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Ultrastructural Changes of Large Rabbit Blood Vessels Following Mild Mechanical Trauma

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Gefäßveränderungen nach mildem mechanischem Trauma

Zusammenfassung. Morphologische Veränderungen der Aorta abdominalis und der Vena cava inferior ein bis sieben Tage nach mildem mechanischem Trauma (Quetschen der Gefäßwand mit einer von Gummi überzogenen Klemme) wurden untersucht. Silberimprägnierte Totalpräparate werden lichtmikroskopisch und Gefäßschnitte elektronenmikroskopisch durchmustert. Sowohl in der Vena cava als auch in der Aorta abdominalis werden zu allen untersuchten Zeiten folgende morphologische Veränderungen gefunden: Fokale Nekrose, abnorme Überlappung und zum Lumen gerichtete Pseudopodienbildung der Endothelzellen und der medialen glatten Muskelzellen, die oft durch Fenestrae der elastischen Membran in die Intima hineindringen. Intramurale Leukocytenanhäufungen wurden nur in der Vena cava gefunden. Eine Vermehrung der Grundsubstanz war nur im Bereiche der Myointimalzellen der Aorta sichtbar. Herkunft und Bedeutung der oben erwähnten Gefäßveränderungen werden diskutiert.

Summary. The morphological changes of the abdominal aorta and inferior vena cava were studied from one to seven days following application of mild mechanical trauma (compression of the vessel wall with a clamp covered by rubber tubing). Both light microscopy of silver-stained surface preparations and electron microscopy of sectioned vessels were performed. The following morphological changes were observed in both the vena cava and abdominal aorta at all time sequences investigated: focal necrosis, abnormal overlapping of endothelial cells and pseudopod-formation from them usually directed into the lumen; and pseudopod-formation from medial smooth muscle cells, often passing into the intima via fenestrations in the elastic membrane. Leucocytes accumulated in the vessel wall only in the vena cava, whereas the ground substance increased only around myointimal cells of the aorta. The genesis and significance of the above changes are discussed.

Introduction

Earlier investigations by Gottlob and Zinner (1962) demonstrated distinct differences in the morphologically characterized changes between hard and mild trauma to blood vessels using light microscopy of silver-stained surface preparations. Whereas hard mechanical trauma (ligation of the vessel wall with thick silk thread) produced complete destruction of endothelial cells at the ligated area and eventual overgrowth by regenerating endothelial cells, mild mechanical trauma (compressing the vessel wall with a clamp covered by rubber tubing) was characterized by early focal endothelial injury, eventual formation of multinucleated endothelial cells and endothelial phagocytosis of degenerated blood cells. Although such hard mechanical trauma has been used by a number of investigators

as a model for studies of thrombosis and endothelial regeneration (Poole, Sanders and Florey, 1958; Cotton, Harwood and Wartman, 1961; Gottlob and Zinner, 1962), it is felt that mild mechanical trauma mimics more closely pathophysiological phenomena in the human circulation, such as inflammation and surgical injury. Recent investigations have indicated that intense mechanical injury to the intimal lining of the arterial wall enhances atherosclerosis in cholesterol-fed rabbits (Friedman and Byers, 1965). It would be of interest to see if any of the morphological changes characteristic of very early atherosclerosis could be observed at periods following mild mechanical trauma without inducing hyperlipemia.

Whereas studies on surface preparations of blood vessels enable the investigator to assess the overall change in the endothelial lining, including the viewing of focal injury and multinucleated giant cells, electron microscopy of the vessel wall could very well elucidate other changes below the resolution of the light microscope. For this reason an investigation (using the electron microscope) was undertaken to study morphological alterations in the wall of the rabbit abdominal aorta and inferior vena cava at periods ranging from one to seven days following application of mild mechanical trauma, and comparing the results from electron microscopy of sectioned vessels and light microscopy of silver-stained surface preparations.

Material and Methods

Male and female rabbits four months old and weighing between 1500 and 2000 gm were anesthetized with ether. Under aseptic conditions the abdominal cavity was opened with a midline incision and a segment of both the abdominal aorta and inferior vena cava exposed just below the renal bifurcation. A bulldog clamp with jaws covered by rubber tubing of one mm thickness was applied to the two apposing blood vessels concurrently for ten seconds. The clamp was then removed and the abdominal cavity closed. The injured area was labelled by inserting a wire suture into the lumbar muscle at the level of injury. A total of 25 animals was used in this study and from two to three animals for each time sequence.

At periods of 1, 2, 3, 4, 5 and 7 days after initial injury, animals were sacrificed and following an *in situ* fixation, narrow segments of the injured part of the blood vessels were removed for electron microscopy. The entire aortic and venous beds were fixed by an *in situ* perfusion with 5% glutaraldehyde in 0.1 m cacodylate buffer pH 7.4. Briefly the perfusion consisted of infusing the fixative through catheters placed in the thoracic aorta and vena cava close to the heart and allowing the fixative to flow out of the previously incised femoral arteries and veins. Uninjured segments of the two blood vessels were removed at the same location as the test segments from ten rabbits as controls following fixation and then prepared for electron microscopy. The glutaraldehyde fixed segments were then washed in 0.1 m cacodylate buffer and immersed in 1% osmium tetroxide in 0.1 m veronal-acetate buffer pH 7.4 at 4° C for two hours. This post-fixation was followed by dehydration in graded ethanols and embedding in Epon.

Cross- and longitudinal sections of Epon-embedded blood vessels were cut on a Reichert Ultramicrotome. One micron-thick sections were stained with toluidine blue and viewed with the conventional microscope for survey purposes. Ultrathin sections were viewed with the Zeiss EM 9 and Siemens Elmiskop I electron microscopes.

Parallel observations using the same time intervals were also repeated with surface preparations according to the method of Gottlob and Zinner (1965).

