# EXPERIMENTAL ARTERIOSCLEROSIS

## I. Fibrous Plaque Formation in Primates, an Electron Microscope Study*

By MICHAEL B. STEMERMAN and RUSSELL ROSS

*(From the Department of Pathology, University of Washington, School of Medicine, Seattle, Washington 98195, and the Division of Hematology, Department of Medicine, Montefiore Hospital and Medical Center, Bronx, New York 10467)*

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Before the development of the focal lesion of human atherosclerosis, a diffuse thickening of the intima occurs which presumably increases with age (1–6). Recent reports (7, 8)[1] have described a similar diffuse fibrous intimal hyperplasia in rabbits after denudation of, or injury to the endothelial cells. The endothelia were stripped away by an intravascular balloon catheter without disruption of the internal elastic lamina (IEL)[2] and with little injury to the other segments of the vessel wall. The ensuing hyperplastic intima showed changes typical of less specific arterial trauma in which minimal alterations of diet produced atherosclerotic plaques (9–11). The ability to induce this lesion conveniently and reproducibly in primates provided us with the opportunity to systematically examine the factors associated with the genesis of this lesion and with its progression. This report describes the role of the medial smooth muscle cell in the development of the lesion and points to the importance of normally functioning endothelium in its prevention.

### *Materials and Methods*

Eight monkeys (*Macaca nemestrina*) age 10–12 months, male and female, weighing from 1.1 to 1.8 kg were anesthetized by halothane. Under sterile conditions, the right or left femoral artery was exposed and cannulated with a 4F Fogarty arterial catheter (Edwards Laboratories, Santa Ana, Calif.). With the catheter in the artery, the balloon was inflated and maintained at a pressure of approximately 700 mm Hg, was promptly and rapidly pushed through the corresponding iliac artery into the abdominal aorta to the region of the diaphragm and quickly withdrawn. The entire transit time for the inflated balloon was less than 10 sec. The balloon

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[1] Baumgartner, H. B. Repair of vascular endothelium. I. Blood cells interacting with vessel wall selectively denuded of endothelium. Manuscript in preparation.

[2] *Abbreviations used in this paper:* IEL, internal elastic lamina; RER, rough endoplasmic reticulum.

was deflated after it was again in the femoral artery and the catheter was removed. The femoral artery was ligated and the wound closed.

The animals were allowed to recover for 10 min, 1 hr, 24 hr, 2, 3, 4, 5, 7, 14, 28 days, 3 months, and 6 months, after which they were sacrificed and their vessels were examined by light and electron microscopy. A nonballooned artery either from the opposite side or from a similar age animal was used as a control. In some of the animals both sides were ballooned, whereas in others, one side remained as an unoperated control.

### *Electron Microscope Procedures*

At the time of sacrifice under halothane anesthesia, the animals were exsanguinated through the abdominal aorta. The abdominal aorta and both iliacs were either dissected free and fixed by immersion fixation, or were promptly flooded with fixative *in situ*, removed by careful dissection, and placed in cacodylate-buffered 2.5% glutaraldehyde–2% paraformaldehyde (pH 7.3) (12) for 1 hr. The vessels were cut into approximately 1-mm rings and returned to the fixative for another hour at 4°C. They were placed in 0.1 M cacodylate buffer before postfixation. The tissues were postfixed in 1% osmium tetroxide buffered with *s*-collidine, pH 7.3, for 1½ hr followed by en bloc staining with 2% uranyl acetate for ½ hr. The rings were dehydrated through ethanol and were sectioned into semicircles when in 70% ethanol and flat embedded in Epon 812. Only the iliac vessels are included in this study.

1-$\mu$ sections were stained for light microscopy with basic fuchsin-methylene blue (13). Thin sections were taken from regions in each block previously selected by light microscopy. The sections were stained with uranyl acetate followed by lead citrate and examined in an AEI 801 electron microscope.

## RESULTS

*Control Vessels.*—In these primates, the intima was composed of a continuous layer of endothelial cells resting upon the endothelial surface and contiguous with the internal elastic lamella (IEL) as shown in Fig. 1. The subendothelium consisted of a discontinuous basement membrane with the elastic fibers of the IEL. Occasional collagen fibrils were present at the subendothelial surface and where present were intermixed with the components of the IEL. Occasional smooth muscle cells were seen in the intima of the iliac arteries. The internal elastic lamella is a broad (700–1000 A) continuous layer of elastic tissue interrupted by fenestrae containing microfibrils at the periphery of the elastic fiber as well as within their interstices. Areas of fibromusculo-elastic intimal thickening (4) or "cushions" containing a few smooth muscle cells surrounded by collagen and small elastic fibers were observed usually associated with branchings of the artery. The media consists of smooth muscle cells surrounded by connective tissue. No vasa vasorum were present.

*10 min and 1 hr after Endothelial Injury.*—Endothelium was absent from large areas without apparent disruption of the internal elastica or media. At 10 min and 1 hr, extensive regions of the denuded internal elastica were covered by platelets that were in close association with elastic fiber microfibrils or with basement lamina. Degranulated platelets were spread upon the surface, and other platelets adhered to these forming a layer of varying thickness. Fibrin was not seen between the adhering platelets and the vessel surface. A few swollen medial smooth muscle cells could be seen immediately beneath the internal

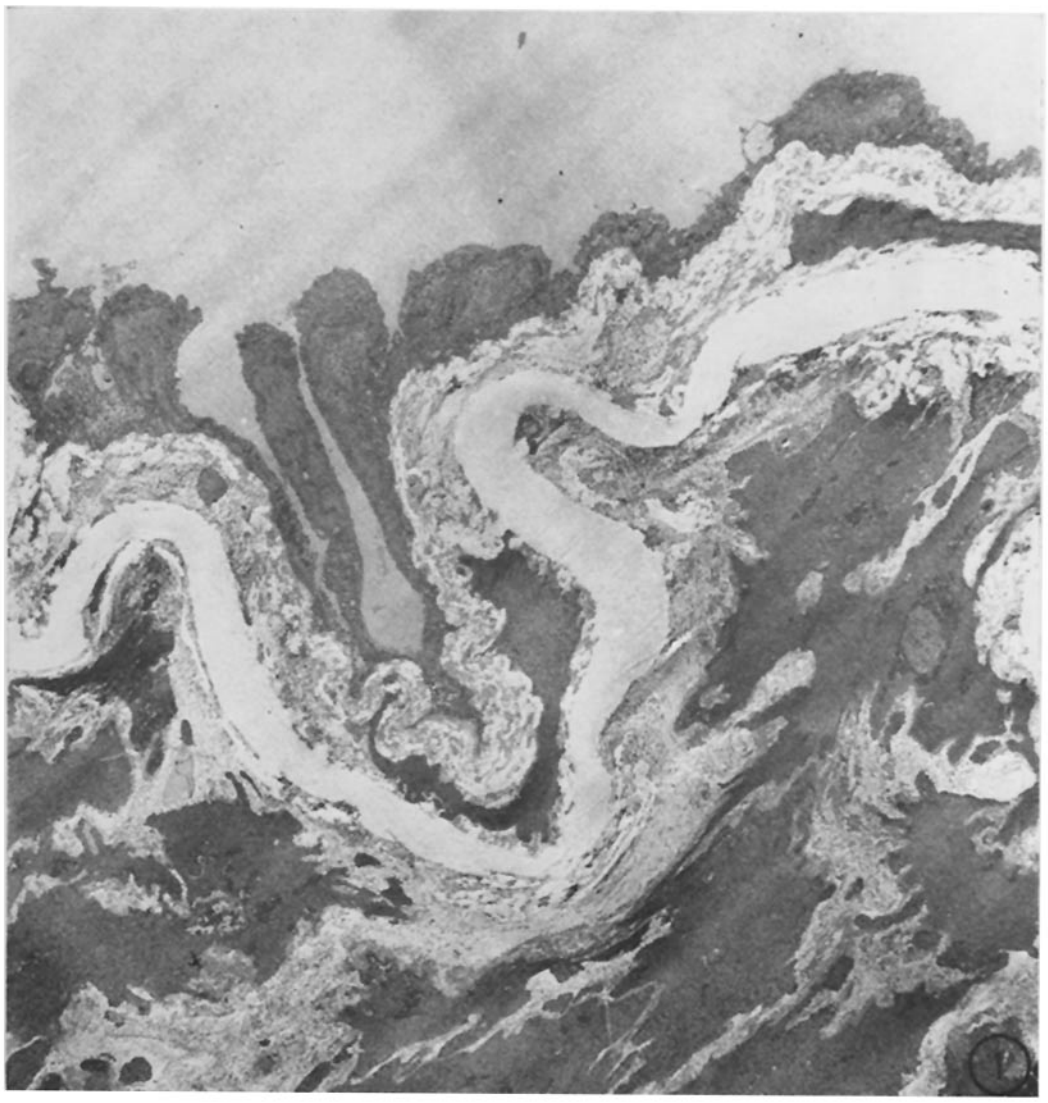

FIG. 1. This low-power electron micrograph is taken from a noninjured iliac artery. The narrow intima contains occasional single smooth muscle cells covered by endothelium and delimited by the IEL. Medial smooth muscle cells lying below the IEL are sectioned somewhat diagonally. This micrograph is characteristic of the intact common iliac vessel of the monkey where the intima is comprised of endothelium, isolated smooth cells, or rare collagen fibrils and small elastic fibers. Magnification × 3000.

