguson, 1968; Hoff and Gottlob, 1968; Willms-Kretschmer and Majno, 1969).

In conclusion, it has been shown that when the rabbit common cartoid artery is doubly ligated, filled with a physiologic saline, and viewed at time intervals ranging from one to seventeen days, a series of morphologic changes relative to the contralateral cartoid and to the blood-filled doubly ligated artery were observed. Essentially only the intensity and localization of the intracellular lipid droplets differed between ligated arteries filled with lipids or lipoproteins and those filled with physiologic saline. Of particular interest would be to further investigate the source and genesis of these endogenously-derived lipid droplets.

Acknowledgments. The author would like to thank Mrs. Falkenberg for technical assistance and Dr. Gershey for helpful suggestions in the preparation of this manuscript. Dr. Hoff is a recipient of an Advanced Research Fellowship of the American Heart Association.

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