Results

The following morphological changes relative to the controls were found at all the time sequences in this study. No specific progression of alterations could be found:

1. Leucocytes were localized within and adhering to the walls of the vena cava only. Polymorphonuclear neutrophils (PMN) could be seen resting closely apposed to the basal side of the endothelial lining (Fig. 1a), surrounded by endothelial cytoplasm (Fig. 1b) or entrapped in the elastin-mucopolysaccharide matrix between smooth muscle cells (Fig. 1b). In some areas (Fig. 1a and b) mitochondria were drastically swollen in all cells. In these areas the nuclear chromatin of PMN was diffusely spread and extremely electron-dense. Usually a gap containing fragmented elastica existed between the endothelial lining and a leucocyte (Fig. 1c). It was curious that the PMN only rarely demonstrated pseudopodia. The large number of leucocytes found just basal to the endothelial layer suggests an interendothelial passage rather than via vasa vasorum, although no leucocyte diapedesis could be demonstrated. Venous smooth muscle cells were also in a most intense state of contraction in areas filled with PMN. The rare finding of a PMN completely surrounded by endothelial cytoplasm as in Fig. 1b together with the oblongshape of endothelial cells protruding into the lumen, suggests that the sections in Fig. 1a and b were cut tangentially to the vessel axis. The PMN apparently surrounded or engulfed by an endothelial cell could in reality be merely an adhering cell which has pressed forward into the plane containing the endothelial cell. Some intramural leucocytes (Fig. 1c) contained numerous autophagic vacuoles, implying that this cell has phagocytosed some material just before or during intramural penetration. Intramural localization of leucocytes was never found in arteries. Thrombocyte adhesion to the endothelia was seen only immediately after injury in preliminary experiments using surface preparations but not at later times.

2. The following changes in the endothelial lining were observed in both the vena cava and abdominal aorta, and were characterized by their extreme focal nature.

a) Isolated necrotic endothelial cells (characterized by ruptured organelle and cell membranes) were often seen between normal endothelia, as were endothelial cells with an abnormal but non-necrotic appearance. Whereas the normal endothelial cell has a more electron-dense ground cytoplasm, and narrow cisternae of rough surfaced endoplasmic reticulum (upper cell in Fig. 2c), the abnormal but non-necrotic cell (lower cell (Fig. 2c) had a much more electron-translucent ground cytoplasm and contained swollen cisternae of the endoplasmic reticulum. Often as in Fig. 2c these cisternae were filled with a granular substance more electron-dense than the cell's ground cytoplasm. The individual necrotic cells observed with the electron microscope appeared to have their surface preparation analogue in the darkly stained cells (uptake of silver) in Figs, 2d and e, lying in narrow bands parallel to the vessel axis. In surface preparations and in an earlier study (Gottlob and Zinner, 1962) these narrow bands of necrotic endothelial cells become narrower with progressing times following injury. No such progression could be seen by electron microscopy.

b) Another ultrastructural alteration was the frequent observation of cells lying just basal to the endothelial lining forming intercellular gaps with the lining morphologically identical to the interendothelial junction. These cells (Fig. 2a) were usually more electron-translucent than the endothelial lining, but otherwise had an appearance identical to the endothelial cell. Sections through