elastic lamina indicating that some of the cells may have been injured either as a result of de-endothelialization or due to the mode of tissue preparation. The extent of this injury appears to vary with the inflation of the catheter. Fig. 2 illustrates an iliac artery 1 hr after removal of the endothelium demonstrating the exposed elastic lamina and the extent of de-endothelialization.

*24 hr after Endothelial Injury.*—After 24 hr, platelets and leukocytes were present in many regions of the de-endothelialized surface and the platelets formed an almost continuous layer. Polymorphonuclear leukocytes and mononuclear leukocytes were present in the platelet thrombi in close association with the adhering platelets and erythrocytes (Fig. 3). Fibrin was intermixed with these cells within the lumen of the vessel but was not observed between the platelets and the elastic fibers (Fig. 4). The IEL was intact and some of the medial smooth muscle cells showed evidence of minimal changes in the form of mild cellular edema. No leukocytes were observed penetrating the elastica nor was fibrin seen beneath the IEL.

*3–5 Days after Endothelial Injury.*—Regenerating endothelial cells were observed at the luminal surface of the vessel, presumably migrating over scattered platelet remnants (Figs. 5 and 6). The new endothelium contained rough endoplasmic reticulum (RER), many clusters of free ribosomes, and few pinocytotic vesicles. Endothelial cell filaments were located principally at the periphery of these cells, and dilated mitochondria were distributed throughout their cytoplasm which was less dense than that of mature endothelium. These regenerating endothelial cells were in close association with a morphologically denser basement lamina (Figs. 7 and 8).

At this time, the medial smooth muscle cells contained greatly expanded RER and were seen penetrating fenestrae of the IEL, and some were found to be located beneath and regenerating endothelium. Leukocytes were no longer present at this time.

*7 Days after Endothelial Injury.*—By 7 days the luminal surface was almost completely recovered by the endothelium. Several layers of smooth muscle cells with dilated RER were present within the intima. Small intermittent regions appearing like tight junctions were seen between endothelial cells for the first time. The predominant connective tissue surrounding these cells morphologically resembled basement membrane (Figs. 9 and 10). A few platelet remnants were still present.

*14–28 Days after Endothelial Injury.*—Between 14 and 28 days there was a gradual and progressive thickening of the intima consisting of increased numbers of smooth muscle cells surrounded by connective tissue fibers. The original IEL was clearly seen abluminal to the thickened intima. Endothelial cells lined the lumen (Fig. 9) and by 28 days contained the usual numbers of pinocytotic vesicles and junctional complexes at the same time retaining relatively large amounts of RER.

At 14 days a large part of the thickened intima consisted of basement mem-

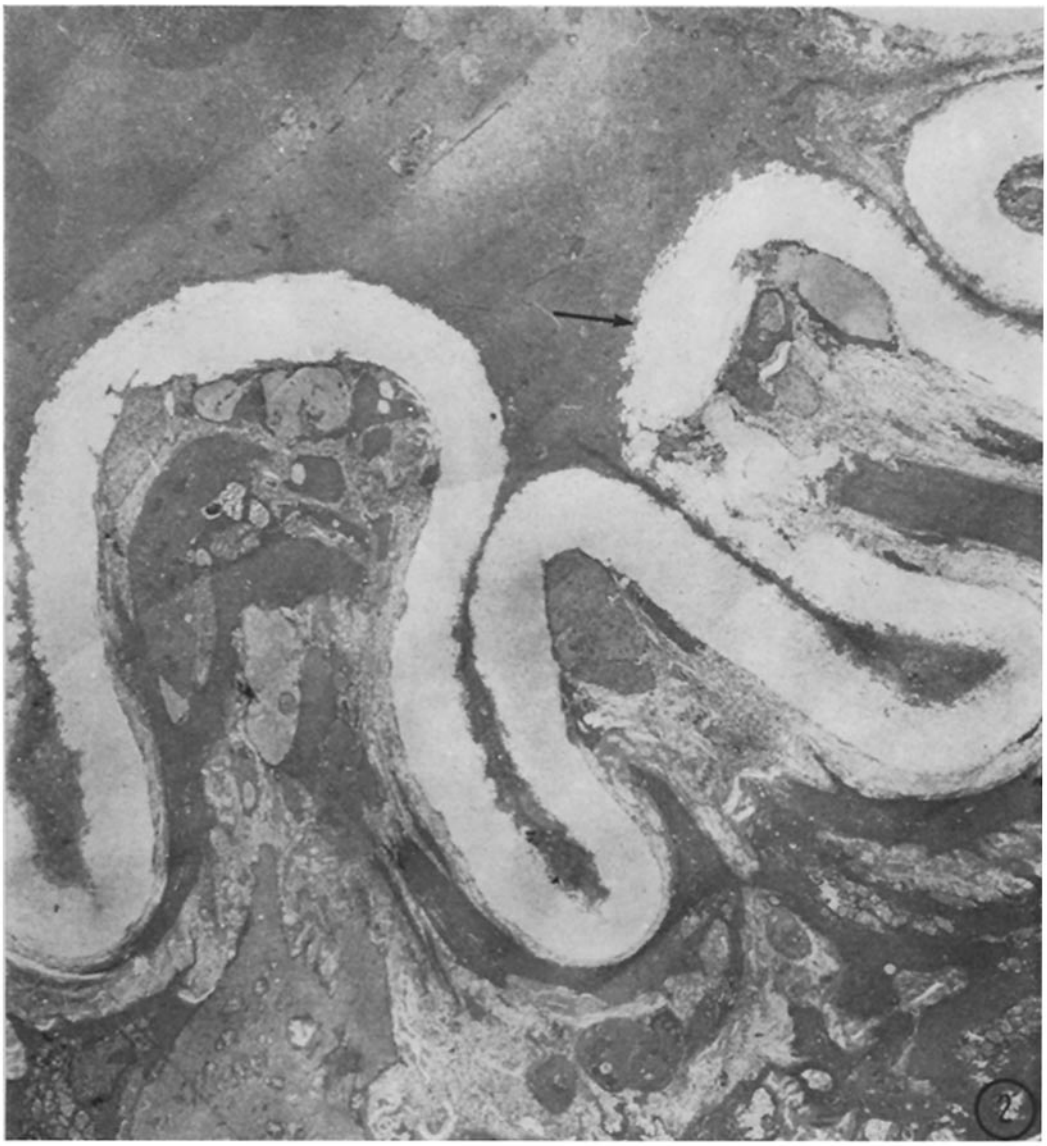

FIG. 2. This low-power electron micrograph should be compared with that in Fig. 1. It demonstrates the common iliac artery of a monkey 1 hr after removal of the endothelium and other intimal elements with an intravascular balloon catheter. The lumen is filled with blood elements and no endothelium remains on the exposed IEL (arrow). Some distortion of underlying smooth muscle cells is seen as evidenced by cellular edema. The IEL is intact and there is no extrusion of cellular material through the fenestra of the lamina. Magnification × 3400.

