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Electron Microscopy of Intimal Plaques Following Induction of Large Superficial Mechanical Injury (Transverse Injury) in the Rabbit Aorta*

Hans-Jürgen Knieriem, Göran Bondjers, and Sören Björkerud

Department of Pathology, University of Düsseldorf
and Departments of Histology and Internal Medicine I, University of Göteborg

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Summary. As has been shown previously, the induction of a superficial injury extending over a large area in the aorta of normolipidemic rabbits is followed by characteristic and reproducible sequential patterns of light-microscopic changes. After a short phase of thickening of the intima and partial regeneration of injured endothelium and injured media, repair is arrested and a phase of delayed repair ensues. The latter is characterized by delayed reendothelialization, excessive thickening of the intima, and accumulation of lipids, mainly extracellularly, in the non-reendothelialized regions.

Characteristic electron microscopic findings in 3- and 7-week lesions were (1) a provisional discontinuous lumenal lining of flattened smooth muscle cells in the central area of the plaques, (2) abundant extracellular fibrin-like osmiophilic material, scarce and immature elastic and collagenous components, and mature smooth-muscle cells in the underlying superficial zone of the intimal thickening, and (3) larger amounts of more mature elastin and collagen fibers and smooth muscle cells with signs of immaturity and proliferation in a basal zone towards the media. As extracellular fibers are soon formed in rapidly reendothelialized, regressive aortic lesions, the present results suggest deficient formation of stress-bearing structures as a factor related to incomplete healing in the non-regressive type of arterial lesions. Increased load of serum constituents due to increased transfer through the discontinuous lumenal smooth muscle lining could interfere with the capacity of the repair tissue for elastin and collagen formation.

Changes indicating cellular injury were apparent in the margin of the regenerating endothelium, in the smooth muscle cell lumenal lining, and in the basal layer of the plaque. It is suggested that shedding of smooth muscle cells from the surface may be a factor favouring elimination of mural thrombotic material.

Introduction

In recent years it has become increasingly accepted that injury to the arterial tissues and the reactions of these tissues to injury constitute major, perhaps central, components of the atherosclerotic process.

The gross characteristics of arterial tissue response patterns following different *defined* types of mechanical injuries are reasonably well known (Björkerud, 1969a, 1969b; Björkerud and Bondjers, 1971b, 1973) but corresponding fine structure data are scarce. The present electron microscopic study of sequential changes following the specific mechanical induction of non-regressive atherosclerotic

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lesions in normolipidemic rabbits was undertaken in an attempt to analyze further the possible atherogenic factors operating in this type of experimental atherosclerosis.

Material and Methods

Eight male, albino rabbits of the Danish country strain, obtained from the same breeder, weighing between 2.4 and 4.3 kg were fed *ad libitum* on standard rabbit pellets (Ewos, Södertälje, Sweden).

Superficial mechanical injury with a large surface area (transverse injury) was induced in the aorta with a microsurgical instrument (Björkerud, 1969; Björkerud and Bondjers, 1973). The animals received i.v. injections of 3 ml of sterile 0.3% Evans blue solution on 2 successive days before sacrifice. The rabbits were killed under sodium pentobarbital anaesthesia at the following time intervals after the induction of the injury: 3 weeks (4 animals; 13 lesions), 7 weeks (2 animals; 3 lesions), and 13 weeks (2 animals; 4 lesions). The abdominal aorta was exposed and the larger branches of the aorta from the coeliac artery to just above the trifurcation were ligated. The aorta was perfused with Ringer's solution, at a pressure of 100 mm of mercury through a catheter introduced into the ascending thoracic aorta by way of the left ventricle. Perfusion was continued until the venous return to the heart was unstained (usually 5–6 min), and an incision was made in the aorta proximal to the coeliac artery, and the catheter was moved to this incision and secured. After 1 min of additional perfusion with Ringer's solution the abdominal aorta was perfused at a pressure of 100–120 mm of mercury with phosphate-buffered 4% glutaraldehyde solution (pH 7.4) in sucrose solution for about 20 min. After the perfusion fixation the abdominal aorta was gently removed, immersed in fresh buffered glutaraldehyde solution and cut open. The lesions and the Evans blue-stained regions of the lesions were studied with a dissecting microscope. Blocks of a total of 19 lesions in unbranched regions from the eight aortae were cut out, divided into small, ca. 1×3 mm segments, and the presence or absence of Evans blue-stain noted. The samples were placed in fresh buffered glutaraldehyde solution at 2–4°C for 2 hours to complete the fixation. They were then washed in sucrose-containing phosphate buffer and postfixed in 1% buffered osmium tetroxide solution for 1 hour. After washing in phosphate buffer the samples were dehydrated in graded acetone and embedded in Durcupan (Fluka AG, Chemische Fabrik, Buchs S.G./Switzerland).

Ultrathin and semithin sections were cut on a Porter-Blum MT 2 or on a Reichert Om U2 ultramicrotome. The ultrathin sections were stained with uranyl acetate and lead citrate and examined with a RCA-EMU 3 G or with a Siemens 101 electron microscope at 50 or 60 kV. The semithin sections were stained with toluidine blue and Azur II (Richardson *et al.*, 1960) and studied with a conventional light microscope to localize the different defined regions of the lesions (pond, bank, land, see Result section).

Results

Macroscopically, the injured segments were characterized by the presence of irregular intimal thickenings. The margins of these thickenings were slightly more elevated (designated "bank") than the central area (designated "pond", the surrounding non-thickened regions are designated "land"; Fig. 1). The macroscopic properties of the lesions were consistent with those reported in a previous paper (Björkerud and Bondjers, 1973).