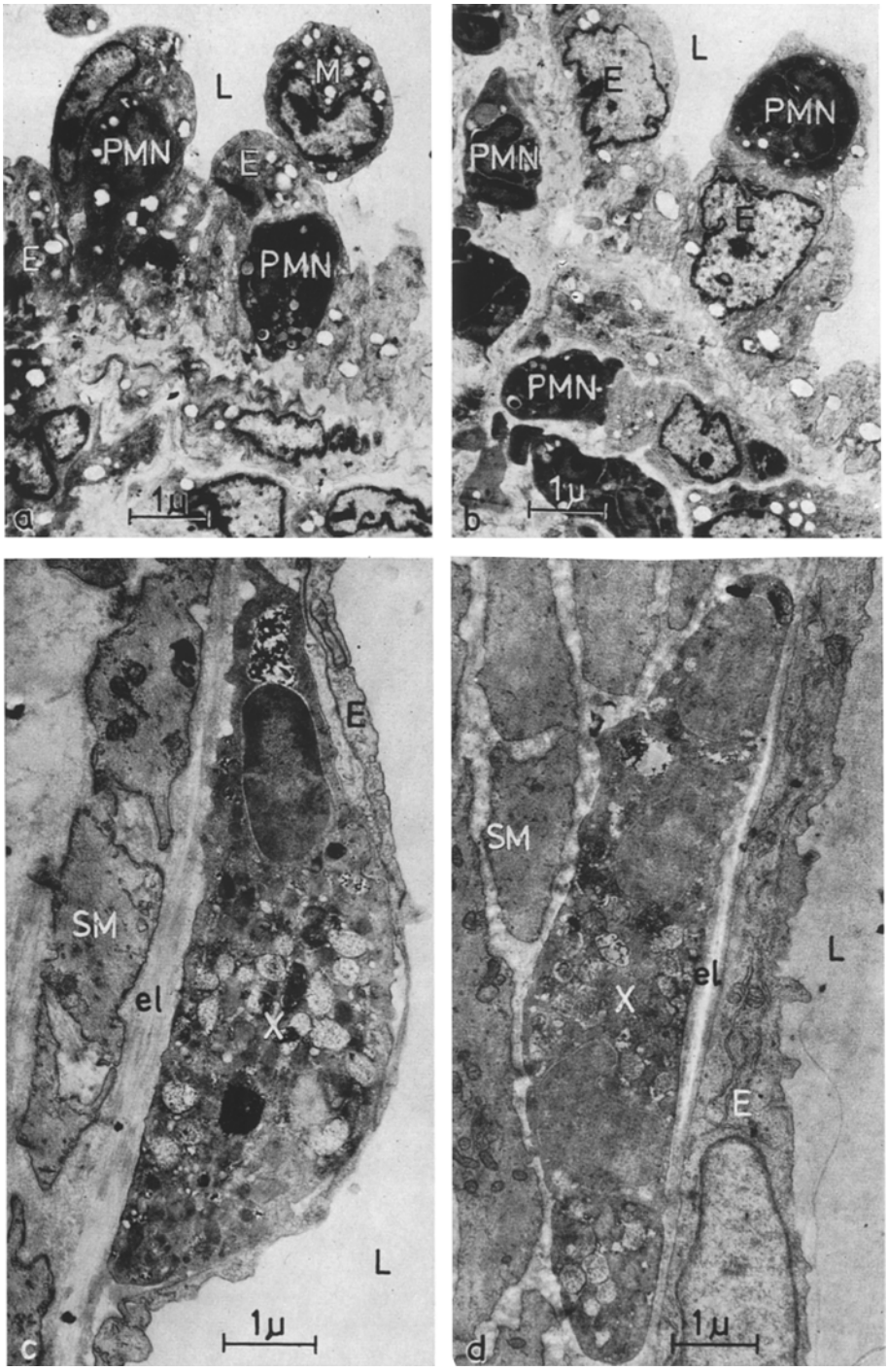

Fig. 1a—d. Intramural localisation of leucocytes in the rabbit vena cava. (*L* lumen, *E* endothelia, *M* monocyte, *PMN* polymorphonuclear neutrophil, *X* leucocytes, *el* elastic membrane, *SM* smooth muscle cell)

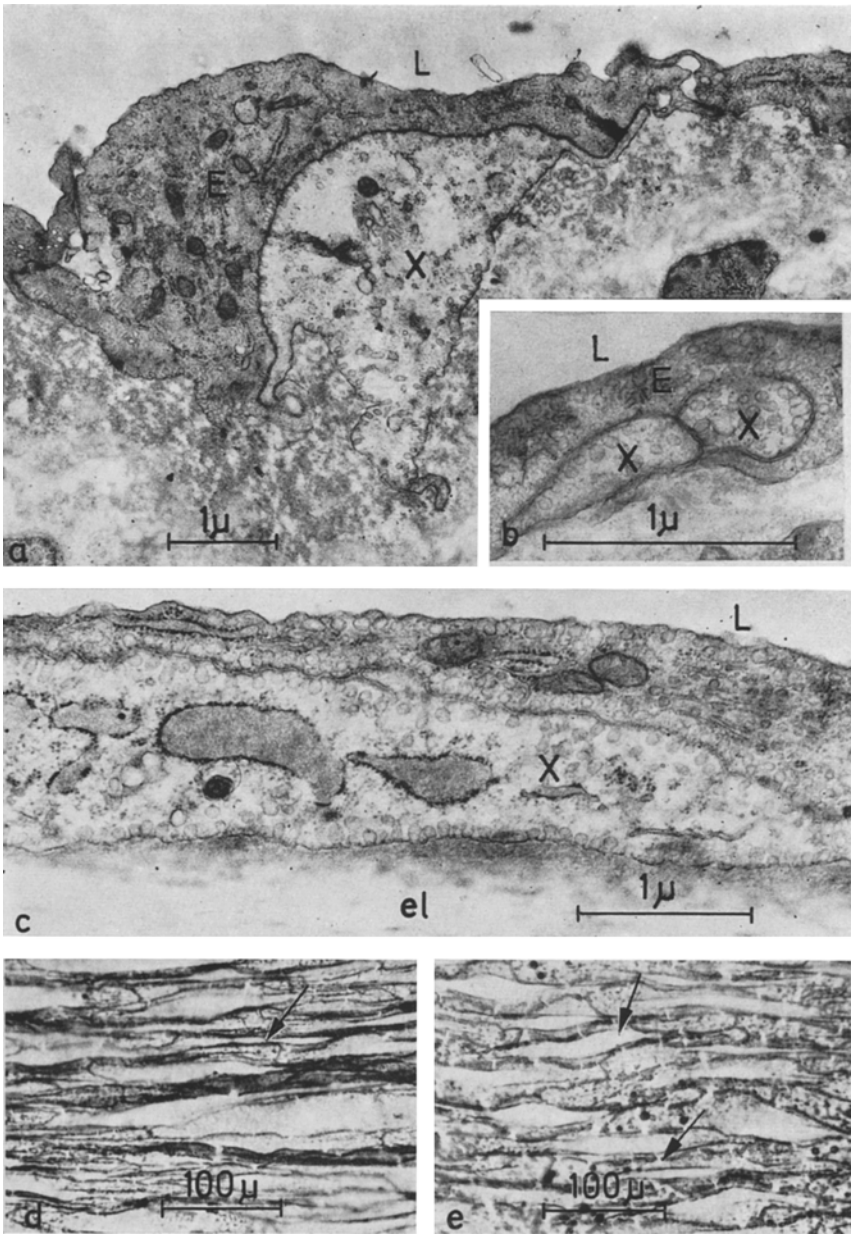

Fig. 2. a Cell with electron-translucent ground cytoplasm (*X*) just basal to the intact endothelial lining (*E*) of the rabbit aorta four days following mild mechanical trauma. b Same as (a) except that the cytoplasmic matrix of two cytoplasmic extensions (*X*) is as electron-dense as the endothelial cell (*E*). Note abundance of micropinocytotic vesicles. c Illustrates an electron-translucent endothelial cell with swollen cisternae of the endoplasmic reticulum (*X*) closely apposed to a morphologically normal endothelial cell. (*L* lumen, *el* elastic membrane) d—e Silver-stained surface preparation of the rabbit vena cava three days following trauma. Note the isolated bands of injured cells in (d) (dark areas) and the appearance in (d) and (e) of one network of silver lines above another (arrows)

nuclei of such cells were rare but present. They appeared to run with their length parallel to the vessel axis since longitudinal sections showed long stretches of the