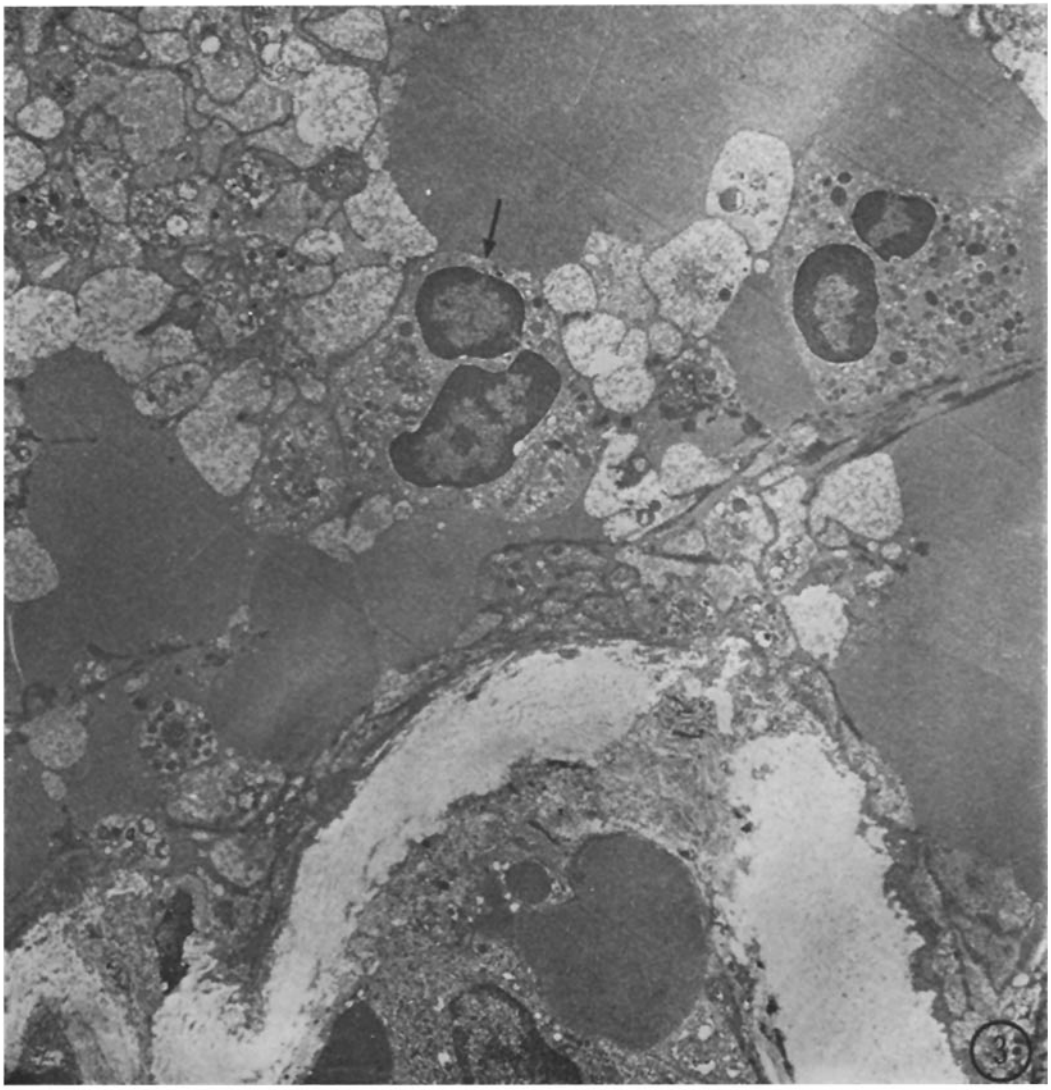

FIG. 3. In this electron micrograph of a 1 hr de-endothelialized vessel, a platelet thrombus is seen attached to the exposed IEL. No endothelial cells are present and masses of platelets and occasional neutrophils (arrow) are seen in close association with each other and the IEL. They are surrounded by numerous erythrocytes. Fibrin strands are present between many of the cells within the platelet thrombus. Magnification × 5200.

brane-like material (Fig. 9). With older lesions, there were increasing numbers of elastic fibers in the thickened intima. Collagen fibrils were seen more frequently in the older lesions and appeared to be distributed close to the original IEL.

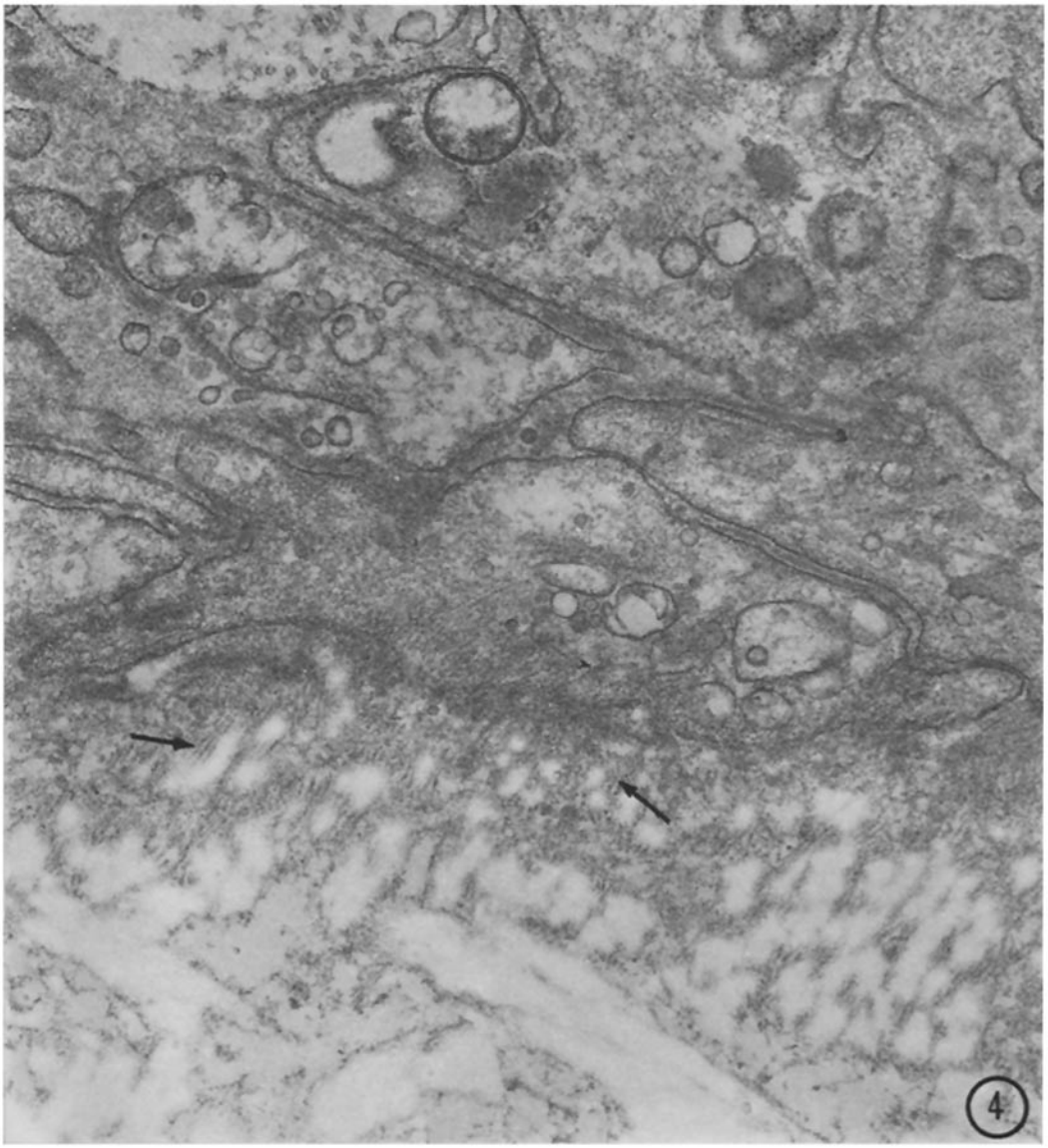


FIG. 4. 24 hr after endothelial removal, platelets adhere to the denuded surface and are seen in this electron micrograph to be closely apposed to the microfibrils (arrows) of the elastic fibers as well as to the basement membrane-like material. Fibrin is seen on the luminal side of the vessel between the platelets. Magnification × 36,000.