Lesions at 3 Weeks after Injury

Most of the lesions consisted of relatively large, semilunar or almost circumferential intimal thickenings. The different regions of the lesions (pond, bank, land, see above) were identified. In a few lesions only bank regions could be found. On semi-thin sections two or three concentric layers of the intima were

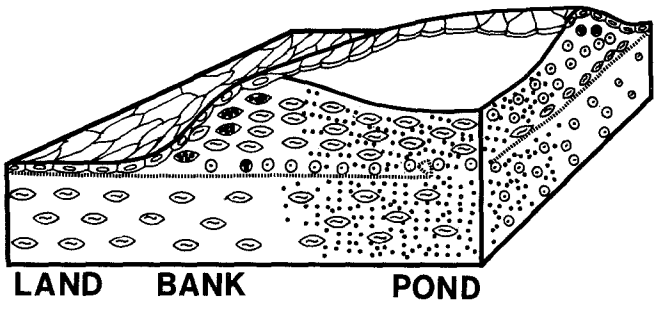

Fig. 1. Schematic illustration of the macroscopic properties and the designations used for different regions of a lesion following the induction of a superficial injury with a large area in the rabbit aorta

discerned (Fig. 2a). Towards the lumen there was a superficial zone with the smooth muscle cells predominantly arranged circumferentially. Towards the media the cells were arranged longitudinally as concentric palisades. In the intermediate zone, if present, the cells were arranged more irregularly (Fig. 2a). One lesion was dilated to a small aneurysm; the media in this lesion was calcified. The light microscopic characteristics of the lesions in this phase of development have been described recently (Björkerud and Bondjers, 1973).

Several types of surface lining cells were present three weeks after the induction of the injury. Cells with morphological properties conforming to the traditional concept of endothelial cells, i.e. flat cells with bulging nucleus and rather osmiophobic cytoplasm appeared on bank and land regions. Some of these cells had a loose, swollen cytoplasm (Fig. 3). On the inner slope of the banks towards the ponds, rounded cells, protruding into the lumen (Fig. 4), were also observed. Numerous very small processes were present on their lumenal side (Fig. 4). They had the properties which have been described for injured endothelial cells (see Discussion).

On the ponds and parts of the banks the surface layer consisted of smooth muscle cells which were arranged as a discontinuous sheet with large intercellular gaps (Fig. 5). The gaps were either filled with tentacle-like processes extending from the underlying smooth muscle cells or with fibrin-like osmiophilic material (Fig. 5). The smooth muscle cells at the surface frequently had numerous, often branched or vesiculated processes extending into the lumen (Fig. 5). Numerous vesicles were present in these cells at the ablumenal side and towards the intercellular gaps (Fig. 5). Some of the vesicles were in direct continuity with the extracellular space (Fig. 5).

No red blood cells, monocytes or platelets were observed.

The intimal plaques consisted most commonly of two zones of smooth muscle cells, as also noted on the semi-thin sections (see above). The cells of the zones were slightly different with regard to shape and arrangement. The cells of the superficial layer were usually oriented circumferentially, were roughly spindle-shaped and had several finger-like processes (Figs. 2a, 4–5).

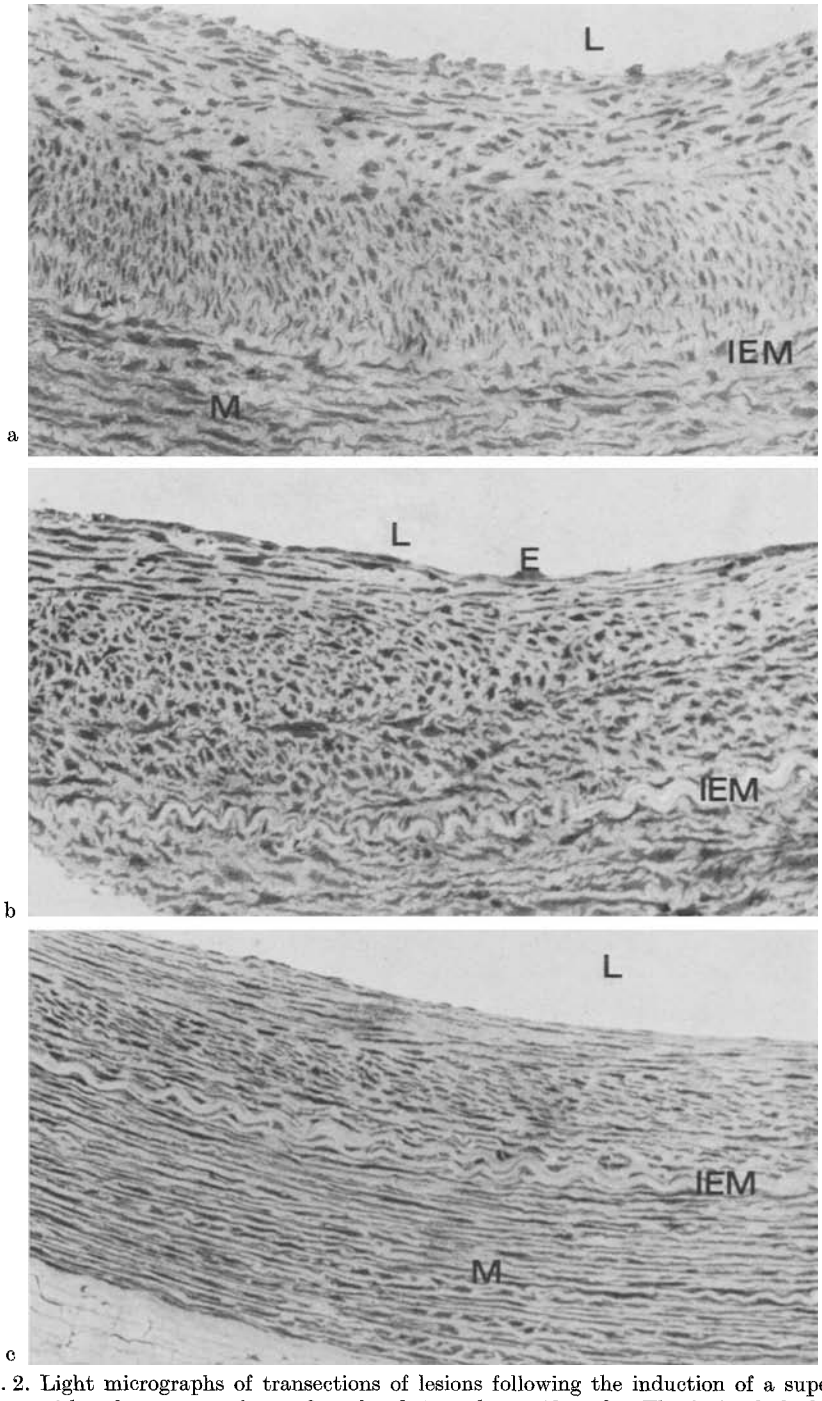

Fig. 2. Light micrographs of transections of lesions following the induction of a superficial injury with a large area after a 3 weeks; b 7 weeks; c 13 weeks. The intimal thickenings have 2–3 welldefined layers. Semithin sections. *L* lumen; *IEM* internal elastic membrane; *M* media. ×310

