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Repair in Arterial Tissue*

A Scanning Electron Microscopic (SEM) and Light Microscopic Study on the Endothelium of Rabbit Thoracic Aorta Following a Single Dilatation Injury

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Summary. Mechanical lesion of the descending thoracic aorta of male albino rabbits was performed with an embolectomy catheter and observed as the basic lesion in a correlative study by scanning electron microscopy (SEM) and light microscopy of injury and repair of arterial wall. Fixation and silver-staining were carried out with the aortas *in situ*. Specimens collected 3, 6, 14, 30, 60, and 180 days after injury were studied *en face* by SEM and light microscopy, and sectioned tissue was submitted to histochemical investigations, reaffirming previous observations on embolectomy catheter lesion. Shamoperated animals were used as controls.

The observations by SEM and by surface light microscopy were the same: initial, severe trauma of the intimal surface followed by re-endothelialization from preserved endothelium on the aortic surface and around the intercostal arteries. In silver-stained specimens three main types of cellular patterns were recognized: hexagonal cells, foam-like patterns of cells, and polarized cells. The hexagonal cells were "true" endothelial cells, whereas the origin of the foam-like patterns of cells was less clear. From a comparison of the observations made in the present study and those recorded in morphological studies by other authors it is concluded that 1) re-endothelialization of rabbit thoracic aorta following extensive endothelial injury is slow, 2) re-endothelialization with "true" endothelium takes place from pre-existing endothelial areas, 3) the exposed arterial surface seems to be covered with sheets of cells from two sources, viz. pre-existing endothelium and cells originating from the blood and/or from the underlying tissue.

Introduction

Embolectomy catheter lesion of the rabbit thoracic aorta has been thoroughly investigated with histochemical and light microscopic means (Helin *et al.*, 1971). The present work is a study on the same experimental model by a correlative study involving use of SEM and light microscopy.

Application of SEM on silver-stained aortic endothelium (Garbarsch and Collatz Christensen, 1970; Collatz Christensen and Garbarsch, 1972) provided information on the morphology of the normal intimal surface viewed *en face*, however, artifacts might confuse the evaluation of details. Reproduciability and simplicity of the methods and fair accordance between observations from SEM and light microscopy encouraged studies on the regeneration of the aortic endothelium.

Applied on the embolectomy catheter lesion detailed information was expected from the high resolution "Three dimensional" pictures of SEM compared with light microscopy.

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Materials and Methods

Male albino rabbits of the Danish country strain in a number of 101 aged about 5 months and weighing about 3 kg were studied. In general anesthesia by Nembutal and propanidid the thoracic aorta was cannulated by a Fogarthy embolectomy catheter (12-080-5F) advanced to the aortic arch through a small incision in the left femoral artery. The balloon of the catheter was inflated with 3 ml air, and the catheter violently redrawn till it was arrested by the resistance, offered by the diaphragm. Following deflation of the balloon and withdrawal of the catheter, the femoral artery was ligated, and the skin incision closed. The object of this operation performed on 61 animals was an abrasion of the thoracic aorta from the left subclavian artery to the aortic opening in the diaphragm. As controls served 40 animals submitted to a shamoperation, viz. simple ligature of the left femoral artery. For further details see Table 1.

The animals were sacrificed 3, 6, 14, 30, 60 and 180 days after injury and the aortas perfused according to the method previously described (Garbarsch and Collatz Christensen, 1970; Collatz Christensen and Garbarsch, 1972) based on silver-staining and perfusion of the aorta *in situ* with 2.5% buffered glutaraldehyde for SEM and neutral buffered 4% formaldehyde for light microscopy.

The descending thoracic aorta was employed for the study as this part of the aorta exhibits few spontaneous arteriosclerotic changes (Garbarsch *et al.*, 1970). The experiments were carried out outside the shedding period (Helin *et al.*, 1969).

Preparation for SEM

Samples from the thoracic aorta were selected as five 8 mm long annular segments, the adventitia was removed by sharp dissection, and thereafter four segments were prepared for SEM and one segment for light microscopy. The specimens for SEM were fixed in buffered glutaraldehyde for 18 hours, dehydrated in acetone, dried in the air, cut into half-cylinders, mounted on copper blocks with silver paste, and finally coated with coal and gold. The SEM investigations were performed by a JSM-2 (JEOL) scanning electron microscope.

Preparation for Light Microscopy

For light microscopic study *en face* two cylindric specimens, approximately 15 mm in length, were sectioned from the middle of the descending thoracic aorta of all animals and were further postfixed for 48 hours in neutral buffered 4% formaldehyde at 4° C. After washing in distilled water and clearing in glycerine the adventitia was gently removed, and the specimens mounted on slides with Apathy's sirup (Pearse, 1968) as mounting medium.

For transmission light microscopy three samples cut from the upper, middle, and lower part of the thoracic aorta as 2 mm broad transversal strips were fixed in 0.5% cetylpyridinium chloride in 4% formaldehyde (Williams and Jackson, 1956) and paraffin embedded.