Migration of smooth muscle cells from the media into the intima was seen as late as 14 days (Figs. 10 and 11). There continued to be a progressive increase in the number of smooth muscle cells within the intima beyond 28 days. These cells were randomly arranged and contained a well-developed RER (Figs. 9 and

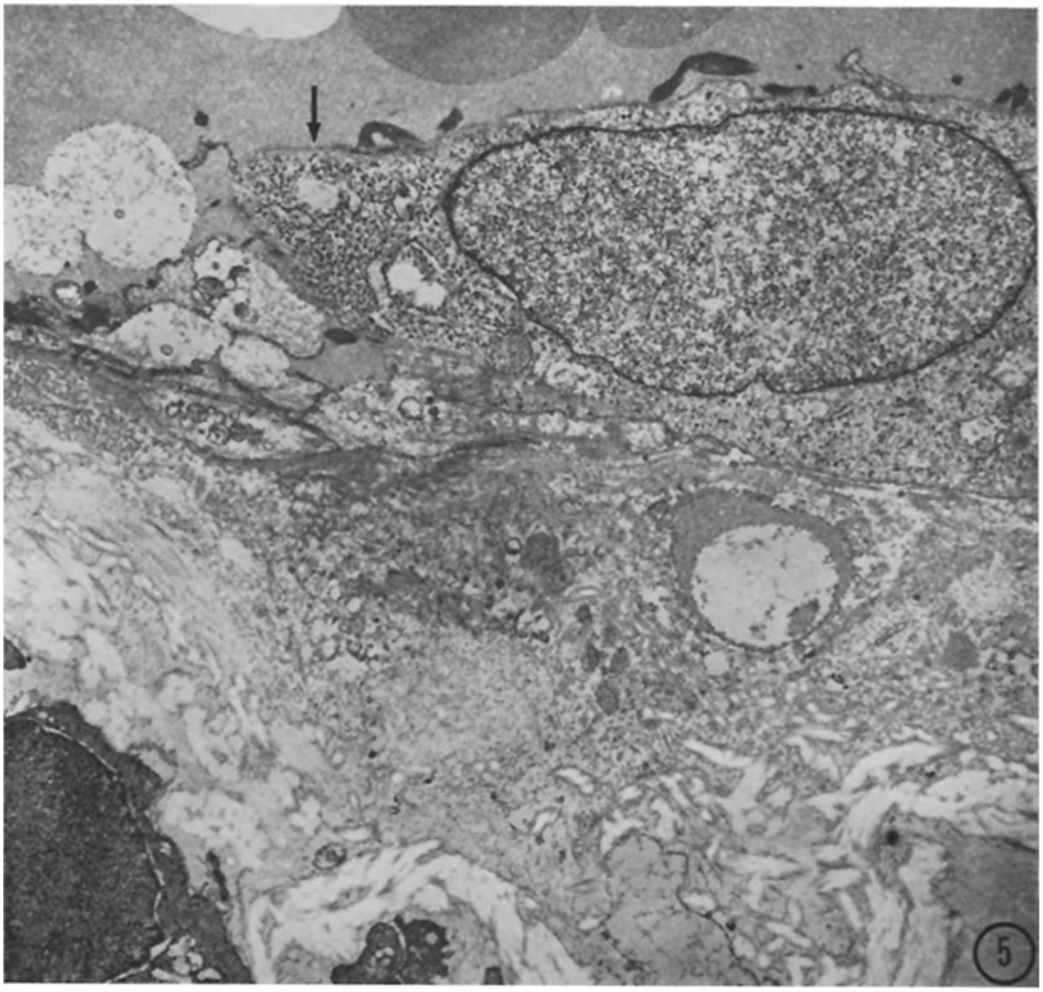


FIG. 5. This electron micrograph is representative of a lesion 3–5 days after removal of the endothelium. A regenerating endothelial cell is seen partially covering the denuded surface. The cell is rich in free-ribosomes and has a nucleus of relatively low density. The cell (arrow) overlies both basement membrane and platelet remnants. Collagen bundles and elastic fibers are seen on the vessel side of the endothelium. Magnification × 8400.

12). The media remained intact and the medial smooth muscle cells were morphologically unaltered and retained their original orientation to the vessel during the entire period of the experiment.

*3 Months after Endothelial Injury.*—The 3 month lesion was similar to the 1 month lesion. The intima was hyperplastic and ranged in thickness from the equivalent of 5–15 cell layers (Fig. 13). Relatively less basement membrane-like

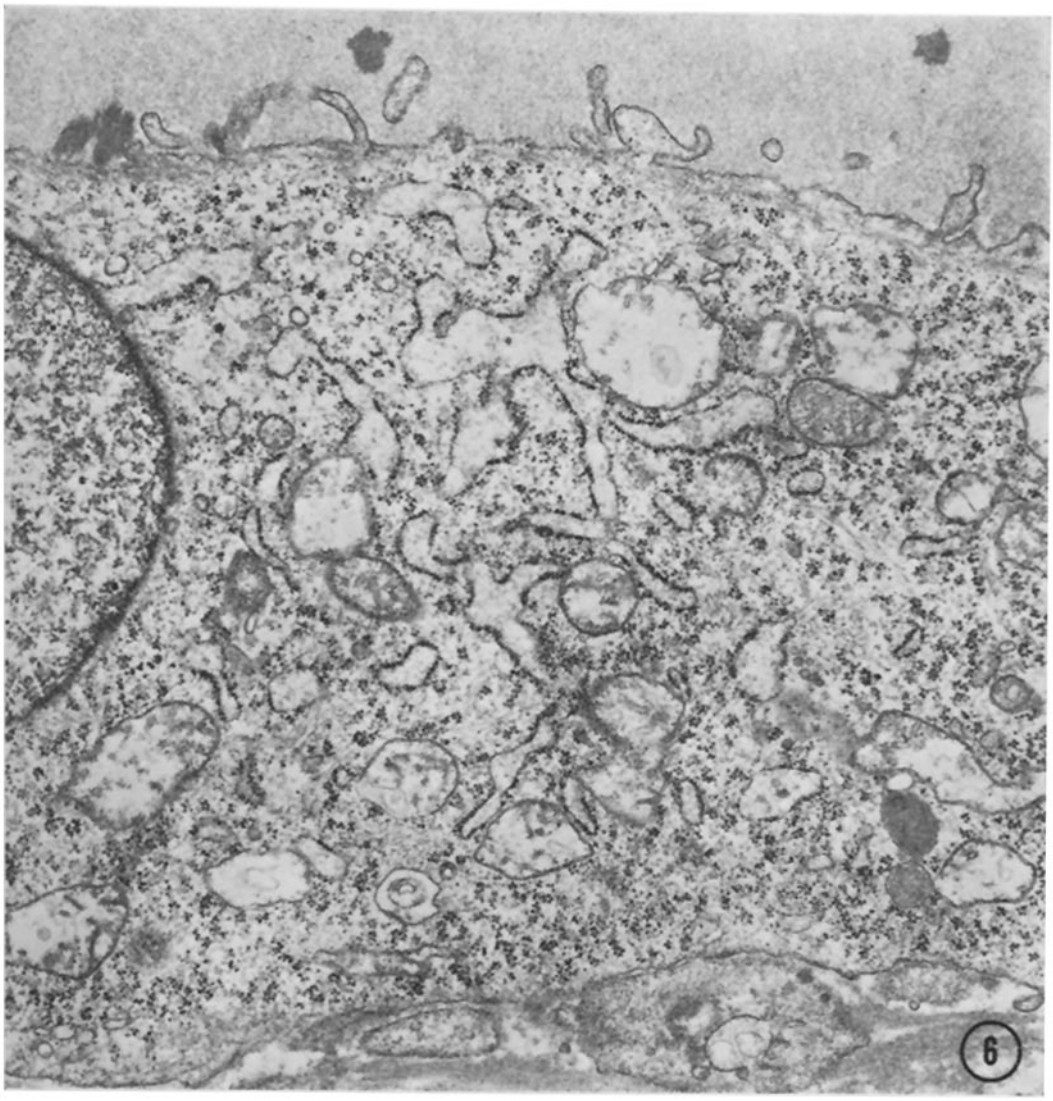


FIG. 6. This is an electron micrograph of a regenerating endothelial cell from a 7 day lesion This cell is characterized by having a large number of cisternae of RER as well as numerous free cytoplasmic ribosomes. The mitochondria are markedly dilated and the cell can be seen to overlie several platelets that are interposed between the endothelium and the basement membrane of the previously exposed intima. Filaments approximately 60 A in diameter are seen scattered throughout the cell but are predominant at the periphery of the cell. Projections of the cell surface are seen on the luminal side. Magnification × 21,000.