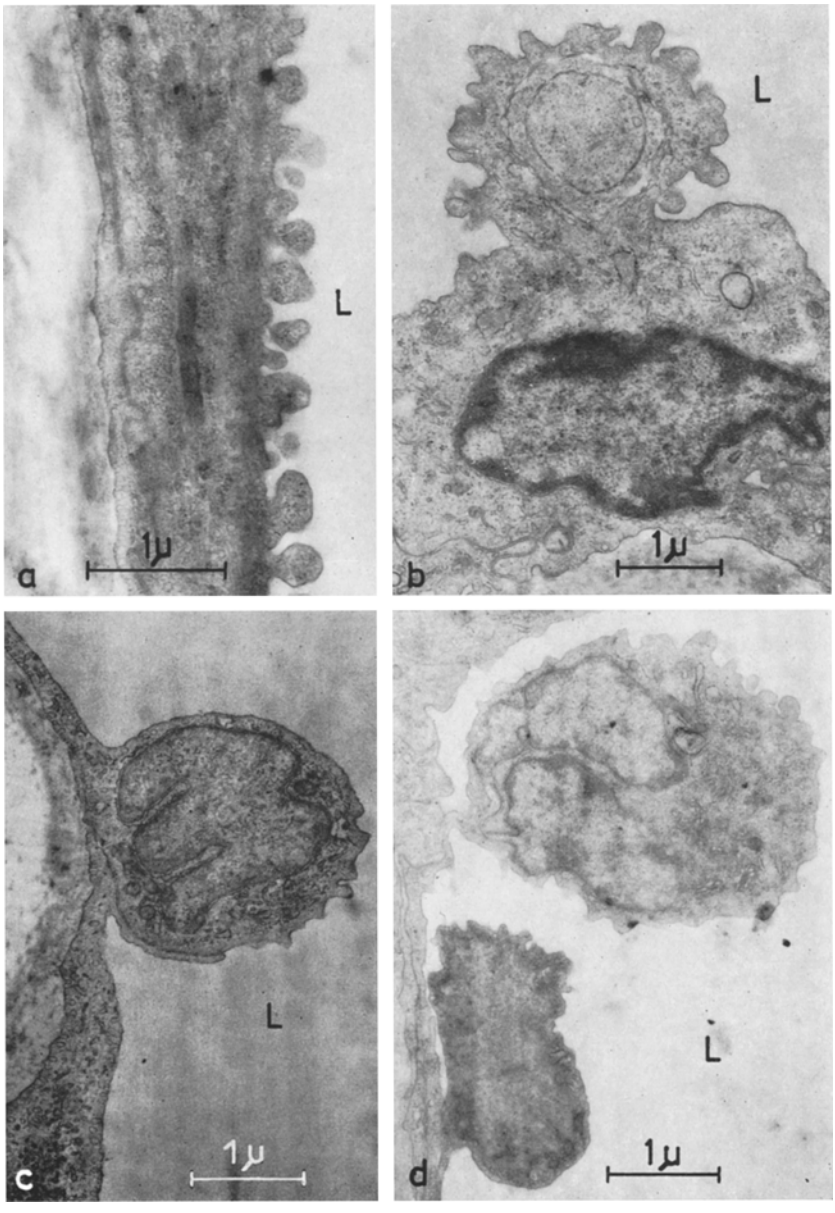

Fig. 3a—d. Various forms of endothelial cytoplasmic extensions protruding into the lumen in large blood vessels following mild mechanical trauma. a Shows cytoplasmic foot processes in endothelia of the aorta, b Shows a large bizarre-shaped polyp budding off an arterial endothelial cell, c Demonstrates the nucleus of an arterial endothelial cell lying at the peak of the convoluted elastica and extending into the lumen, while d Demonstrates two arterial endothelial cell nuclei protruding into the lumen (*L* lumen)

cell, while cross-sections (Fig. 2b, insert) often showed very small sections of cytoplasm. In surface preparations one layer of silver lines was often seen just above another (Fig. 2c), indicating focal areas in which two layers of endothelial