Table 1. Material

Days after lesion with embolectomy catheter	Scanning electron microscopy				Light microscopy		
	Number of rabbits				Number of rabbits		
	Embolectomy catheter lesion	Sham-operation	Total	Silver-stained / Unstained	Embolectomy catheter lesion	Sham-operation	Total
3 days	5	2	7	4 / 3	5	2	7
6 days	4	1	5	4 / 1	5	1	6
14 days	4	2	6	2 / 4	5	2	7
30 days	9	6	15	10 / 5	5	5	10
60 days	4	5	9	5 / 4	5	5	10
180 days	5	5	10	8 / 2	5	4	9

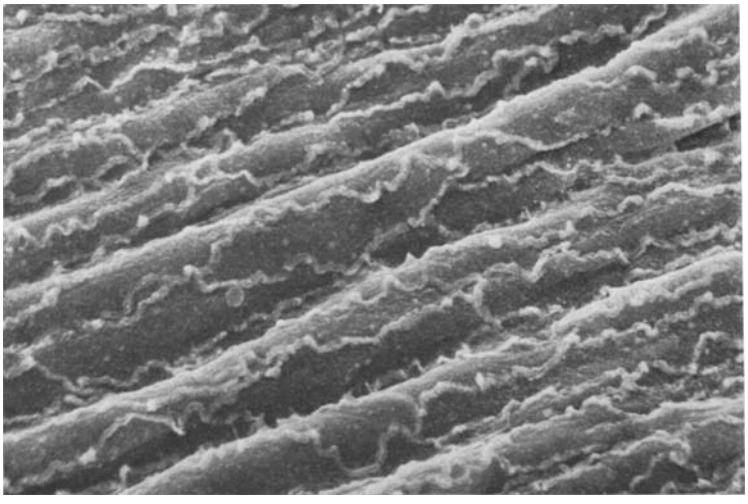


Fig. 1. Scanning electron micrograph of silver-stained rabbit thoracic aorta. The endothelial cells are bounded by silver lines and lying on coarse parallel folds, produced by the inner elastic lamella. Shamoperated animal after 180 days. ×800

The sections were stained as follows: hematoxylin and eosin, 0,3% Alcian blue 8 GX (G. T. Gurr) at pH 1 (0.1 N hydrochloric acid) and pH 2.5 (3% acetic acid) (Pearse, 1968). The concentrations of Alcian blue modified. 0.1% toluidine blue 0 (Merck) in 30% ethanol (Kramer and Windrum, 1955), v. Gieson staining (Lillie, 1965), Mallory's PTAH-staining (Pearse, 1968), Adams DMAB method for tryptophan (Adams, 1957) and a combined method (Alcian blue-Orcein-Iron Hematoxylin-v. Gieson) for acid glycosaminoglycans, elastin, collagen fibers and cells as described elsewhere (Garbarsch, 1973).

Results

The results below will be based on the descriptions of SEM pictures, whereas surface light microscopy will be referred to only briefly, and if specially relevant. This simplification of descriptive terms was justified by the accordance between observations from SEM and light microscopy in previous works (Garbarsch and Collatz Christensen, 1970; Collatz Christensen and Garbarsch, 1972), as well as in the present work, and by the fact that light microscopy of sectioned tissue reaffirmed the changes induced by the embolectomy catheter lesion previously described (Helin *et al.*, 1971).

Shamoperated Animals

All specimens were macroscopically normal, and whatever surfaces or sections were studied, light microscopy showed normal endothelium and normal vascular wall.

SEM and light microscopy in every detail revealed the features of normal anatomy as well as artifacts described and discussed earlier (Collatz Christensen and Garbarsch, 1972). Generally the quality of preparations was good, and all shamoperated animals presented normal intimal surfaces (Figs. 1 and 2a).

