Virchows Arch. A Path. Anat. and Histol. 363, 33—46 (1974)

Repair in Arterial Tissue

A Scanning Electron Microscopic (SEM) and Light Microscopic Study on the Endothelium of the Rabbit Thoracic Aorta Following Noradrenaline in Toxic Doses

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Received January 25, 1974

Summary. The effects of severe toxic doses noradrenaline were evaluated by surface light microscopy and SEM of silver-stained thoracic aortae from young male albino rabbits.

Noradrenaline 1 mg a day was given i.v. for 14 days; the rabbits were killed on the 17th day. Rabbits injected by physiologic saline served as controls. Noradrenaline induced excessive blood pressure elevation, exitement and pulmonary edema, and at autopsy ascites, hydrothorax, dilatation of the aortae with plaques and calcifications was prominent. The thoracic aortae, silver-stained and fixed in situ, were studied by surface light microscopy and by SEM. Besides normal endothelium the following changes were observed alone or in combination: hexagonal patterns of silverlines, change of individual endothelial cells, and various miscellaneous changes. The hexagonal endothelium was considered genuine endothelium adapted to a mechanical dilatation of the aorta, but it also might reflect reparative processes and the effects of hemodynamic forces on endothelial morphology. The changed endothelial cells seemed by light microscopy to be permeable to silver, which stained the cell body and left the nuclear zone free. By SEM these cells were intensively silver-stained, folded and shrunken with nuclear swelling, and they looked severely damaged. A direct toxic effect of noradrenaline on endothelial cells was suggested, however, the damaging effect probably was composite. A causal relation between changed endothelium and hexagonal endothelium i.e. the hexagonal endothelium compensates loss of damaged cells by proliferation or by expansion, presupposes a fast expulsion of damaged cells, and remains open to question. The chronology of the changes induced by noradrenaline so far remains unknown.

Intravenous injections of adrenaline (Josué, 1903, 1905; Anitschkow, 1914, 1933; Lange, 1924; Lorenzen, 1963b) and of noradrenaline (Friedman *et al.*, 1955; Lorenzen, 1963a, 1966; Helin *et al.*, 1970) into rabbits may induce severe gross arteriosclerosis in the aorta. SEM investigations of the aortic intima for changes induced by catecholamines so far are scarce (Sunaga *et al.*, 1969; Shimamoto *et al.*, 1971).

The present work is a study on the experimental model of Helin *et al.*, 1970, which uses toxic doses of noradrenaline. The gross and microscopic lesions as well as the biochemical changes of the aortic wall are known to be similar to the changes induced by adrenaline (Lorenzen, 1963b, 1966) and by exposure of rabbits to repeated periods of systemic hypoxia (Lorenzen and Helin, 1967, Helin and Lorenzen, 1969; Garbarsch *et al.*, 1969; Helin *et al.*, 1969). The changes induced in the aortic wall are basically unspecific processes of injury and repair, well known from wound healing.

The involvement of the intimal surface in these processes is studied by SEM and surface light microscopy in the work here presented.

Materials and Methods

Male albino rabbits of the Danish Country strain in a number of 36 aged about 5 months and weighing about 3 kg were studied. Thirteen died during the first week of the experiment, and were discarded, whereas 23 rabbits survived the planned procedure, and were included in the material; 12 animals were injected with noradrenaline, and 11 with physiologic saline over a period of 14 days. The animals were killed on the 17th day.

The experimental animals had daily i.v. infusions of noradrenaline bitartrate in two periods of 15 minutes with an interval of 5 minutes. One hundred mg of noradrenaline bitartrate were dissolved in 100 ml physiological saline containing 5000 U of heparin. The rate of the infusion pump was 4 ml/h, corresponding to 66.6 μg noradrenaline bitartrate = 33.3 μg noradrenaline/min. The total daily amount of noradrenaline was 1 mg, and the entire daily infusion period was 30 minutes. The control animals were injected in the same way, using physiological saline in stead of noradrenaline solution. The animals were sacrificed on the 17th day using general anesthesia by nembutal and propanidid. Silverstaining and perfusion of the aorta in situ with 2,5% buffered glutaraldehyde was performed according to methods previously described (Garbarsch and Collatz Christensen, 1970; Collatz Christensen and Garbarsch, 1972).

Preparation for SEM

Samples from the thoracic aorta were selected as 8 mm long annular segments. Two segments from the proximal, and two segments from the distal part were used for SEM. A middle segment was used for transmission light microscopy, and the rest of the thoracic aorta for surface light microscopy. The specimens for SEM were fixed in buffered glutaraldehyde for 18 hours, dehydrated in acetone, dried in the air, cut into halfcylinders, mounted on cupperblocks with silverpaste, and finally coated with coal and gold.