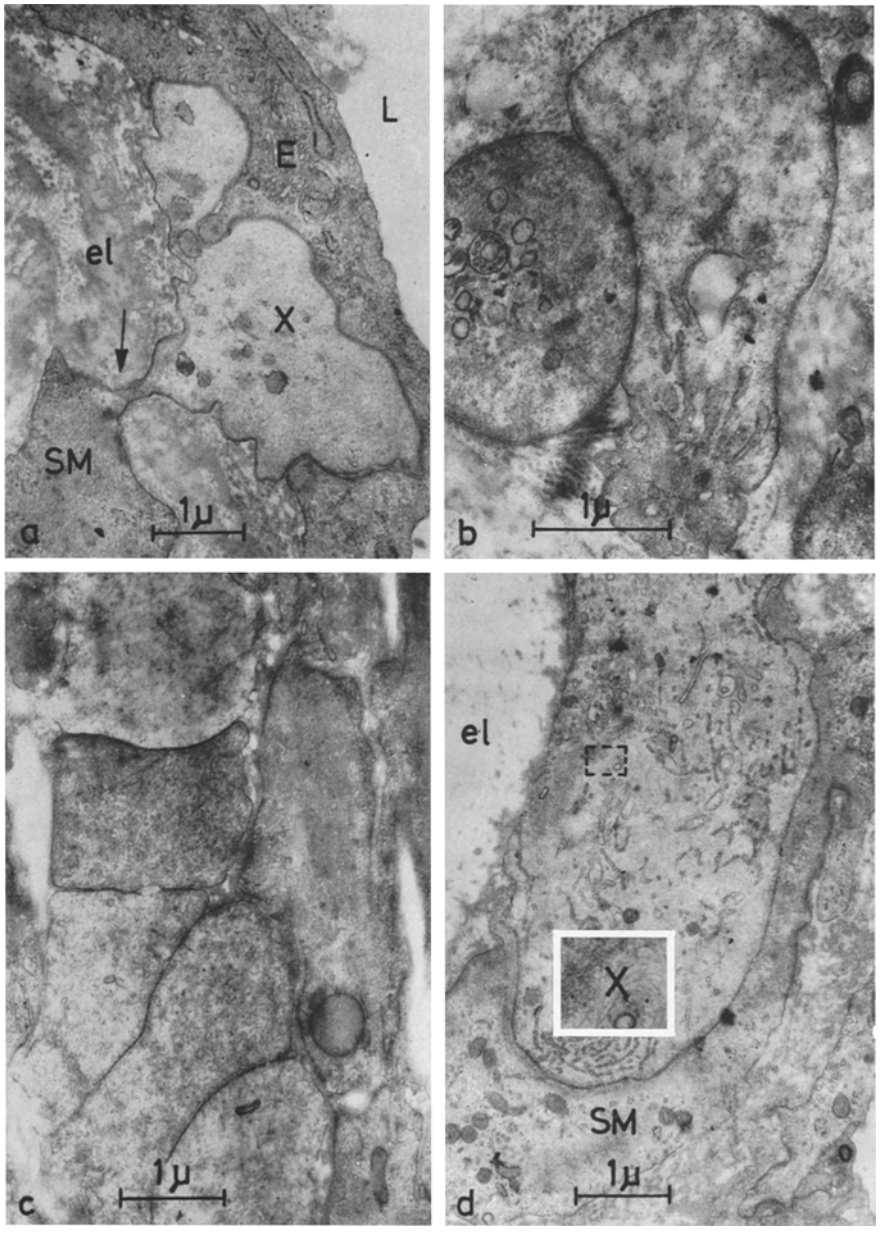

Fig. 4a—d. Smooth muscle cell pseudopods seen from different perspectives. a Shows such a pseudopod (*X*) extending into the arterial intima via a fenestration in the elastic membrane (arrow). Note the granular ground cytoplasm of the pseudopod and its close apposition to the endothelial lining. b Demonstrates the transition area between morphologically normal smooth muscle cytoplasm (presence of myofilaments) and a pseudopod with its thin, granular cytoplasm. c Shows a group of pseudopods grouped together. Their squamous shape suggests that the pseudopods are very distensible when compressed. d Demonstrates the cytoplasm of a smooth muscle cell poor in myofilaments but rich in small vesicles often beaded together. Insert (*X*) demonstrates the squared area at higher magnification (×30.000). One sees that the filamentous structures are myofilaments which have spread out in the cytoplasm that has increased in volume. Note the difference between this cell and the neighbouring normal smooth muscle cell. It is suggested that this abnormal cytoplasm represents a section through a pseudopod. (*L* lumen, *E* endothelial cell, *SM* smooth muscle cell, *el* elastica)