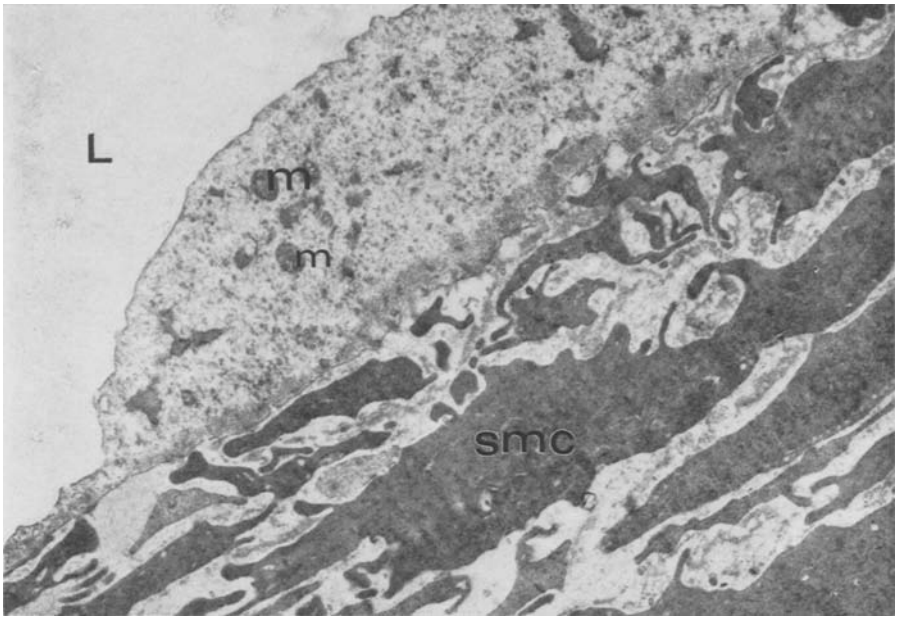

Fig. 3. Distended endothelial cell overlying intimal smooth muscle cells (*smc*) in a 3-week lesion. Small mitochondria (*m*) are present within the edematous cytoplasm. *L* lumen. ×12000

The smooth muscle cells of the basal portion of the intimal plaque were more densely packed and showed a more rounded or oval shape (Fig. 6). They had prominent nuclei (Fig. 6) and a prominent rough endoplasmic reticulum (Fig. 6). As a rule a few short processes extended from the cell body.

In the border zone between the two layers and in the basal layer, smooth muscle cells were found which contained lipid inclusions (Fig. 7) and phagocytic material as hemosiderin granules (Fig. 7).

Throughout the intimal plaque the smooth muscle cells had closely packed vesicles in the superficial part of the cytoplasm (Fig. 5). Some of the vesicles were in direct continuity with the extracellular space. The vesicles were most numerous in the superficial zone of the intimal plaque.

In the superficial layer the wide extracellular clefts contained increased amounts of ground substance with large amounts of fibrin-like material which was very osmiophilic (Fig. 5). The markedly increased osmiophilia was particularly pronounced in the central non-reendothelialized portion of the lesions, i.e. in the pond region. The basal zone contained ground substance, loosely arranged collagen fibrils, immature elastic matrix and, occasionally, fine elastin filaments.

Although scattered foam cells were observed (see above) accumulations of large numbers of such cells were not seen.

The smooth muscle cells of the media underlying the intimal plaque had a more pronounced, rough, endoplasmatic reticulum than medial smooth muscle

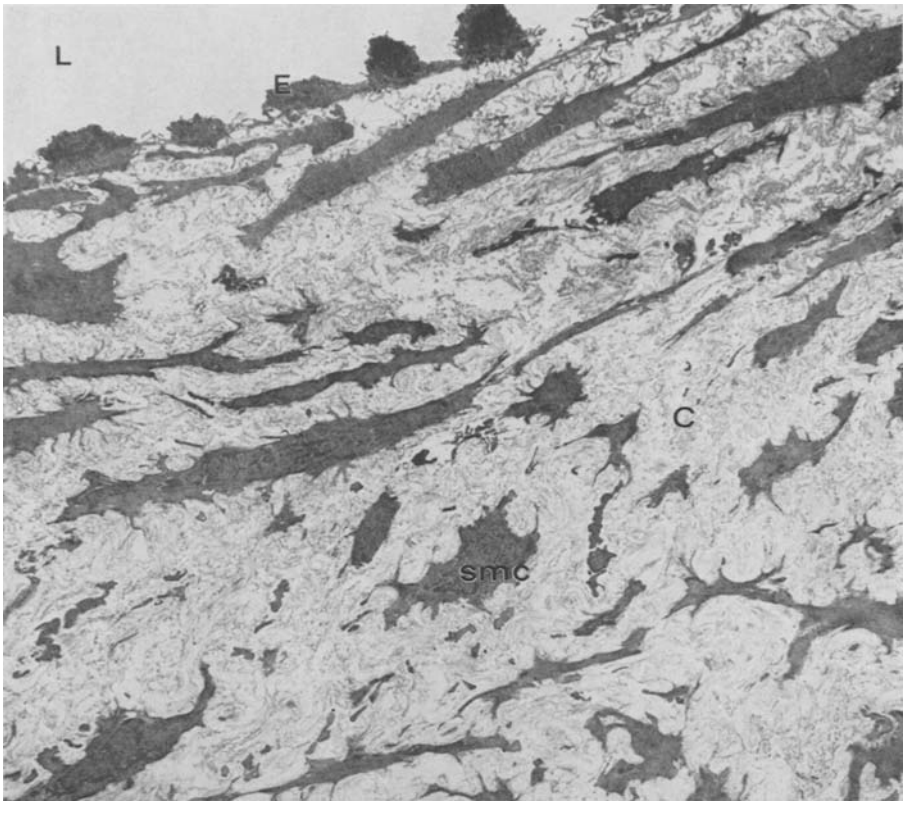

Fig. 4. Section through the superficial and middle intimal zones of a 3-week lesion. The surface layer consists of endothelial cells (*E*) with signs of injury, *viz*, distension (budding) and rough surface. The underlying smooth muscle cells (*smc*) have numerous finger-like processes. The wide extracellular clefts contain ground substance and collagen fibrils (*C*), *L* lumen. × 1900