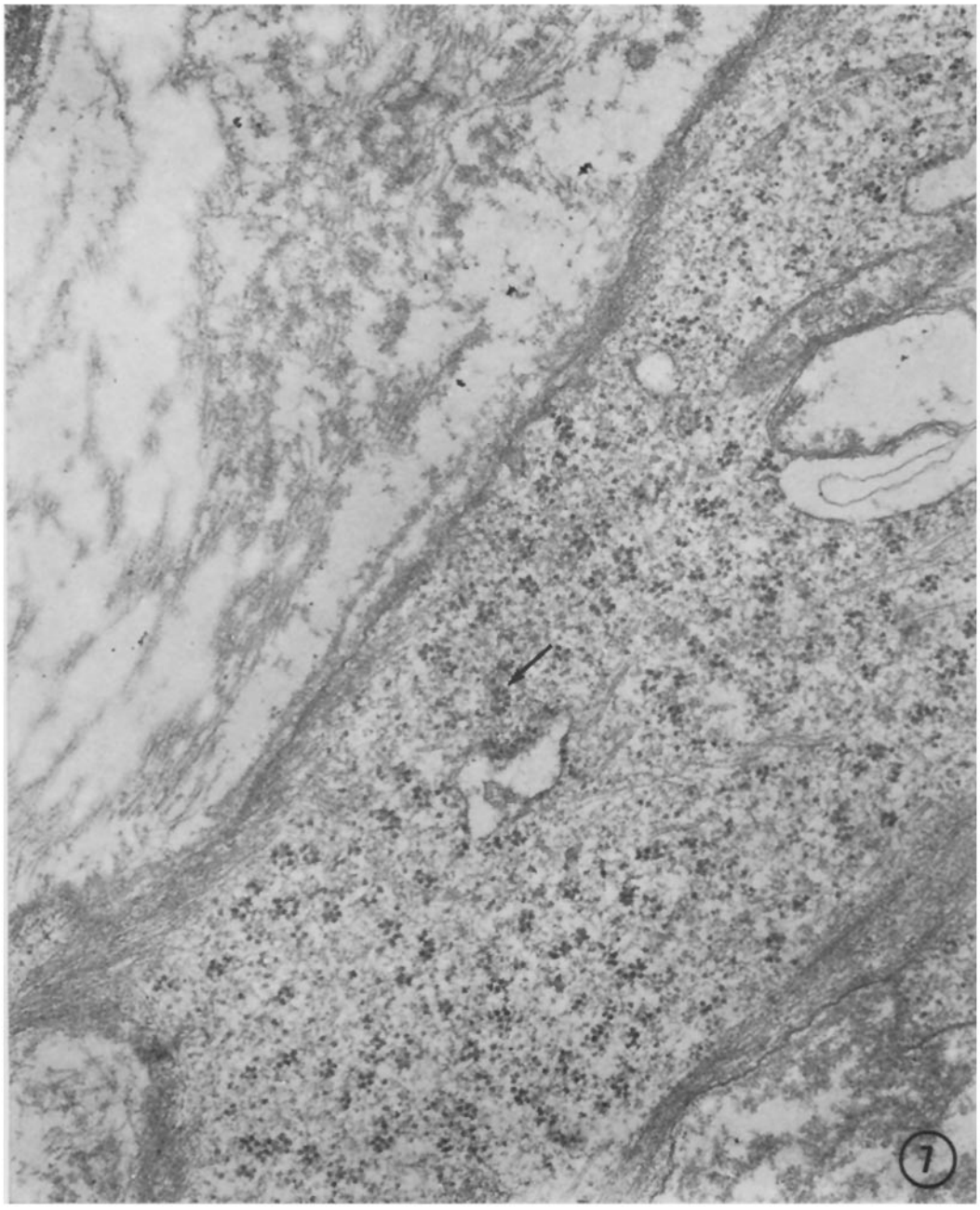


FIG. 7. This electron micrograph illustrates a regenerated endothelial cell from a 7 day lesion. The large numbers of free cytoplasmic ribosomes (polysomes) (arrow) and peripheral filaments are clearly evident in this higher magnification of this cell. On the vessel side of the endothelial cell is seen basement membrane and elastic fibers with microfibrils. Magnification × 32,000.

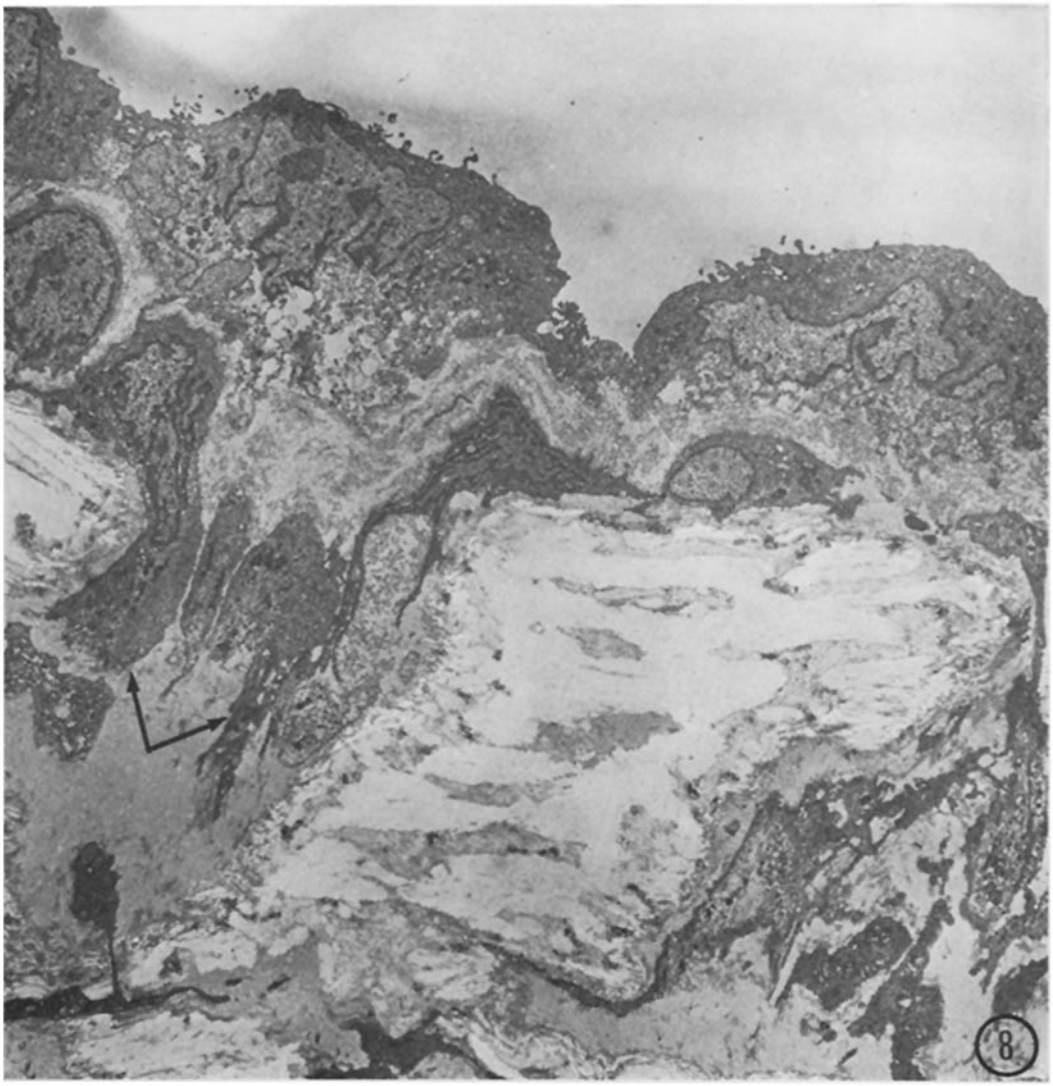

FIG. 8. This is a low-power electron micrograph of a 2 wk old lesion. By this time the endothelium is regenerated and beneath the endothelium is seen a segment of the IEL with two fenestrae. In these fenestrae are smooth muscle cells (arrows) migrating from the media into the intima. Several of these cells are partially extended into the intima whereas others are completely within the intima. Observations such as this are common between 7 days and 2 wk. Magnification × 3400.