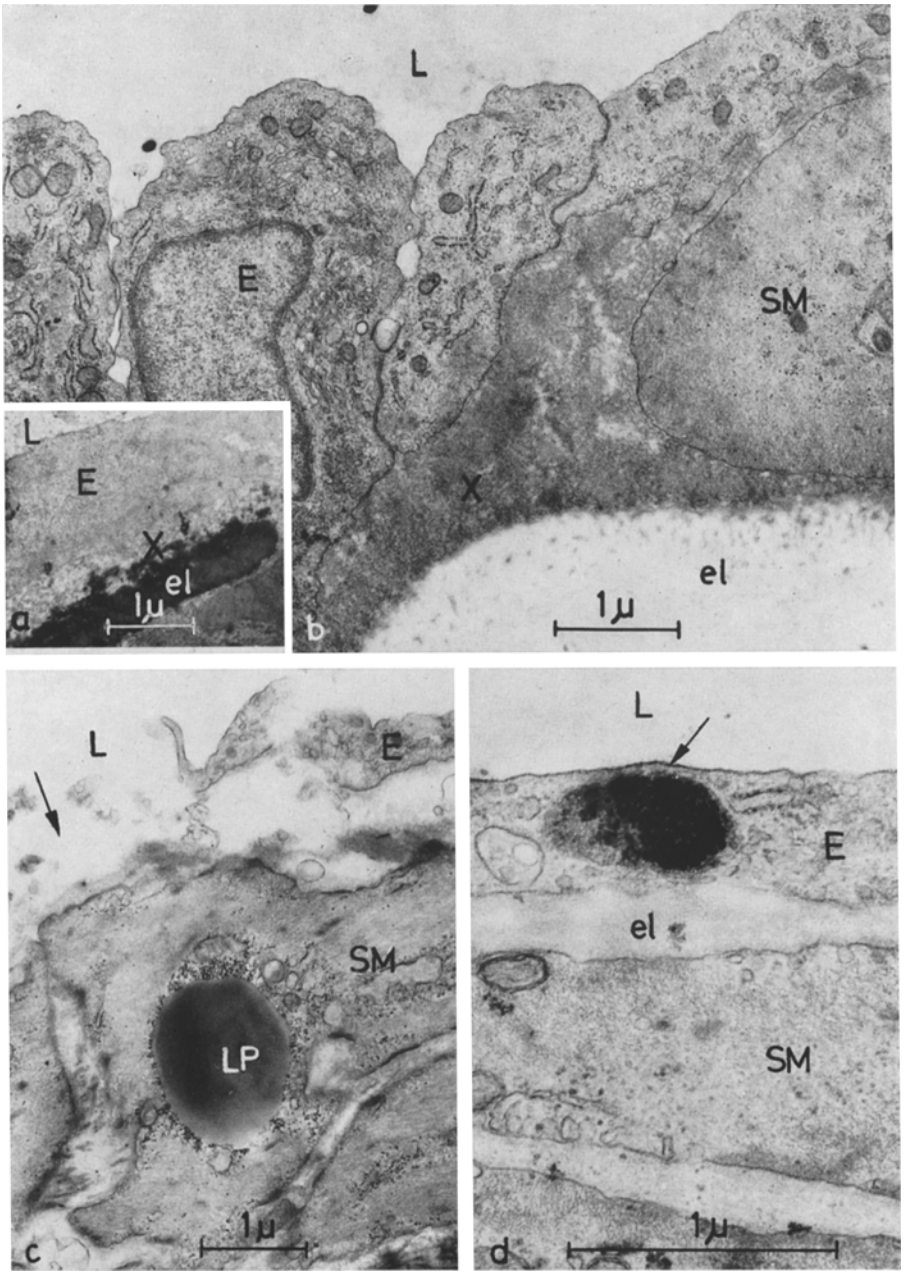

Fig. 5. b Subendothelial space of the rabbit abdominal aorta filled with a granular material (*X*) demonstrated in (a) not to be stained with phosphotungstic acid. c Illustrates lipid droplets (*LP*) in venous smooth muscle cells in the vicinity of denuded endothelia (arrow). d Shows an electron-dense organelle (arrow) in an arterial endothelial cell seen following mild mechanical trauma. *L* lumen, *E* endothelial cell, *SM* smooth muscle cell, *el* elastic membrane)

cells were present. This could have its electron microscopic analogue in the cells lying just basal to the endothelial lining (Fig. 2a).

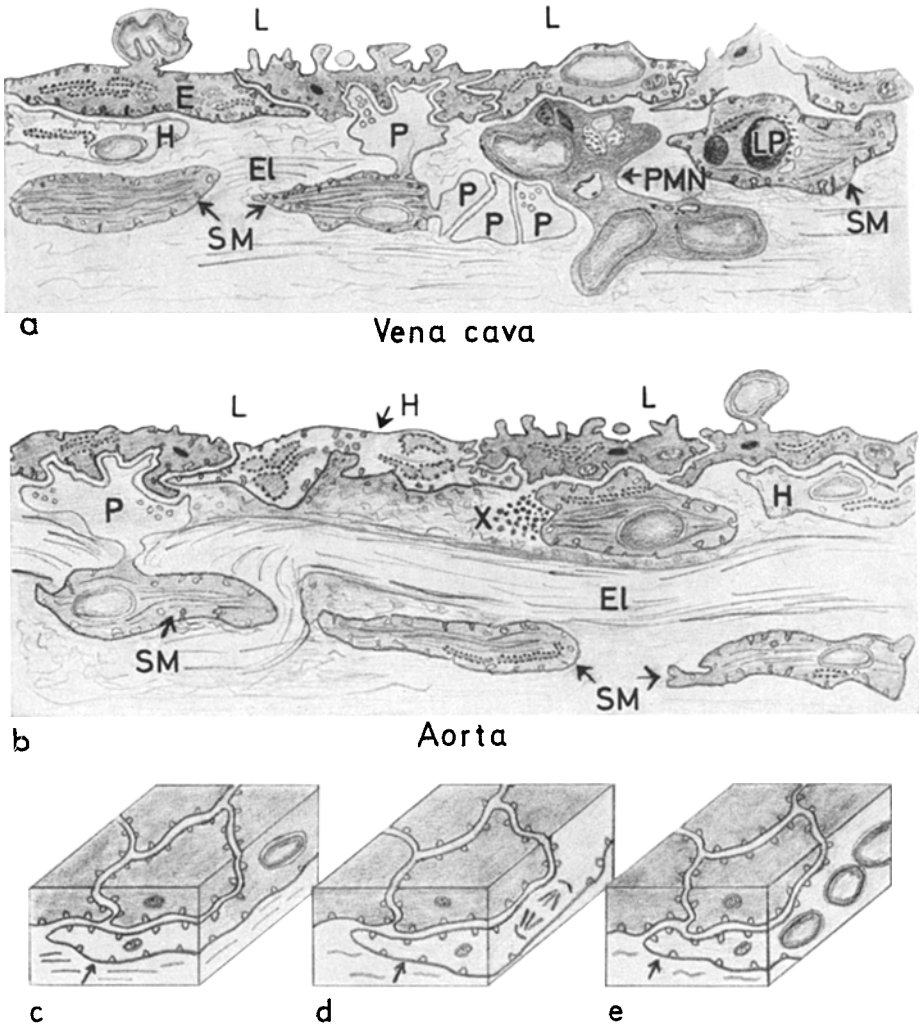

Fig. 6a—e. Schematic drawings illustrating most of the major alterations in the blood vessel ultrastructure following mild mechanical trauma. a Vena cava whose endothelial cells (*E*) demonstrate various forms of extensions into the lumen (*L*). Electron-translucent cells (*H*) lie just basal to the lining as do polymorphonuclear neutrophils (*PMN*). Smooth muscle cells (*SM*) demonstrate pseudopods (*P*) and sometimes contain lipid droplets (*LP*) usually near partially denuded endothelia. b Abdominal aorta with similar morphological changes as in the vena cava. The elastic membrane (*EL*) is thicker in the former and myointimal cells are often accompanied by an extracellular granular material (*X*) presumably mucopolysaccharide. Some endothelial cells have a more electron-translucent cytoplasm (*H*) and the cisternae of the endoplasmic reticulum are widened. c Demonstrate in perspective three possible derivations of the cytoplasmic arms seen just basal to the endothelial layer (darker cells). c Shows this lighter cytoplasm to be an extension from a neighbouring endothelial cell. d Shows this cytoplasm to be part of an endothelial cell on the surface in mitosis, or as one of the two daughter cells. e Depicts this cytoplasm to derive from a giant multinucleated endothelial cell on the surface

c) Endothelial cell extensions into the lumen were frequently observed and took a variety of forms as illustrated in Fig. 3. Some were in the form of short

foot-processes (Fig. 3a), others as odd-shaped pseudopods (Fig. 3b) and still others as entire cell nuclei attached to the vessel wall by a narrow neck of cytoplasm (Fig. 3c and d). This last phenomenon was seen mostly in the aorta. In some cases (Fig. 3d) the bulging of the nuclei with very dense chromatin extended out into the lumen, although the elastic membrane was not convoluted.