cells of non-thickened segments. Accumulations of lipids were not found in these cells. In one lesion the internal elastic membrane was ruptured, and several polymorphic, distorted smooth muscle cells were present in the gap. Vasa vasorum were not found in the media or intima.

In some lesions the capillaries of the adventitia were dilated and filled with numerous red blood cells (despite the postvital elimination of blood; see Material and Methods). In two sections small infiltrations with lymphocytes were seen in the adventitia.

Lesions at 7 Weeks after Injury

The 7-week lesions were more flattened than the 3-week lesions. Layering of the intimal thickening could still be distinguished.

In the non-endothelialized regions, the smooth muscle cells towards the lumen were flat, had a very dense osmiophilic cytoplasm, and a small, elongated

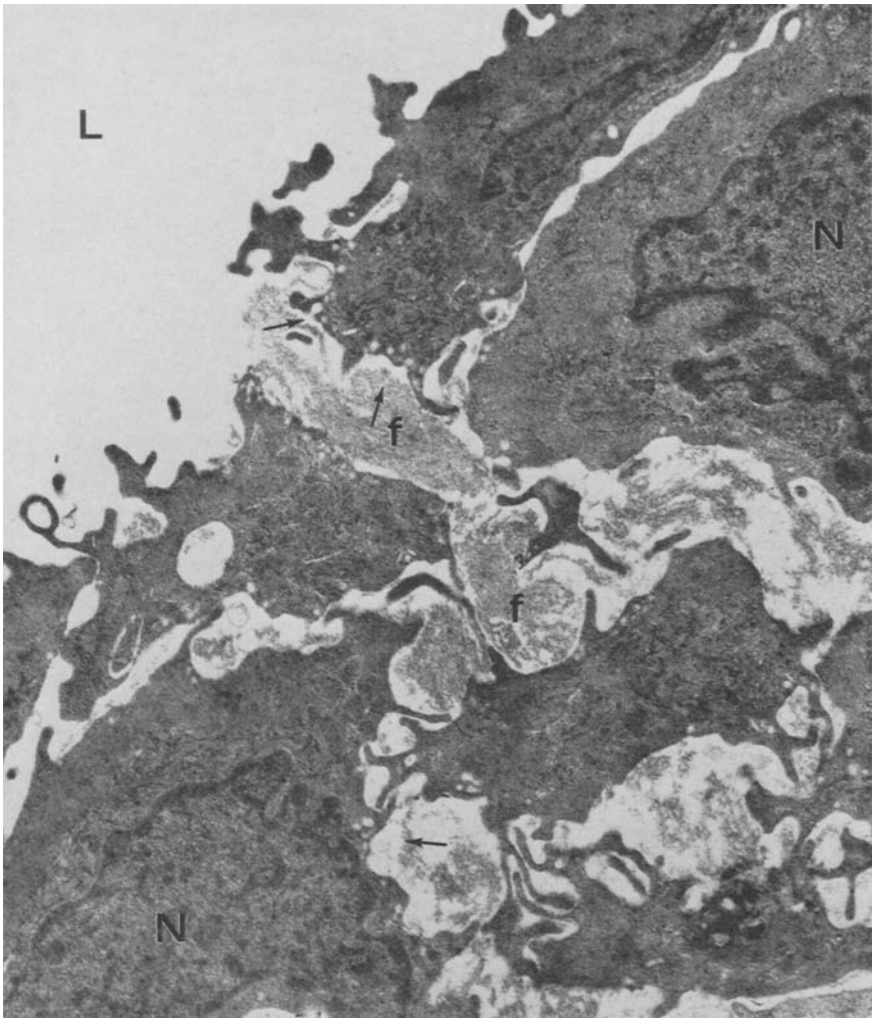

Fig. 5. The superficial intimal cell layer of the central non-reendothelialized region (pond) of a 3-week lesion. Slightly flattened smoth muscle cells constitute a surface lining. The smooth muscle cells have numerous surface vesicles (arrows), some of which are in direct continuity with the extracellular space. Numerous smooth muscle cell processes extend into the lumen and into the extracellular space of the intima. Fibrin-like osmiophilic material (*f*) is present in intercellular clefts of the surface lining and in the underlying tissue. ×19800

nucleus. The intercellular gaps were in general more narrow than those 3 weeks after injury (Fig. 8). As for the smooth muscle surface cells observed 3 weeks after injury the superficial portion of the cytoplasm contained mainly ablumenally, numerous small vesicles, some of which were in direct continuity with the extracellular space or lumen. Some surface cells exhibited signs of injury. Their cytoplasm was markedly vacuolated; the surface was rough and the cells were attached