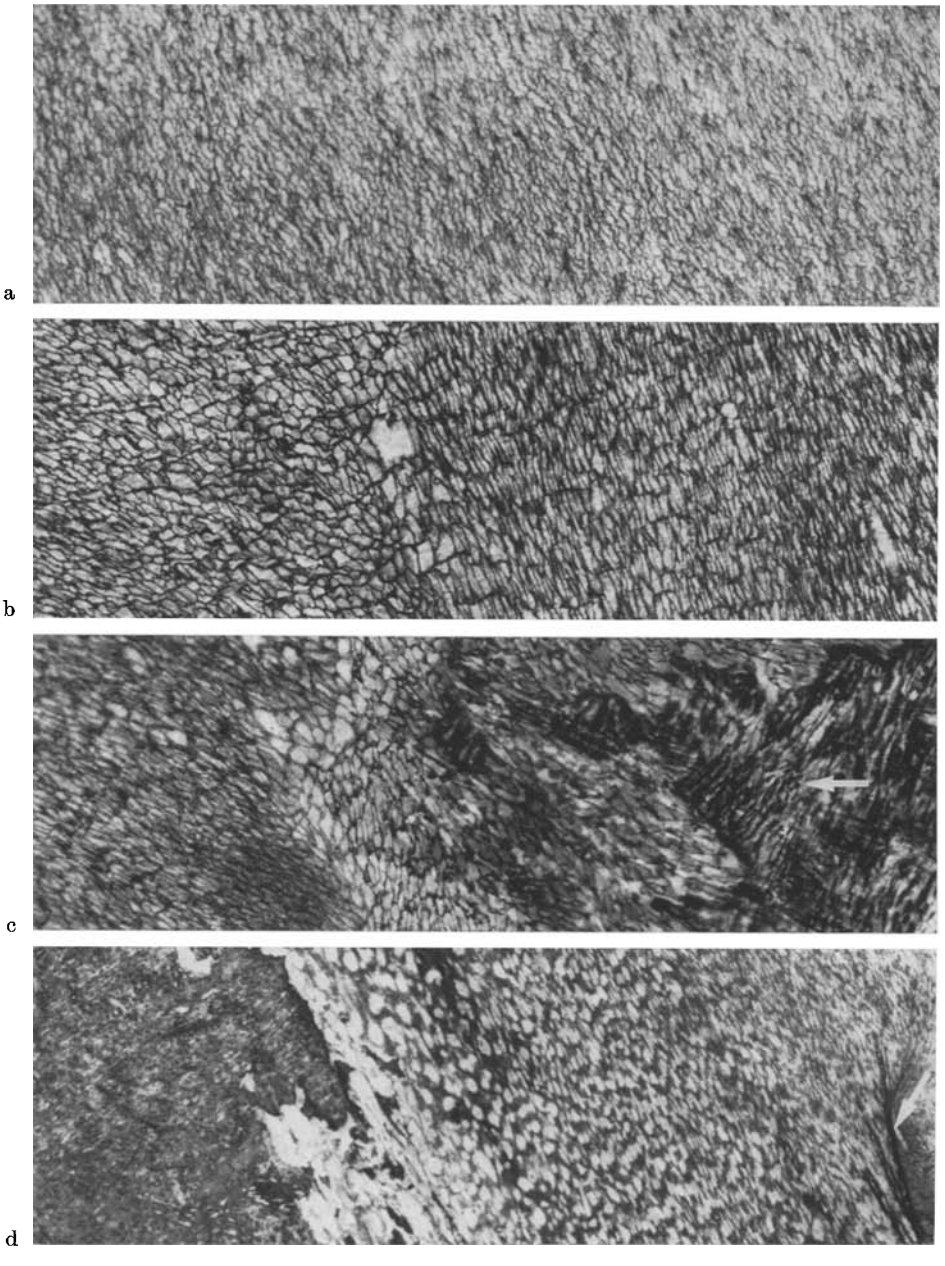


Fig. 2a—d. Light microscopy of silver-stained non-dilated a and dilated (b, c, d) rabbit descending thoracic aorta (×95). b Six days after dilatation injury. From the right to the left: normal endothelium, giant cells, borderline to foam cells, polarized foam cells. c Six days after dilatation injury. Cellular polymorphy in the central area. To the right bared area (arrow), to the left normal looking endothelium. d Fourteen days after dilatation injury. To the right intercostal artery (arrow), to the left bared area. In the mid-zone edge of the endothelium with endothelial giant cells

Embolectomy Catheter Lesion

Macroscopic changes, prominent after fourteen days were as follows: dilatations, confluent plaques and bean-shaped bulges, longitudinally arranged serpentine elevations lateral to the intercostal arteries and dispersed calcifications. The observations were in accordance with those of Helin *et al.* (1971).

Light Microscopic Review

Three days after the dilatation injury large denuded areas were prominent, in which smaller solitary islands of normal looking endothelium had apparently escaped abrasion from the balloon. Normal endothelium was always present inside and around the openings of intercostal arteries and at the intact edges of the endothelium above and beyond lesion. At these edges large flat, giant cells were observed.

After six days (Fig. 2b and c) conspicuous areas were covered with cells in hexagonal or foam-like patterns of silver lines, and until the end of the observation period 180 days later these patterns persisted. At the 60 days stage large parts of the surfaces were uncovered with endothelium, and still after 180 days uncovered areas were present.

At several spots of re-endothelialized surfaces cells in whirled patterns might take a course with their longitudinal axes transverse to the long axis of the aorta.

The denuded areas were covered with an amorphous brownish material (Fig. 2d). However, at the edges of proliferating endothelial flakes this substance was often absent leaving a surface beset with tiny silver rings.