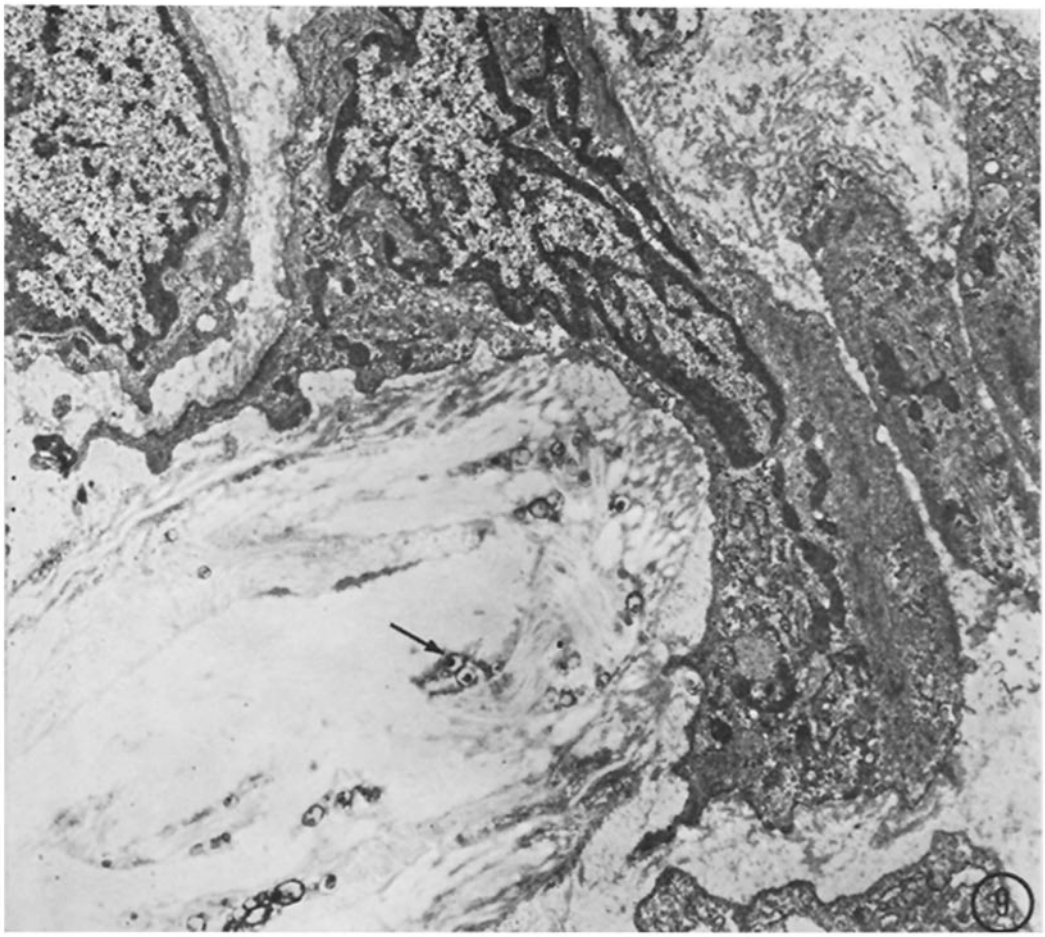

FIG. 9. This is a higher magnification of one of the smooth muscle cells seen in Fig. 8 in the process of migrating from the media into the intima. Since these cells are not commonly present in the intima in these quantities, the assumption that the migration is in this direction is reasonable. Small membrane-bounded structures (arrow) are also seen intermixed between the element of the IEL suggesting cellular damage or debris from the injury. Magnification × 4500.

material was present in the extracellular spaces, which contained many more collagen fibrils and small or immature elastic fibers. The endothelial cells appeared more mature at this time. They contained rod-shaped tubular bodies and numerous tight junctions were apparent.

*6 Months after Endothelial Injury.*—After 6 months the lesions had markedly decreased in size to one to three layers in thickness (Fig. 14). This marked decrease in thickness was apparent throughout the vessel wall, although the intima

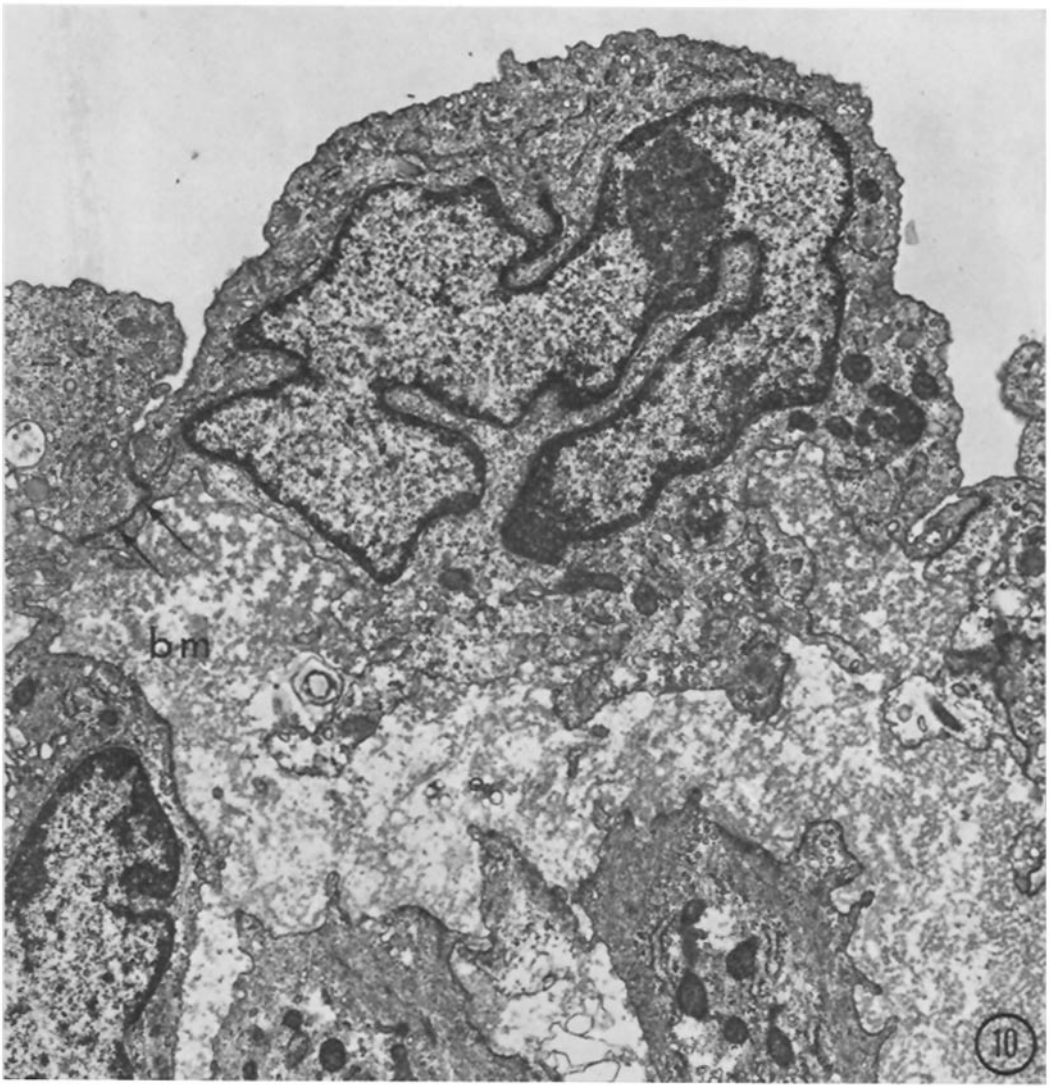


FIG. 10. This electron micrograph demonstrates the appearance of a regenerated endothelial cell 14 days after injury. Below this cell are several smooth muscle cells that have migrated into the lesion thickening the intima. Junctional sites are seen between two endothelial cells (arrows) and at higher magnification in Fig. 11. This cell appears to rest on basement membrane-like material (*bm*) and has a reasonably well-developed RER, numerous mitochondria, and peripheral filaments. Magnification × 20,000.