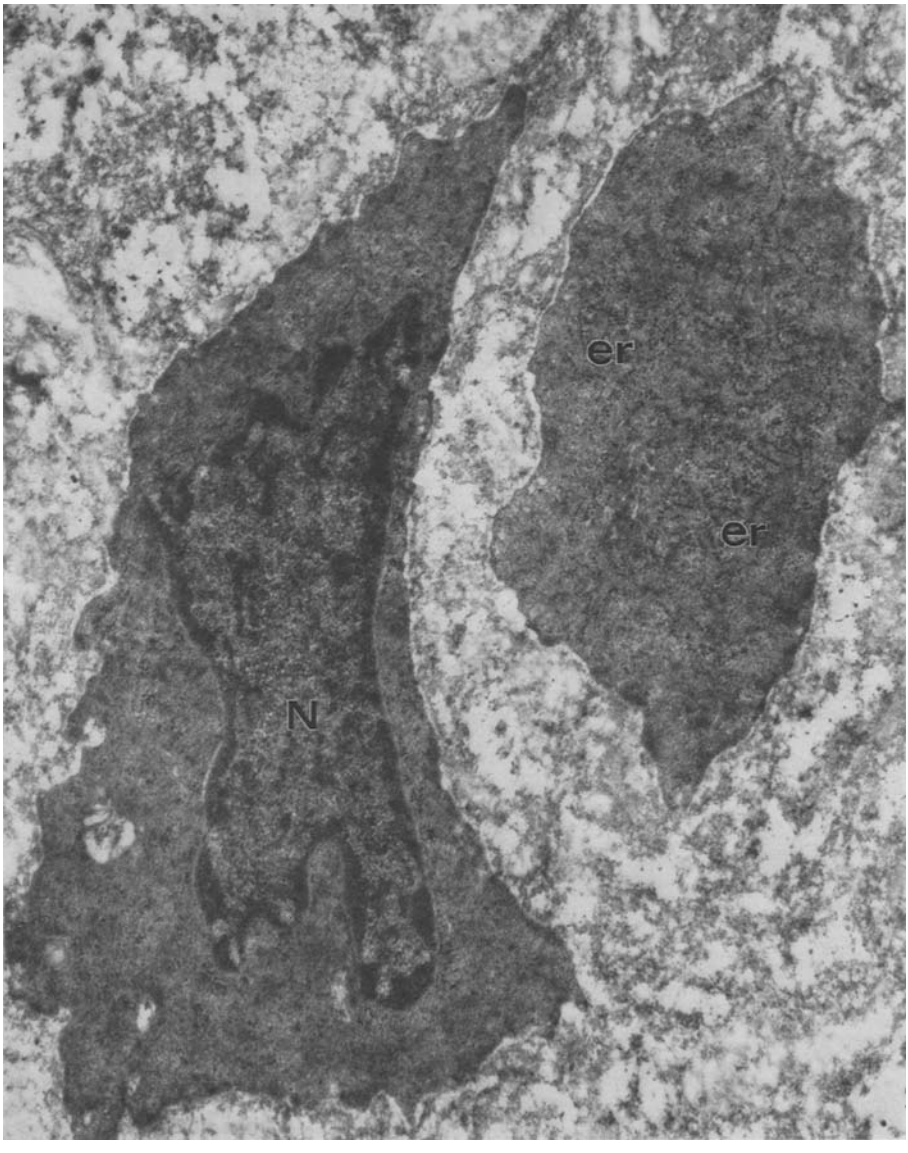

Fig. 6. Smooth muscle cells of the basal intimal layer with few processes and surface vesicles in a 3-week lesion. Note the prominent rough endoplasmic reticulum (*er*). ×2800

to the underlying cells with numerous short processes or partially desquamated (Fig. 8).

The smooth muscle cells of the pond regions of the thickened intima were elongated and had numerous processes (Fig. 8) and superficial vesicles. The rough endoplasmic reticulum was less prominent than 3 weeks after injury. Some cells contained accumulations of hemosiderin-like material or lipids. The

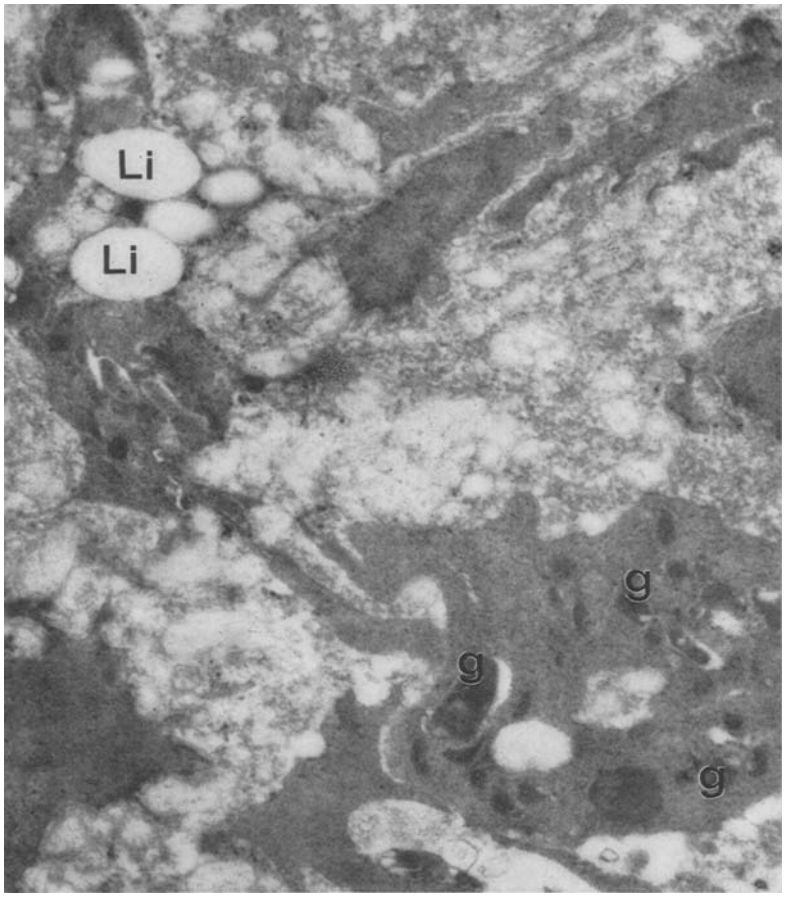

Fig. 7. Cells containing lipid droplets (*Li*) and hemosiderin granules in the middle intimal zone of 3-week lesion (cf. Fig. 2a). ×14800

cleft between the cells were wide and contained increased amounts of immature elastic material and of collagen fibers (Fig. 8). Occasionally, small pieces of more mature elastic lamellae were seen. Small accumulation of cell debris or small foci containing calcified collagen fibers were present, predominantly in reendothelialized parts of the plaques.