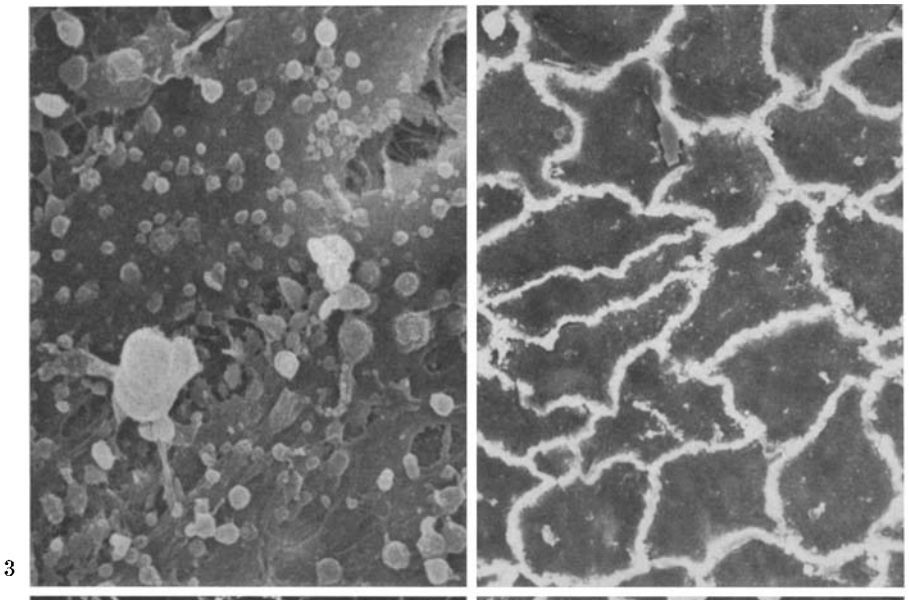
SEM Observations

At lower magnification the state of endothelium corresponded to light microscopy. The uncovered internal elastic lamellae visible from the third to the sixth day appeared fenestrated, and the torn endothelium presented fissures and cracks with openings into the subendothelial tissue. In later experiments the subendothelial tissue appeared uneven with fibrillar structures intermingled with amorphous substances, and varying amounts of platelets, mononuclear cells and cells of blood formed a thin layer, which never concealed the surface (Fig. 3), but tended to crowd on and around the bared subendothelial tissue.

Three main patterns of silver lines termed hexagonal patterns, foam-like patterns, and patterns of polarized cells were revealed.

Hexagonal patterns were present from the third day and throughout all later stages (Fig. 4). The cells were uniform and of equal size, the cell surface was beset with small rounded elevations, and the faint contour of the nucleus round or oval. The silver lines were coarse, uniform and straight. Hexagonal cells typically covered the bottoms of the bean-shaped bulges in the arterial wall, and continuity with intact endothelium on the aortic surface and around intercostal arteries was evident.

Foam-like patterns were present from the sixth day and were in a state of expansion till the end of the observation period (Fig. 5a and b). The meshes delineated by the silver lines were of unequal size, the silver lines were thinner, and followed a sinuous, slightly bent, track between neighbouring meshes. While large



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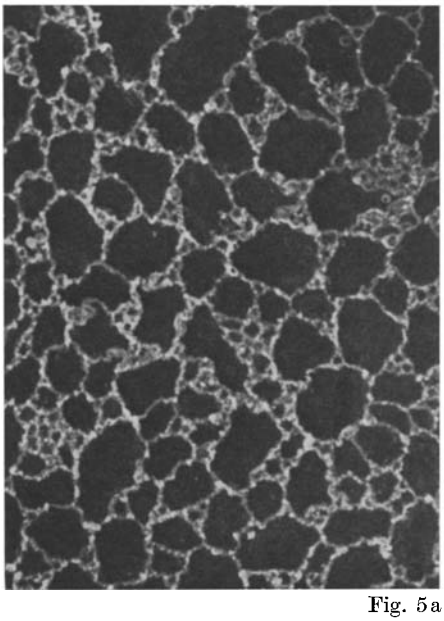

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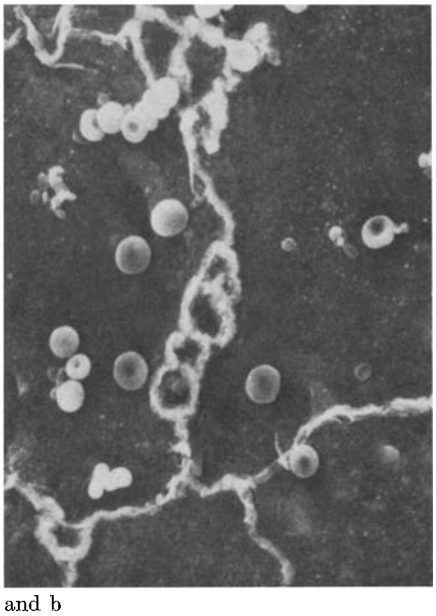