3. Smooth muscle cells were frequently seen to extend pseudopods into the intima via fenestrations in the elastic membrane (Fig. 4a). They occurred both in the vena cava and abdominal aorta. In control blood vessels such cytoplasmic arms found extending into the intima possessed an ultrastructural morphology identical to the cell body and characterized by myofilaments and cell membrane invaginations. The smooth muscle cell pseudopods found in this present study had a thin and granular cytoplasm, contained very few organelles, rarely exhibited membrane invaginations, and appeared to be quite distensible, as suggested by their squamous shape when grouped together (Fig. 4c). The granular cytoplasm seemed to occur through a thinning out of the myofibrils (insert Fig. 4d). Numerous microvesicles were seen attached together as beads on a string. Nowhere were there any signs of necrosis such as organelle swelling or membrane disruption.

4. The following focally located morphological changes were seen on occasions:

a) Large accumulations of extracellular granular material could be seen around myointimal cells within the aorta (Fig. 5a). This material failed to stain with phosphotungstic acid (Fig. 5b), and therefore does not appear to be fragmented elastica or infiltrated serum protein. Its similarity in structure and close proximity to the basement membrane of the myointimal cells suggests that it may be of mucopolysaccharide nature and presumably synthesized by the myointimal cell. The neighbouring endothelial cells seemed to contain a more dense population of organelles, in particular vesicles, presumably belonging to the Golgi zone.

b) Frequently lipid droplets were seen in the smooth muscle cells of the vena cava (Fig. 5c). This focal localization of intracellular lipid accumulation occurred particularly in areas of denuded endothelia. The fat droplets were often circumscribed by glycogen particles and swollen mitochondria.

c) Lysosomal organelles containing very electron-dense particles or aggregates whose diameters ranged from 20—30 mμ were occasionally seen in all blood vessels. They appeared to be localized to the narrowest stretches of the endothelial lining.

Discussion

Large numbers of morphological changes in the blood vessel ultrastructure have been described following mild mechanical trauma. Contrary to what would be expected, no changes in the injury picture could be found between one and seven days. This would imply that those changes occurring after one day were still present after one week, although there is no assuring that certain alterations may return to normal while new changes appear in other areas. Some of these alterations such as focal necrosis of endothelial cells and leucocyte diapedesis suggest degenerative changes. Others like endothelial and smooth muscle cell pseudopod formation may just be reversible changes. Still others such as endothelial proliferation and phagocytosis, and increased synthesis of mucopolysaccharides by myointimal cells appear to be constructive changes.

Adsorption and diapedesis of white blood cells into the vessel wall is one of the prime characteristics of inflammation and has been described in detail by Mar-

CHESI (1961) and by COTRAN, LA GUTTA and MAJNO (1965) in rat venules following injury; and by ROBERTSON, MOORE, and MERSEREAU (1959), by STEHBENS (1965) and by TS'AO and SPAET (1967) in the rabbit vena cava following mechanical irritation. Although no leucocyte penetration was observed in the aorta, HACKENSELLNER, DAVID and UERLINGS (1965) found such intramural accumulation following double ligation of the rabbit carotid artery. Diapedesis into the aortic wall of only mononuclear blood cells was found by STILL (1967) just proximal to a ligature applied to induce hypertension. The large accumulations of leucocytes usually found may be an indication of the difficulty of these cells to penetrate the elastin and mucopolysaccharides between the vascular smooth muscle cells. BLINZINGER (1965) demonstrated that the endothelial basement membrane formed a barrier to leucocyte penetration in small meningial veins during Colimeningitis. The failure to find adhesion of platelets to the vessel wall at times after a few hours could be attributed to their consequent removal by the blood, perhaps when endothelial proliferation reduces the area covered by necrotic endothelial cells.

The focal nature of endothelial injury following mechanical trauma, visualized both with light microscopy of silver-stained surface preparations and with electron microscopy has previously been reported by HOFF and GOTTLOB (1967) in the rabbit aorta within minutes following mechanical and chemical injury, and by GOTTLOB and ZINNER (1962) up to several weeks following mild mechanical trauma. Focal necrosis has also been observed by HACKENSELLNER, DAVID and UERLINGS (1965) weeks following double ligation of the rabbit carotid artery. Why certain cells should be more prone to injury than others is still not understood. Cells generally resembling endothelial cells and localized just basal to the intact endothelial lining are believed to be either extensions of endothelial cells growing underneath their neighbours or possibly new endothelial cells created by mitosis. One could also suggest that the normal endothelial lining grows over the necrotic areas. This could explain the narrowing of the bands of necrotic endothelial cells with time (GOTTLOB and ZINNER, 1962) and the focal areas of two endothelial layers as observed in silver stained surface preparations. GOTTLOB and ZINNER (1962) have suggested that this endothelial overlapping prevents the formation of microthrombi by minimising the area presented by necrotic cells. Although no such overgrowth was seen with the electron microscope, one cannot rule out this possibility. The fact that the cells just beneath the endothelial layer showed no signs of necrosis as observed in electron micrographs (Fig. 2a), reduces the feasibility of this theory. SPAET and LEJNIEKS (1967) have clearly demonstrated mitosis by aortic endothelial cells of rabbits following mechanical trauma using autoradiographic techniques. Cells undergoing mitosis but no cytoplasmic division could also produce giant multinucleated cells. Numerous multinucleated giant cells were seen in the surface lining of injured vessels as viewed on silver-stained surface preparations (GOTTLOB and ZINNER, 1962). They have also been observed by POOLE, SANDERS and FLOREY (1958) on the denuded aorta of rabbits, and by SINAPIUS and RITTMEYER (1966) on the chemically injured rabbit jugular vein. Perhaps such giant cells having undergone multiple mitosis have a less dense cytoplasm. This could then explain the electron-translucence of the ground cytoplasm in intimal cells of this study. The dissimilarity in ultrastructure between these intimal cells and smooth muscle cells or their pseudopodia

make it rather unlikely that these cells originate from the media. There is indeed much speculation in the above interpretation of these intimal cells but unfortunately the available data does not permit a more precise evaluation.