The media underlying the thickened intima contained smooth muscle cells with moderately increased endoplasmic reticulum and moderate numbers of vesicles. No fatty changes, nor necroses, were encountered.

Lesions at 13 Weeks after Injury

The intimal thickenings were flattened (cf. Fig. 2c). Despite the attenuation of the plaques two intimal zones could often be discerned.

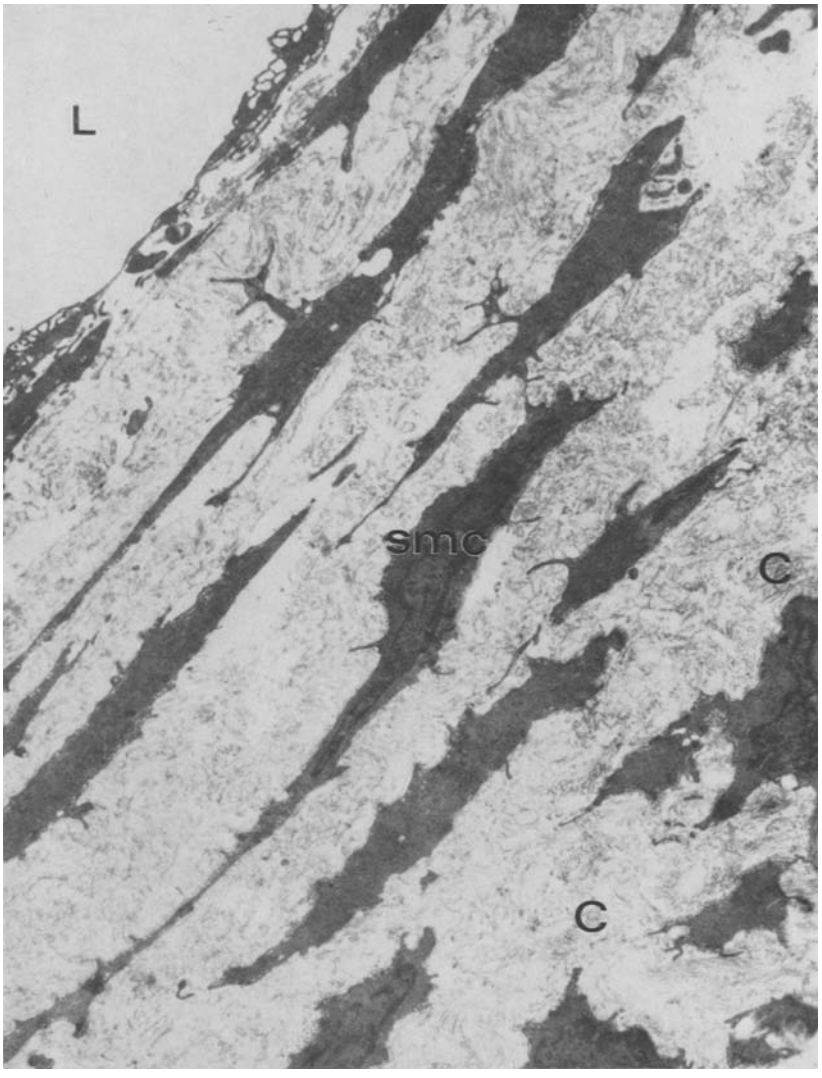

Fig. 8. Intima of 7-week lesion. The smooth muscle cells on the surface are markedly vacuolated, and partially detached. The underlying smooth muscle cells have numerous processes and surface vesicles. The wide extracellular space contains collagen fibrils (*C*) in its basal portion. ×7700

Fig. 9a and b. Electron micrographs of intimal tissue of 13-week lesions. a Well-developed collagen fibrils (*C*) and irregular portions of elastic matrix are present in the intercellular space. b The superficial zone of the intima in a reendothelialized area contains granular calcifications (arrows) of collagen fibrils (*C*) and welldeveloped elastin lamellae. a ×12000; b ×5800