b

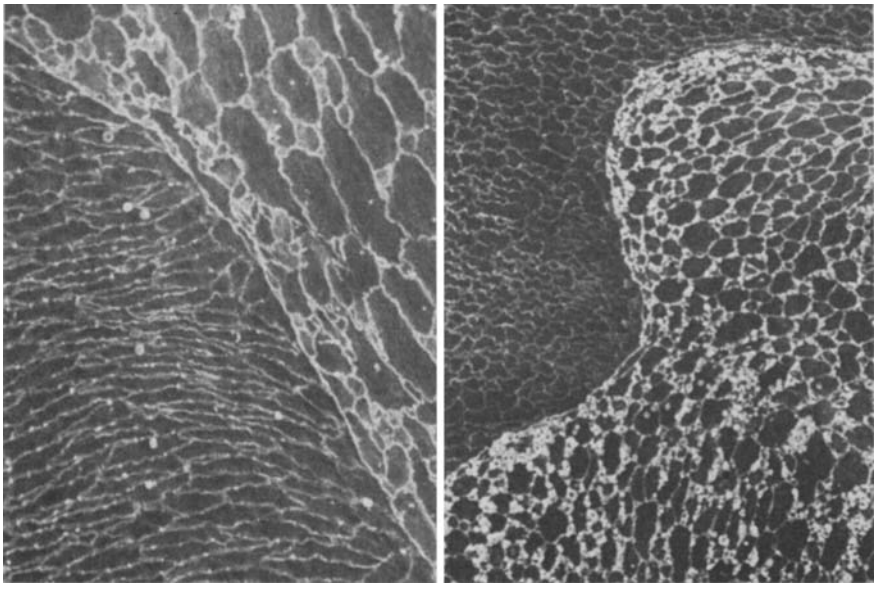
Fig. 5a and b

Figs. 3—5. Scanning electron micrographs of descending rabbit thoracic aorta after a single mechanical dilatation injury

Fig. 3. Aortic surface, 30 days after injury. Denuded endothelial surface with cracks, platelets, and mononuclear cells. ×2400

Fig. 4. Aortic surface, 60 days after injury. Silver-stained endothelial surface with hexagonal cells. ×800

Fig. 5. a Aortic surface, 180 days after injury. Silver-stained surface with foam-like patterns of cells. ×240. b Detail from the same specimen as Fig. 5a. ×800



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Figs. 6—7. Scanning electron micrographs of silver-stained rabbit thoracic aorta following a single mechanical dilatation injury

Fig. 6. Aortic surface, 60 days after injury. Polarized foam-like patterns of cells (right), polarized endothelial cells with axes transverse to the long axes of the vessel (left), sharp boundary between the two cell patterns. ×240

Fig. 7. Aortic surface, 180 days after injury. Intimal thickening covered by a foam-like pattern of cells (right). ×80

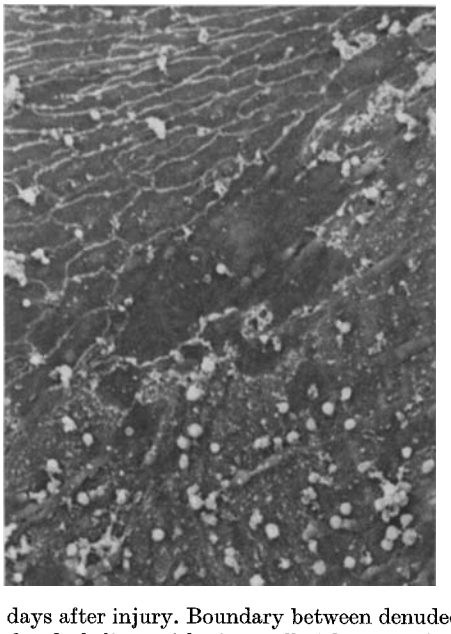


Fig. 8. Aortic surface, 6 days after injury. Boundary between denuded area (below), and edge of endothelium with giant cells (above). ×240

meshes, sometimes presenting one or several slightly elevated nuclei, probably represented mononuclear and multinuclear giant cells, the smaller meshes of unequal size were too small to represent individual cells (Fig. 5b). Small meshes were interspersed as pincenez-like eyes within silver lines (Fig. 5b), and they were crowded between contiguous corners of neighbouring large meshes. The whole pattern presented a foam-like appearance, and although conforming to a general morphologic pattern in the same way as the hexagonal pattern described above, the foam-like patterns were more varied and pleomorphic than the hexagonal pattern. Typically foam-like patterns covered large thickened areas, and they never presented transitions to areas covered by normal endothelium or their derivatives.

Patterns of polarized cells were present in areas of preserved normal endothelium and in fields of new-formed endothelium, hexagonal or foam-like in appearance (Fig. 6). The silver lines here assumed an appearance much like that of normal cells, but they could always be recognized as new-formed by a coarse or lacking nodulation and a greater thickness of the silver lines, and by the fact, that the long axes of new-formed cells were frequently perpendicular to the long axis of the vessel. Polarized cells fading into patterns of hexagonal cells were more uniform than polarized cells related to foam-like patterns; the latter always presented cells of varying extension and plenty of small meshes of unequal size (Fig. 6).

Hexagonal and foam-like patterns were always separated by clear-cut, extremely sharp, boundaries, and generally the two cellular patterns were flush in the horizontal level. However, varying levels of neighbouring cellular patterns were sometimes observed, and in that case the foam-like pattern always seemed to represent the area of a higher level (Fig. 7). Clear-cut borderlines also existed between destroyed surfaces and cellular sheets with normal, hexagonal, or foam-like patterns (Fig. 8).