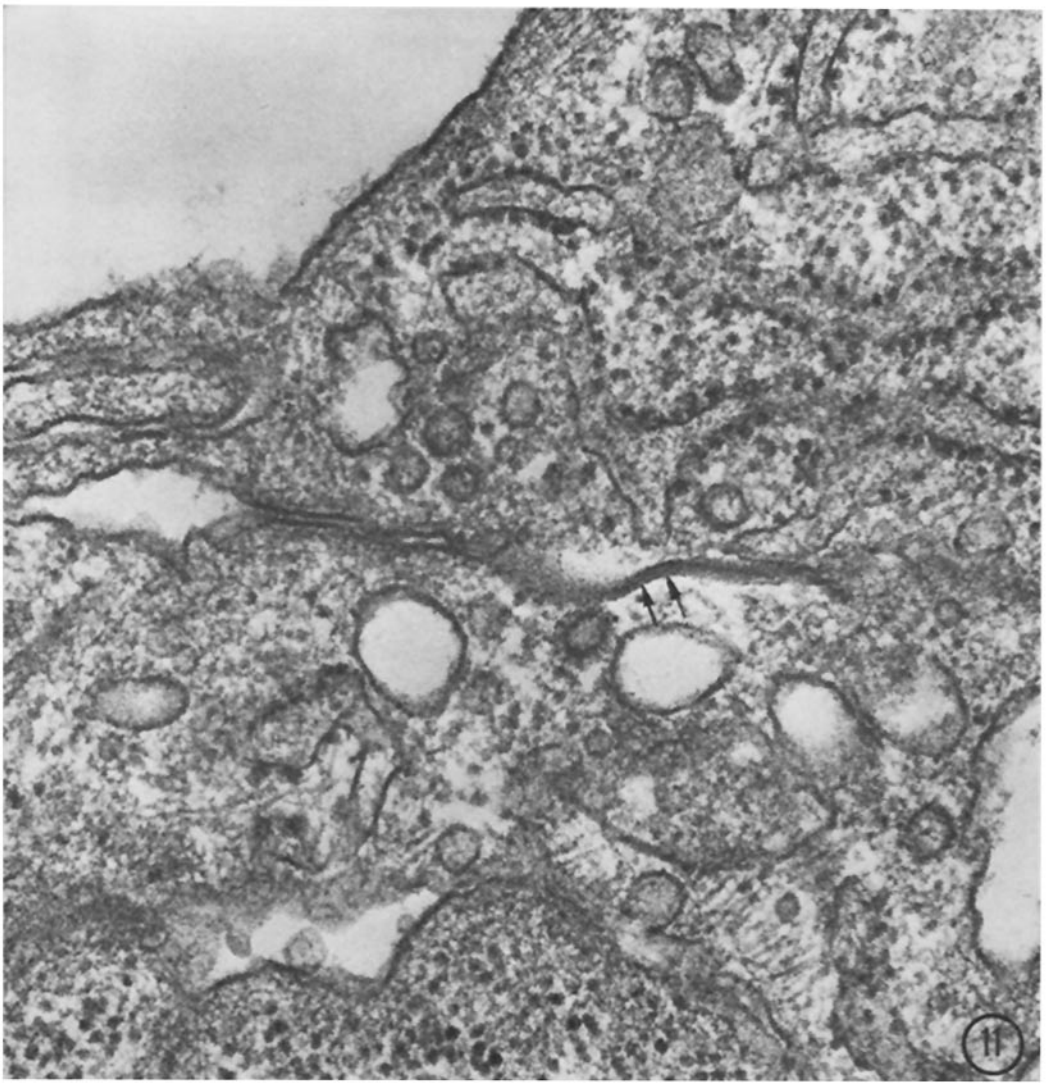

FIG. 11. This electron micrograph is a higher magnification of a junctional complex between two regenerated endothelial cells from a 2 wk old lesion. In one region a gap-junction can be seen (arrows). Several elements of RER as well as smooth surface membrane can be seen in both of these cells. Magnification × 76,000.

still appeared thickened in contrast to control uninjured arteries. No evidence of smooth muscle degeneration or necrosis was observed at this time. The endothelial cells were somewhat lower in profile and contained all of the organelles and junctional complexes usually associated with arterial endothelium.

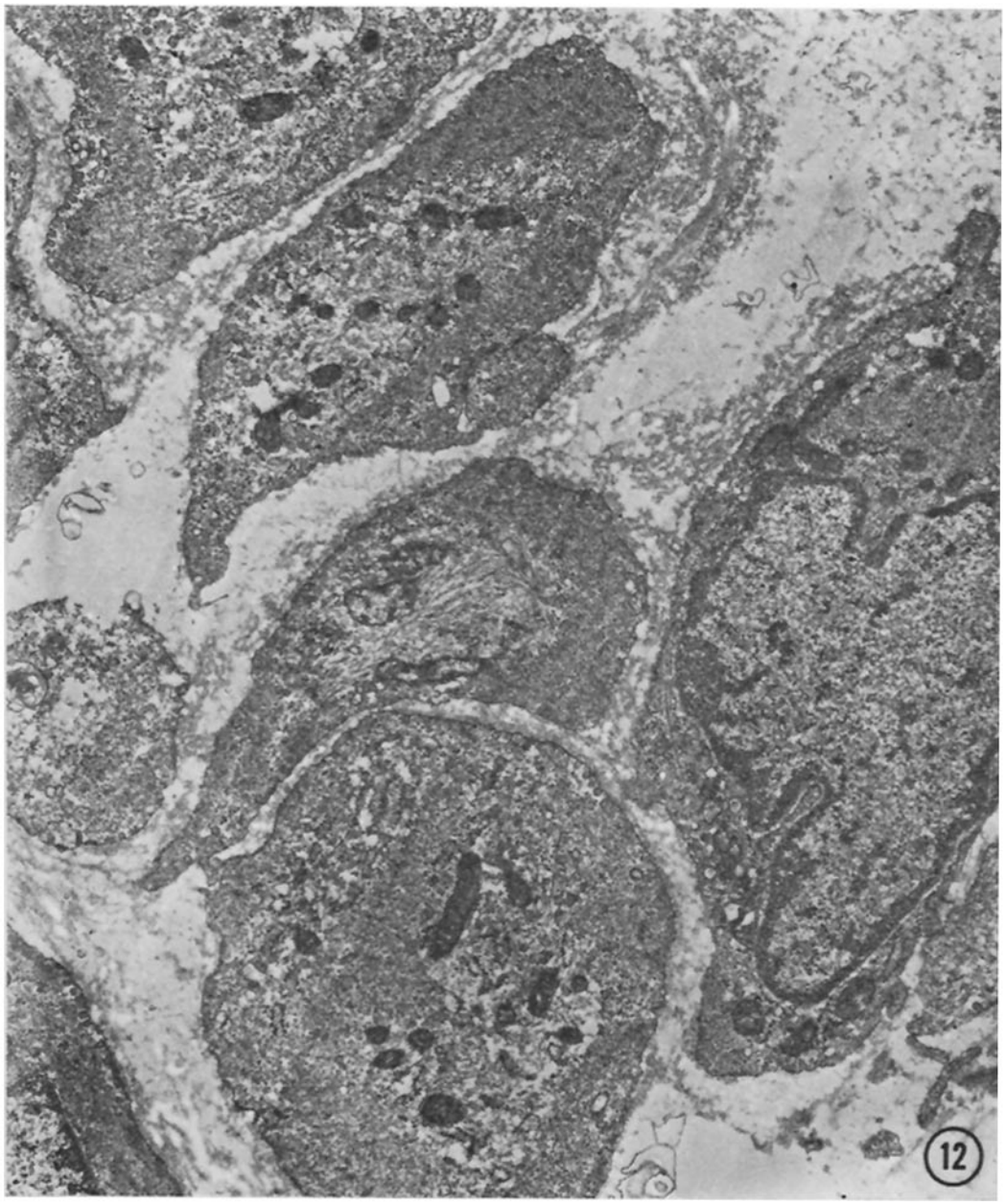


FIG. 12. This electron micrograph is taken of several cells in the center of the intimal lesion 2 wk after injury. These cells have characteristics typical of smooth muscle including myofilaments, and an incomplete basement membrane. The young elastic fibers and basement membrane-like material can be seen in the extracellular matrix surrounding these cells. Magnification × 13,000.