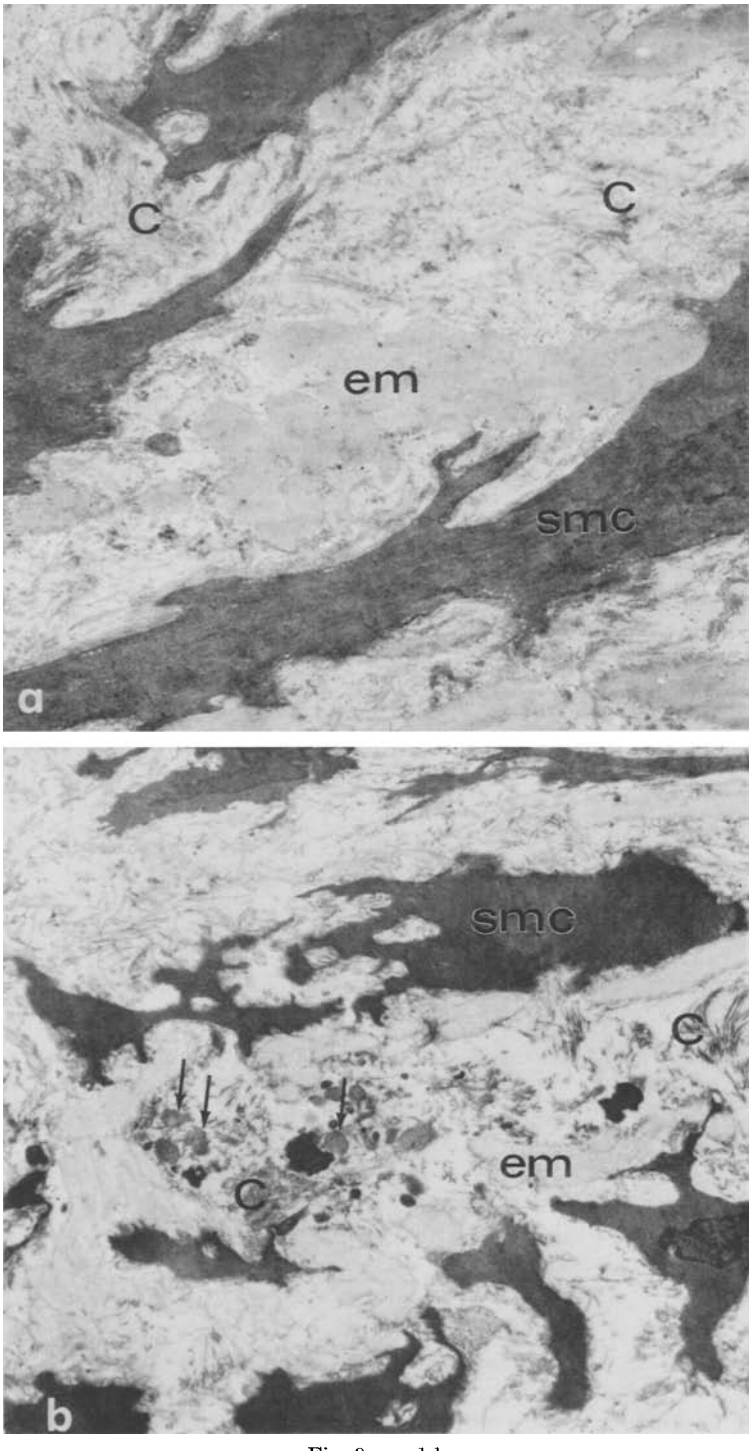

Fig. 9a and b

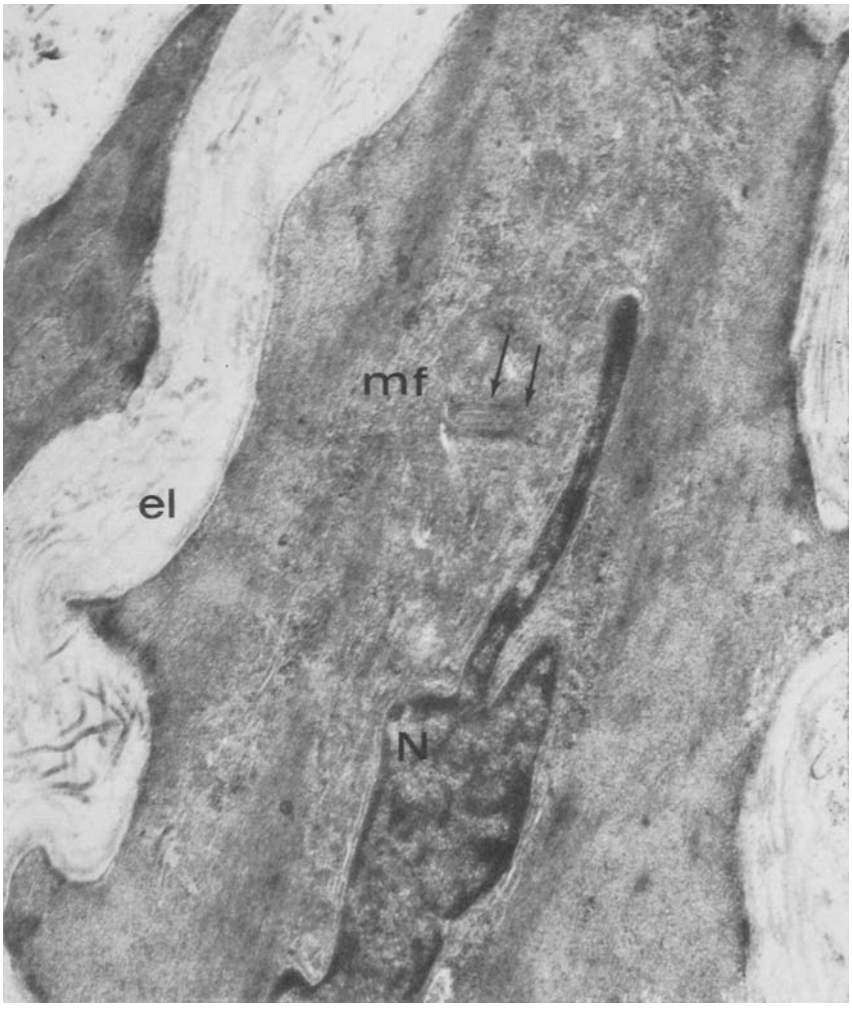

Fig. 10. Smooth muscle cell of the media of a 13-week lesion with a focal disintegration of myofilaments (*mf*) and of the perinuclear cytoplasm (double arrow). Elastin lamellae (*el*) and collagen are present extracellularly. ×5800

The surface layer of all lesions was composed of elongated attenuated cells. In the central region of the plaque (pond), the surface cells had the characteristics of flat, smooth muscle cells, while the periphery was covered with endothelial cells. Although rare, distended protruding cells were also observed in the surface layer. The surface layer did not contain large intercellular gaps in contrast to the lesions at 3 weeks after injury. Mural thrombi were not seen.

The smooth muscle cells of the intima were very elongated. The cellularity of the tissue was decreased as compared to that at shorter time intervals after injury. The cells were surrounded by large amounts of extracellular material,

containing mature collagen fibers and irregular pieces of elastic matrix and elastin filaments (Figs. 9a, 10). Foci with finely granular calcification of collagen, cell debris with myelin figures, and with whorls of fine elastin filaments were also seen (Figs. 9a, b). No apparent difference was observed between the cells of the media of the lesions and those in non-thickened regions. In one lesion, necrotic smooth muscle cells were observed in the media of an endothelium-covered bank.

Typical monocytes or fibroblasts were not encountered in the lesions at any of the time intervals studied.

Discussion

The electron microscopic properties of the intimal thickenings induced by superficial injury with a large area verified the smooth muscle nature of the thickenings (Björkerud, 1969b; Björkerud and Bondjers, 1973). At three weeks after the induction of the injury the plaque consisted of proliferating smooth muscle tissue. A provisional lining of the non-reendothelialized areas was provided by flattened smooth muscle cells at this and later stages. It is probable that the discontinuities present in the smooth muscle cell lining, i.e. the wide intercellular gaps or clefts, may represent a morphological background for increased permeability for serum components, such as albumin (Björkerud and Bondjers, 1971a) and lipoproteins (Bondjers and Björkerud, 1972, 1973) in the non-reendothelialized regions. A further factor which could contribute to increased permeability may be the enlargement of the surface area due to the presence of numerous processes and surface vesicles which might facilitate transcellular migration of serum constituents.