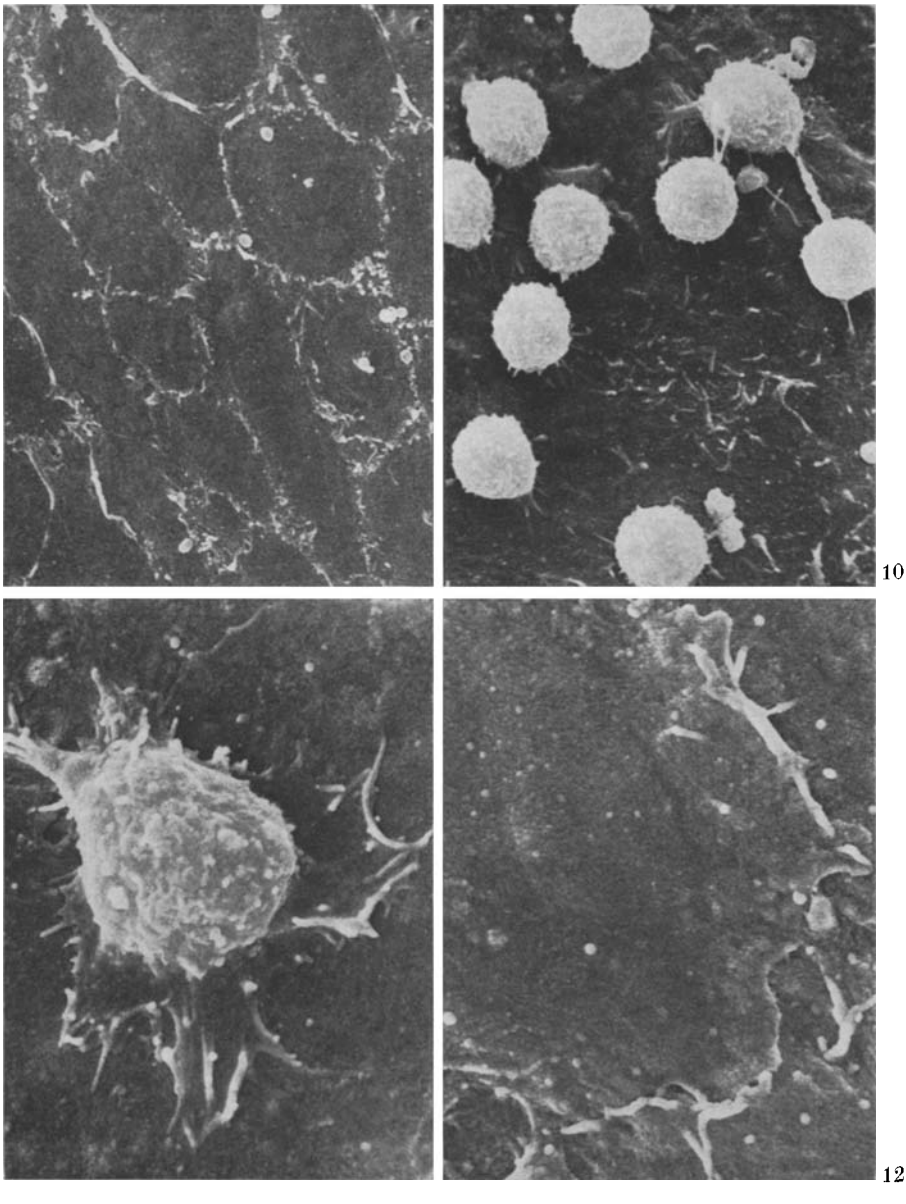
Unstained specimens supplied the same information as silver-stained ones, however, the silver-staining was of paramount importance in differentiating cellular patterns and boundary zones.

Special description is necessary concerning the 30 days experiment and some observations during the 180 days experiment.

Specimens from the 30 days experiment and, to a certain degree, from the 60 days experiment were difficult to stain with silver for SEM. While silver-stained fields covered smaller parts of the specimens studied, unstained areas with nuclear swelling and destroyed areas were prominent, and occurrence of mononuclear cells was evident. In the 60 days experiment these changes yielded to a dominance of silver-stained patterns.

Surfaces with nuclear swelling presented an almost identical appearance in stained and unstained specimens. Sheets of varying cellular patterns were separated by clear-cut borderlines and were easy to distinguish by varying shape, orientation, and crowding of the swollen nuclei. Cellular boundaries varied in appearance, some being almost invisible, others conspicuous with characteristic features, viz. serrated edges with overlapping borders and interdigitating processes (Fig. 9).

Destroyed areas seemed to consist of similar cellular ground patterns, and presented changes varying from superficial coating with thrombocytes and mono-



Figs. 9—12. Scanning electron micrographs of unstained rabbit thoracic aorta 30 days after a single mechanical dilatation injury

Fig. 9. Cells with serrated interdigitating borders. ×800

Fig. 10. Leucocytes on the aortic surface. ×2400

Fig. 11. Free cell with pseudopodes on the aortic surface. ×6560

Fig. 12. Detail of interdigitating cell borders of surface cells. ×6560

nuclear cells to almost total destruction with extensive uncovering of subendothelial tissue. Free cells were dispersed on destroyed surfaces sometimes in small clusters, whereas a linear crowding occurred on the boundary zones of intact and destroyed areas.