Endothelial protrusions into the lumen have been shown to take many forms. Such pseudopod extensions have been illustrated by Hoff and Gottlob (1967) to occur within minutes after application of a number of mechanical or chemical stimuli to the endothelial lining of the rabbit abdominal aorta. Their appearance in this present study even after one week suggests that, whereas their formation is very quick, they remain for quite some time. Whether the processes are reversible or not cannot be ascertained from this study, but they do not appear to be a form of necrosis, since the rest of the cell often had a normal appearance. The bulging of endothelial nuclei into the lumen have been previously described by Altschul (1957) in arteries after mechanical irritation and by Majno and Leventhal (1967) in venules following injection of vasoactive mediators. Altschul attributed the bulging to a wandering of the endothelial nuclei to the peaks of loops formed following vessel constriction after injury. Majno and Leventhal ascribed this bulging to constriction of individual endothelial cells induced by these mediators. The bulging nucleus seen in Fig. 3c and found at such peaks of convolution may very well represent such a mechanism described by Altshul. The two nuclei seen in Fig. 3d, and attached by narrow strands to the remaining endothelial layer appear to be pushed into the lumen by a violent contraction of the cell's cytoplasm. The abnormal convolution of the interendothelial gaps may also be a manifestation of such a process. Such isolated contractions of individual endothelial cells is evidently not an artifact of fixation, since otherwise presumably all endothelial cells would be in a state of contraction. Remarkably, however, such bulging nuclei occurred several days after injury suggesting a rather long state of contraction. The jagged silver lines of injured and regenerating endothelia as described by Poole, Sanders and Florey (1958) may represent the surface preparation analogue of such apparent contractions.

Migration of smooth muscle cells have been observed to occur from the media to the intima via fenestrations in the internal elastic membrane under various forms of irritation (Murray, Schrodt and Berg, 1966; Ts'ao and Spaet, 1967) and in such chronic processes as atherosclerosis (Parker, 1966). These extending arms contained myofilaments and otherwise resembled their cell body. On the other hand the smooth muscle extensions in this report had a morphology characteristic for pseudopods as found in leucocytes (Cotran, la Gattuta and Majno, (1965). Smooth muscle cell pseudopods in the abdominal aorta have been previously shown to occur within minutes by Hoff and Gottlob (1967) after irritating the vessel wall mechanically and with vasoactive agents, and by Hoff, McDonald and Hayes (1968) after application of a ligature distal to the area observed. Like the endothelial pseudopods previously described, smooth muscle pseudopods appear to be formed very quickly, triggered by some irritating stimuli, but then remain for periods at least up to one week. Whether such cytoplasmic extensions are reversible cannot be clearly stipulated from this study, although no signs of necrosis in their cell bodies were ever seen. This thinning out of the myofilaments and loss of cell membrane invaginations in such pseudopods may be explained by the increase in the room now available to the myofilaments, and

by the utilisation of the cell membrane invaginations for construction of new membrane.

Besides inducing migration of medial smooth muscle cells into the intima, acute and chronic irritation has been demonstrated in the past to bring about intimal accumulations of mucopolysaccharides in rabbit brain capillaries (Mc Donald and Hayes, 1967) and in arteries from hyperlipemic rabbits following irradiation (Kirkpatrick, 1967); in atherosclerotic arteries of rabbits (Still and Marriott, 1964); and in grafted dog veins (McCabe, Wyatt and Cunningham, 1965). The only sparse frequency in appearance of this change in this study may merely represent the mild nature of the mechanical trauma. Those areas exhibiting mucopolysaccharide accumulation may have been within the area of maximal pressure exerted on the vessel wall.

The lipid accumulation in smooth muscle cells seen in this study may represent an enhanced uptake of lipid in injured vessels as described by Still and Dennison (1966), and Friedman, Byers and Saint-Georges (1966) in hyperlipemic rabbits; or it may reflect an increase in endogenous lipid synthesis often observed in cells undergoing some form of irritation (Mölbert, 1957). The intraendothelial localization of blood cell degradation products in rabbit veins has previously been described by Gottlob and Zinner (1962) and Sinapius and Niemann (1959) as studied on surface preparations.

In reflecting on the battery of morphological changes found after mild mechanical trauma we must agree with Tedder and Shorey (1965) that there is a general morphological response of the vessel wall to injury. The observation that only the vena cava demonstrated characteristics for inflammation, namely leucocyte diapedesis, whereas only the aorta showed characteristics of early atherosclerosis, namely intimal accumulations of mucopolysaccharides, tempts one to the following speculation: both inflammation and early atherosclerosis may both be the general manifestation of injury on the various forms of blood vessels, and that differences in morphology may be due to differences in vessel anatomy and external physical factors. Forinstance, the thick elastic membrane of arteries and the high flow rate of blood within them might inhibit leucocyte diapedesis. The thin smooth muscle sheet in veins might not function like in arteries as a matrix for intima proliferation of smooth muscle cells which are believed to synthesize the ground substance found in the subendothelial space.

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