Some of the smooth muscle lining cells had properties indicating cellular injury, i.e. cellular swelling with budding into the lumen and markedly vacuolated cytoplasm (Björkerud and Bondjers, 1972). Such cells were often weakly attached to the underlying wall by fine processes, or were partly detached (cf. Fig. 8) which suggests the shedding of smooth muscle surface cells into the blood stream. Mural thrombi were rare, especially in the central part of the non-endothelialized regions (Björkerud and Bondjers, 1973), and it is possible that shedding of the surface layer may be a factor acting against mural thrombotization.

The peripheral parts of the plaques, i.e. the banks, were partly covered with endothelium, which was in continuity with the endothelium of the non-thickened segments. The structure of the majority of the endothelial cells was in conformity with that reported earlier by others (Ashford and Freiman, 1967; Tsao, 1970). However, cells with signs of injury, e.g. vacuolation of the cytoplasm, combined with the presence of processes towards the lumen, and distension of the cell with budding into the lumen, were not uncommon, especially on the banks at the border towards the non-reendothelialized regions (cf. Fig. 4). The major retardation of the reendothelialization of the lesions occurred on the banks, where also mural thrombi or flaplike detachments of endothelium have been found (Björkerud and Bondjers, 1973). The factors responsible for the retardation of endothelial regeneration are unknown. However, as the lumenal width decreases abruptly at the banks, the surface of the banks might be subject to increased shearing stresses due to increased rate of flow (for review see Glagov, 1972).

In addition, morphological (Björkerud, 1969b; Björkerud and Bondjers, 1973) as well as mechanical (Björkerud, Bondjers, and Viidik, in preparation) properties were different in bank and pond regions with less resistance to distension in the latter. It is conceivable, therefore, that large intramural strains may occur at the border between these regions which also may interfere with the regeneration of the endothelium.

A regular finding was the layering of the intimal thickenings. The basal layer contained densily crowded, activated smooth muscle cells with few processes and surface vesicles. Immature elastin matrix, fine elastin filaments and thin collagen fibrils wer present intercellularly. Such properties are characteristic of young, rapidly proliferating media (Stein *et al.*, 1969, 1971; Karrer, 1961; Björkerud, 1973) of young non-atherosclerotic intimal tissue (Björkerud, 1969a, 1973; Stemerman and Ross, 1972) and of arterial thrombi in the state of organization (Jurukova and Knieriem, 1970; Knieriem, 1972a, b). The superficial intimal layer had a number of cellular properties of old "dormant" arterial tissue, i.e. low cellularity, numerous cellular processes and surface vesicles, and a poorly developed rough endoplasmic reticulum (Stein *et al.*, 1969, 1971; Björkerud, 1973). However, extracellular components characteristic of mature smooth muscle tissue, i.e. elastin fibrils or lamellae and collagen fibers (Haust *et al.*, 1965; Haust and Geer, 1970; Knieriem, 1967, 1970) were lacking in the superficial layer which was also observed in earlier light microscopic studies (Björkerud, 1969b). The sequential development of intimal thickenings after mechanical injury indicates that the inner media represents the source of the intimal smooth muscle cells (Björkerud, 1969a; Hassler, 1970), but multiplication of smooth muscle cells has been observed in the basal layer of plaques (Hassler, 1970; Björkerud and Bondjers, 1973). This would lead to a radial "age" gradient in the intima with increasing age of the cells towards the lumen, a suggestion which is supported by the morphological results of the present study. However, non-atherosclerotic, regressive, intimal thickenings, induced by a slightly different type of mechanical injury, are rich in elastin and collagen fibers (Björkerud, 1969a, 1969b). Therefore, it is possible that deficient formation of these stressbearing structures may be an important factor for the delayed and incomplete healing of the atherosclerotic experimental lesions.

Fibrin-like osmiophilic material was present in the extracellular space of the intima. It was most abundant in the superficial layer and almost filled the intercellular clefts below and the gaps between the smooth muscle surface lining cells (cf. Fig. 5). As the non-reendothelialized regions have an increased permeability for serum constituents, such as albumin and lipoproteins (see above), it is probable that the presence of the fibrin-like osmiophilic material reflects an increase of serum constituents in the tissue. It is noteworthy that elastin and collagen fibrils were virtually absent in such regions where osmiophilic fibrin-like material occurred. This could reflect interference with the formation of collagen and elastin in a repair tissue overloaded with serum constituents.

Certain changes in the intimal tissue seemed to be related to the time elapsed after the induction of the injury. Such changes were: less prominent rough endoplasmic reticulum in the cells, and increased amounts of collagen and mature elastin, the latter also reflected as a decreased cellularity of the tissue. Such

changes are characteristic of mature, less actively proliferating, arterial tissue, as observed in arterial media of un-manipulated (Stein *et al.*, 1969) or growth-retarded (Stein *et al.* 1971) animals and in intimal thickenings of non-atherosclerotic experimental lesions (Björkerud, 1973). Necrotic foci and focal calcifications were more numerous with increasing age of the lesions. However, from the results of the present study it is difficult to evaluate, if the focal decrease of viability of the tissue was a direct expression of increasing age or related to a higher degree of integrity of the overlying surface lining, as suggested by earlier observations (Björkerud and Bondjers, 1973).

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Priv.-Doz. Dr. H.-J. Knieriem
Pathologisches Institut
Universität Düsseldorf
D-4000 Düsseldorf, Moorenstraße 5
Federal Republic of Germany

Doc. S. Björkerud, M.D.
Arterial Biology Section
Utvecklingslab.
Sahlgren's Hospital
S-41345 Göteborg
Sweden

Doc. G. Bondjers, M.D.
Dept. of Histology
Fack
S-40033 Göteborg
Sweden