Cells, presumed to be leucocytes, were rounded with rounded surface protrusions, and they did only present small pseudopods (Fig. 10). Cells with large pseudopods presented an elevated cell body with rounded protrusions, whereas the peripheral part of the cell body was flattened, and the border serrated with branching pseudopods. The flattened border of the cells with pseudopods was morphologically identical to the elements of interdigitating cellular borders of flattened cells described above (Figs. 9 and 12).

Light microscopically were recognized gradual transitions between solitary cells with cytoplasmic processes, probably mononuclear cells, and cells evidently forming foam-like patterns. Otherwise, light microscopy in the 30 days experiment revealed silver-stained and unstained areas identical to the patterns in the other experiments.

In the 180 days experiment silver-stained patterns dominated, and areas with nuclear swelling and mononuclear cells were rare. Another observation was bizarre jig-saw like patterns of silver lines occurring in narrow zones continuous with areas covered by hexagonal patterns of silver lines.

Discussion

Our observations generally conform with the results obtained from light microscopy of silver-stained mechanical trauma produced in a variety of ways (Zahn, 1884), (Poole *et al.*, 1958, 1959), (Cotton *et al.*, 1961), (Gottlob and Zinner, 1962), (Hoff and Gottlob, 1968), (Björkerud and Bondjers, 1971).

In normal intima of great vessels the regular patterns of silver lines were produced by deposits of silver along cellular boundaries (Florey *et al.*, 1959; Gottlob and Hoff, 1967). However, the basic phenomena causing them remained unexplained in the perspective of Sinapius (1952, 1956) and Florey *et al.* (1959). By SEM (Garbarsch and Collatz Christensen, 1970; Collatz Christensen and Garbarsch, 1972; Geissinger, 1972) the light microscopic appearance of the silver lines as broad, nodulated and elevated was confirmed.

In damaged tissue of great vessels silver-staining revealed a basic morphology common to various experimental models: bizarre patterns of silver lines, occurrence of giant cells, partial or complete re-endothelialization as well as modest thrombotic phenomena in the aorta (Poole *et al.*, 1958), (Cotton *et al.*, 1961), (Gottlob and Zinner, 1962), (Björkerud and Bondjers, 1971).

The combined use of SEM and light microscopy confirmed these basic changes, and it was acceptable that the silver lines represented a pattern of cell boundaries, in which three morphological entities could be deduced.

The hexagonal cells probably represented endothelial cells. Outgrowth from intact edges and from preserved endothelium around intercostal arteries was evident, and cells in any stage of transition from normal to hexagonal shape, as well as the capacity of hexagonal cells to polarize and differentiate was open to direct recognition.

The foam-like patterns possibly represented cellular patterns, although the smaller meshes could not represent individual cells. However, no decisive information on the origin of the foam-like patterns was obtained, except negative evidence: transitions were never observed to normal, neither to hexagonal endothelium. The morphological identity between these patterns and the pseudoendothelium described by Pugatch (1964) led to the assumption that the foam-like patterns were derived from macrophages and/or mesenchymal cells, and could accordingly be termed pseudoendothelium or endothelium-like cells. Observations from the 30 days experiment, discussed in the following, may possibly support this suggestion.

The patterns of polarized cells were considered to be caused by changes in the hemodynamic conditions. Experimental work (Flaherty *et al.*, 1972) furnished proof of dependency of cell polarization on hemodynamic conditions, and accordingly the whirled patterns of cells covering bulges and ridges in the arterial wall in our experiments might be attributed to changed hemodynamic conditions. Whatever the explanation, new-formed cells often presented their longitudinal axes perpendicular to the long axes of the vessel in areas prone to severely changed hemodynamics.

Diverging opinions have been advanced on the speed of regeneration after mechanical trauma. In the rabbit small denuded areas of the aorta healed quickly (Gottlob and Zinner, 1962; Björkerud and Bondjers, 1971) whereas larger areas were slowly covered (Pool *et al.*, 1958, 1959; Björkerud and Bondjers, 1971). Besides extension and gravity of lesions, species characteristics seemed to play a role. Thus for example re-endothelialization was quick in the dog (Cotton *et al.*, 1961) and slow in the rabbit (Pool *et al.*, 1958, 1959). However, Björkerud and Bondjers (1971) ascribed the slow regeneration of rabbit endothelium more to methodological factors than to species characteristics.

In our experiment the initial healing was fast, whereas the healing process taken as a whole was slow. In the pleomorphic picture of mixed hexagonal and foam-like patterns, the latter had rather a big share compared to patterns presented by the authors cited above. The pleomorphic patterns of reparative processes, intermingled with areas of destruction, observed as late as half a year after lesion might be taken as overstressed reparative properties of the aortic wall, not unlike the non-regressive atherosclerotic lesions described by Björkerud and Bondjers (1971).