## DISCUSSION

One of the principal problems in studying the evolution of atherosclerosis has been the development of a suitable experimental animal system (4). Although this disease entity has been studied in a number of different animals, in most instances the lesion that forms is either significantly different in its morphologic characteristics from that of the human lesion, or, the conditions required to generate the lesion are sufficiently different from those in man that the ability to

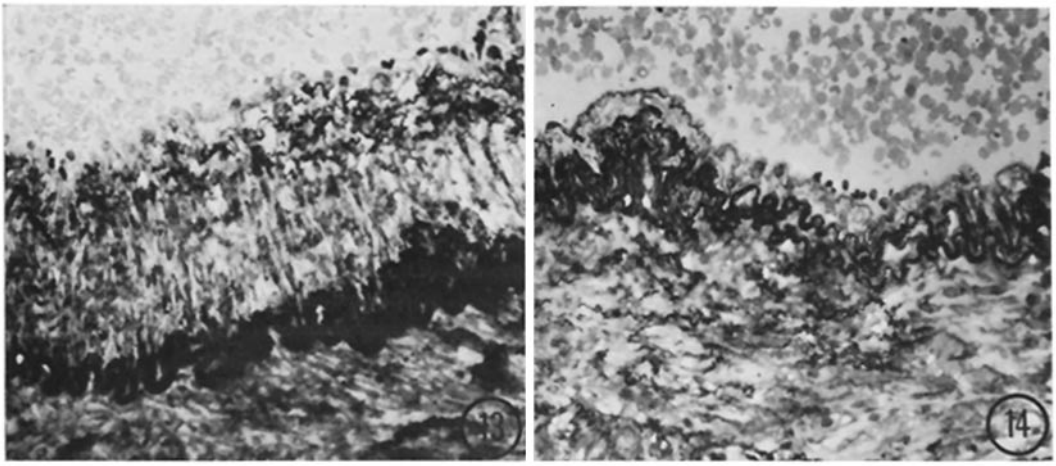

FIG. 13. This light micrograph demonstrates a lesion 3 months after injury. The intima is markedly thickened, the lumen is filled with erythrocytes, and the IEL is apparent. Magnification × 480.

FIG. 14. This light micrograph demonstrates the appearance of a lesion 6 months after injury. The size of the lesion is markedly diminished between 3 and 6 months suggesting the possibility that such a lesion in an animal on a nonlipid diet may be reversible. Magnification × 480.

correlate and study the development of this lesion with that occurring in man is difficult (14). There are perhaps two animal systems that have proven useful for studying the pathogenesis of the atherosclerotic plaque. These are the miniature swine and the nonhuman primate (15). Many species of monkey spontaneously develop atherosclerosis with aging (16–18). They also develop similar lesions when placed on lipid-rich diets, not too dissimilar from the type of diet related to the pathogenesis of the lesion in man (19–21). Because of this, the nonhuman primate will be an extremely valuable animal for the study of the development of the early lesion of atherosclerosis.

*Intimal Trauma.*—It is now widely accepted that the early lesion of atherosclerosis begins as an intimal proliferation of smooth muscle cells (4–6). "Injury" to the endothelium may represent the principal event preceding the onset

of atherosclerotic change. Mechanical injury has long been known to produce a thickening of the arterial wall with narrowing of the lumen (22–25). Recent studies (7, 26, 27)[1] point to the importance of endothelial injury and the possible development of a lesion similar, if not identical, to the human fibromuscular elastic thickening and the fibrous streak (4). Most studies utilized methods that damaged the whole thickness of the vessel wall usually penetrating into the media rather than just the intima (25, 28). Selective removal of the arterial endothelium with an intravascular balloon catheter allows restriction of damage to the endothelial cells and the intima (8, 29–31). After selective de-endothelialization in this manner, a hyperplastic lesion results consisting of proliferated smooth muscle cells within the intima of the artery surrounded by basement membrane-like material, collagen, and young elastic fibers (7, 8, 27).[1]

In the present study, monkey iliac arteries subjected to this form of injury demonstrated an early progressive migration of medial smooth muscle cells into the intima associated with proliferation of these cells. The method is easily reproducible and leads to widespread intimal hyperplasia. The morphologic findings are similar to studies performed in the rabbit (8, 27)[1] although fixation of the monkey tissue subsequent to dissection gave some alteration of some of the cells immediately subjected to the internal elastic lamina. These differences included trapping of blood in the lumen and traces of edema of medial cells immediately subjacent to the IEL in the monkey specimens.

With endothelial removal, subendothelial connective tissue is exposed to blood. The thrombus that forms is composed largely of platelets with some fibrin close to the lumen, and at longer time intervals these thrombi have intermixed monocytes and granulocytes. Reendothelialization is generally complete in approximately 7 days. The source of this new endothelium has not yet been ascertained, but we suspect that it may be derived from accessory vessels that branch from the principal artery or from patches of endothelium remaining after injury.

*The Smooth Muscle Cell.*—Smooth muscle cells first appear within fenestrae of the IEL of the injured or de-endothelialized vessel within approximately 4 days. At this time they can be morphologically identified because of their large quantities of myofilaments together with an extensive development of their RER and associated basement lamina (32). In these early time periods they are the only identifiable cell in the thickened intima.

The smooth muscle cell has been implicated by numerous investigators over a long period of time as being the cell associated with the genesis of the atherosclerotic lesion (33–35) and is also considered to be the cell responsible for the synthesis and turnover of the connective tissue components of the medial arterial wall. Recent evidence from both in vivo studies (36) and in vitro studies (37) have clearly demonstrated that the smooth muscle cell is the connective tissue synthetic cell of the media of the arterial wall, and is responsible for the synthesis of collagen, elastic fiber proteins, and probably proteoglycans. Thus

the factors associated with the genesis of the atherosclerotic plaque need to take into account at least three phenomena. These include the factors responsible for the migration and proliferation of smooth muscle cells from the media of the vessel into the intima, those factors associated with the stimulation of the synthesis and secretion of connective tissue proteins and proteoglycans, and the conditions associated with altered lipid metabolism that result in the lipid accumulation both intra- and extracellular within these lesions.

The principal factors responsible for cell migration in a luminal direction and proliferation of smooth muscle cells are not clear. However, there have been a number of observations from in vitro systems to suggest that there may be components normally present in plasma which may act, at least in vitro, as a stimulus for both migration and proliferation of smooth muscle cells (R. Ross, unpublished data) as well as other connective tissue forming cells such as fibroblasts (38, 39). Conceivably, therefore, injury to the endothelium may permit a concentration gradient of normal plasma constituents to be presented to the medial smooth muscle cells resulting in this migration and proliferation. The permeability characteristics of the regenerating endothelium are not well understood and until they are restored, the presence of plasma constituents may be important in smooth muscle proliferation. There may also be a number of other stimulating factors such as abnormal amounts or types of plasma components. These could include various lipoproteins, plasma triglycerides, or other constituents.

*Intimal Damage.*—The injury in these studies appears to be localized to the intima, principally involving the endothelium. Reendothelialization is the only morphological alteration to the vessels other than the smooth muscle proliferation previously described. The internal elastic lamina was not disrupted in these studies nor were leukocytes seen at any time in the media of the vessel wall. The medial smooth muscle cells retained their usual orientation and there was no increase in connective tissue formation within the media. The connective tissue formation that was observed was associated with newly proliferated intimal smooth muscle cells.

*Potential Reversibility.*—This lesion may also be important in terms of its potential reversibility. A further study to determine other factors associated with the genesis of this lesion other than mechanical injury, such as alterations in plasma constituents including increased levels of cholesterol and cholesterol esters, the reversibility of the lesion, and the interrelationship between the factors that produce the lesion, should provide a much clearer insight into the etiology and pathogenesis of atherosclerosis in monkeys and perhaps ultimately in man.

## SUMMARY

Arteriosclerotic lesions have been produced in monkeys (*Macaca nemestrina*) by selective removal of the vascular endothelium with an intra-arterial balloon

catheter. Immediately after de-endothelialization a platelet layer covers the denuded area. This thrombus is gradually removed and by 7 days the vessel appears to be largely reendothelialized. Beginning at day 4, smooth muscle cells undergo modification and migrate through fenestrae in the internal elastic lamina into the intima where they proliferate. By 28 days, the intimal lesion consists of multiple layers of smooth muscle cells surrounded by collagen and elastic fibers and basement-like material. After 3 months the lesions are markedly hyperplastic and contain new extracellular connective tissue elements. In contrast, with no further injury after 6 months the lesion has decreased markedly in size suggesting that it may be reversible in the absence of continued endothelial injury. The importance of endothelial "injury" exposing medial smooth muscle to plasma constituents may be the principal factors associated with the migration and proliferation of the smooth muscle cells into the intima resulting in the lesion. The smooth muscle cells do not contain lipid. The similarities of this lesion to the fibromusculo-elastic lesion or preatherosclerotic intimal hyperplasia in man makes it a useful model for the further study of atherosclerosis.

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Requests for reprints should be directed to Dr. Ross at the University of Washington.

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