Different points of view concerned the origin of various endothelia (Survey: French, 1966). From studies on arterial grafts and endarterectomia Pool *et al.* (1962) concluded that the endothelia from intact edges and arterial branches were the sources of regenerating intimal surface cells, and MacKenzie *et al.* (1969) attributed to circulating cells an important role in the healing of arterial grafts. Björkerud and Bondjers (1971) attributed to monocytes and macrophages the role of covering parts of their non-regressive lesions. Pugatch (1964) by silver-staining and EM and by investigations on phagocytic properties of the cells presented evidence that some of the cells in the bizarre patterns of pseudoendothelium in his ear-chamber experiments were macrophages.

To these controversial issues the combined use of EM and light microscopy added some information, deserving special attention.

The sharp boundaries between hexagonal and foam-like patterns probably representing endothelium and pseudoendothelium respectively, constituted a conspicuous and repeated observation throughout the material. The distinct borderlines were assumed to represent the zones of meeting of cellular sheets derived from different sites and from different sorts of cells. A sort of linear "basal lamina" was supposed to occur, as two cellular sheets, one endothelial and one mesenchymal, were opposed edge-to-edge. Sharp boundaries were also observed between silver-stained patterns and destroyed surfaces. Giant cells at the borderlines were taken as evidence of proliferation, and different levels of cellular sheets sometimes observed, might indicate overgrowth.

The destroyed surfaces represented torn endothelium, damaged or dead cells, and after the sixth day the unveiled subendothelial tissue in accordance with the diffuse intimal thickening observed by light microscopy of sectioned tissue never consisted of a fenestrated internal elastic lamella. The structure of this subendothelial tissue was not clarified by SEM. In the estimation of thrombotic changes of destroyed surfaces an uncertain factor concerned the possible removal of thrombi from the stream of fluid during the procedure of perfusion staining and fixation. However, not a single large thrombus was recognized in about 800 aortic half cylinders, and only modest thrombotic changes were observed.

In the 30 days experiment the difficulties by SEM in obtaining silver-staining, and the general tendency of surface cells to nuclear swelling remained unexplained. By light microscopy silver-staining succeeded, and conformed with observations from previous experiments in the series.

Consequently, the experiment was reduplicated for SEM in order to exclude artifacts, but nevertheless all observations conformed with the previous 30 days experiment, and basically remained unexplained.

However, two trends at ultrastructural level, and one light microscopic statement pointed to a specific role of macrophages in the healing processes. First, macrophage-like cells in small clusters seemed to flatten out on fields of destroyed surfaces. Secondly, the frayed borderlines of free macrophages were morphologically identical to the serrated patterns of the borderlines between intertwining edges of cells forming continuous sheets of cells with swollen nuclei. Thirdly, by light microscopy transitions between free cells with pseudopods, and cells fixed in the foam like patterns of silver lines were often observed. A certain approbation of the theses of Pugatch (1964) and of Björkerud and Bondjers (1971) was rendered by the present work.

The integrated use of SEM and light microscopy in a correlative study on silver-stained rabbit thoracic aortae generally re-affirmed observations obtained by other authors. With a few exceptions information obtained by SEM and light microscopy also corresponded mutually. However, the high resolution three dimensional pictures of SEM revealed valuable information on details, which could hardly be seen in the light microscope. The macroscopic changes, bulges, ridges, and folds, inconspicuous by light microscopy, were striking by the great magnification of SEM. Pleomorphic cellular patterns, opening a scope of questions concerning the morphology of cells related to dilatation and folding of the vascular wall were unveiled by SEM. Torn surfaces presented deep cracks into the underlying tissue, and the distribution of thrombotic elements might be judged on the background of affinity

to dead tissue, collagen, elastic tissue, and constituents of healing tissue. Nuclear swelling and cellular edema, artificial or real, were easily recognized. Free cells, leucocytes and mesenchymal cells were easy to study, their distribution and detailed morphology as well, and judgement on their functional state was primarily brought about by the observations from SEM. Flattening of mesenchymal cells and interdigitation of their pseudopode-like edges in the suggested formation of pseudoendothelium was supposed from the detailed morphological information obtained by SEM. On the other hand, artifacts and exaggerated interpretation of isolated observations constituted a menace to a sound interpretation of the wealth of morphological details obtained by SEM.

However, in the efforts of classifying the major changes of injury and repair based on minute details in the morphology of cells and cell boundaries, we consider a combined use of light microscopy and SEM indispensable in contributing new knowledge to the problems of arterial repair.

From the present study the following conclusions may be drawn:

1. Re-endothelialization of the rabbit thoracic aorta following extensive endothelial injury is slow.

2. Re-endothelialization with "true" endothelium takes place from pre-existing endothelial areas.

The following conclusion is likely:

3. The denuded arterial surfaces seem to be covered with sheets of cells from two sources, viz. from pre-existing endothelium and from cells originating from the blood and/or from underlying tissue.

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