The SEM investigations were performed in a JSM-2 (JEOL) scanning electron microscope.

Preparation for Light Microscopy

For light microscopic study en face the cylindric specimens, approximately 15 mm in length, were postfixed for 48 hours in neutral buffered 4% formalin at 4°C, washed in destilled water and cleared in glycerine. After removal of the adventitia the specimens were mounted on slides with Apathy's syrup (Pearse, 1968) as mounting medium.

For transmission light microscopy the middle segments were divided into two halfcylinders and fixed in neutral buffered glutaraldehyde 2.5% at pH 7.4 for 18 hours at 4°C, postfixed in osmium tetroxide 1% for 1 hour, dehydrated in acetone, and embedded in Araldite. From each halfcylinder 0.5 μm thick sections were cut on a Reichert Om U2 ultramicrotome transverse and longitudinal to the long axis of the vessel, and finally stained by 1% toluidin blue.

Blood Pressure Recordings

In additional experiments blood pressure recordings were obtained by a needle in the central artery of the ear in two animals, and in one animal by a Bardic I-catheter PD-0066 inserted in general anesthesia into the thoracic aorta via laparatomia and arteriotomia of the abdominal aorta. The catheters were filled with heparinized saline and connected to a EMT 34 pressure gauge, and pressure curves were written by a Mingograf 34, Elema-Schönander AB. The pressure recordings were obtained on unanesthesized animals in physical and psychic steady state before, during and after injection of noradrenaline by the procedure described above.

Results

Clinical and Autopsy Observations

Control Group. All animals were clinically unaffected by the injections of physiologic saline, although exitement sporadically occurred. At autopsy all organs, including the aortae were grossly normal.

Table 1. Results. Control group: 11 male albino rabbits, i.v. injections of 2 ml physiologic saline daily for 14 days, 10 investigated by light microscopy, 11 by SEM. Experimental group: 12 male albino rabbits, i.v. injections of 1 mg noradrenaline daily for 14 days, 10 investigated by light microscopy, 12 by SEM

	Number of rabbits. Total material: 23			
	Physiologic saline i.v.: 11 (control group)		Noradrenaline i.v.: 12 (experimental group)	
	Light micr.	SEM	Light micr.	SEM
Observations	10	11	10	12
Normal endothelium	10	11	10	12
Hexagonal patterns of silverlines	2	0	10	10
Change of individual endothelial cells	0	0	6	4
Miscellaneous changes	5	0	7	3

Noradrenaline Injected Animals. During the injections of noradrenaline the animals were brought into a state of stupor, now and then interrupted by exitement and pulmonary edema, which had a fatal outcome in 13 cases. After the injection steady state and apparent health was obtained in minutes. At autopsy massive hydrothorax and ascites was a general and conspicuous finding. In nine animals the thoracic aortae presented severe macroscopic changes including dilatation, confluent whitish plaques, bean-shaped bulges and dispersed calcifications. In three animals dilatation of the aorta was the only macroscopic change.

Blood Pressure Recordings. As soon as the injection of noradrenaline had started, the blood pressure in a few minutes rose from the normal 100/80 to around 250/150. During the 5 minutes injection-pause the blood pressure assumed normal values in less than two minutes. At the beginning of the second 15 minutes period of injection, the same sequence of events was repeated, and in less than two minutes after closure of injections, the pressure was again normal.

Transmission Light Microscopic Studies. The changes described by Lorenzen (1966) and by Helin *et al.* (1970) were reaffirmed by the present study of very thin araldite embedded sections. This method, however, excluded recognition of metachromasia.

SEM and Light Microscopic Studies en face. SEM and light microscopy in the present as well as in previous works (Collatz Christensen and Garbarsch, 1973) conformed to a degree that a common classification and presentation of the observations obtained by the two methods was feasible. The results are presented in Table 1.

1. Normal Endothelium. All the animals in both experimental groups presented extensive areas of normal intimal surfaces (Figs. 1a and 2). By light microscopy the surface appeared flat with a rhombic pattern of nodulated thin silverlines (Fig. 1a). By SEM (Fig. 2) coarse longitudinal folds produced by the underlying elastic lamella were prominent, whereas the rhombic pattern of endothelial cells randomly covering them (Garbarsch, Collatz Christensen, 1970; Collatz Christensen, Garbarsch, 1972, 1973) closely resembled the light microscopical picture.

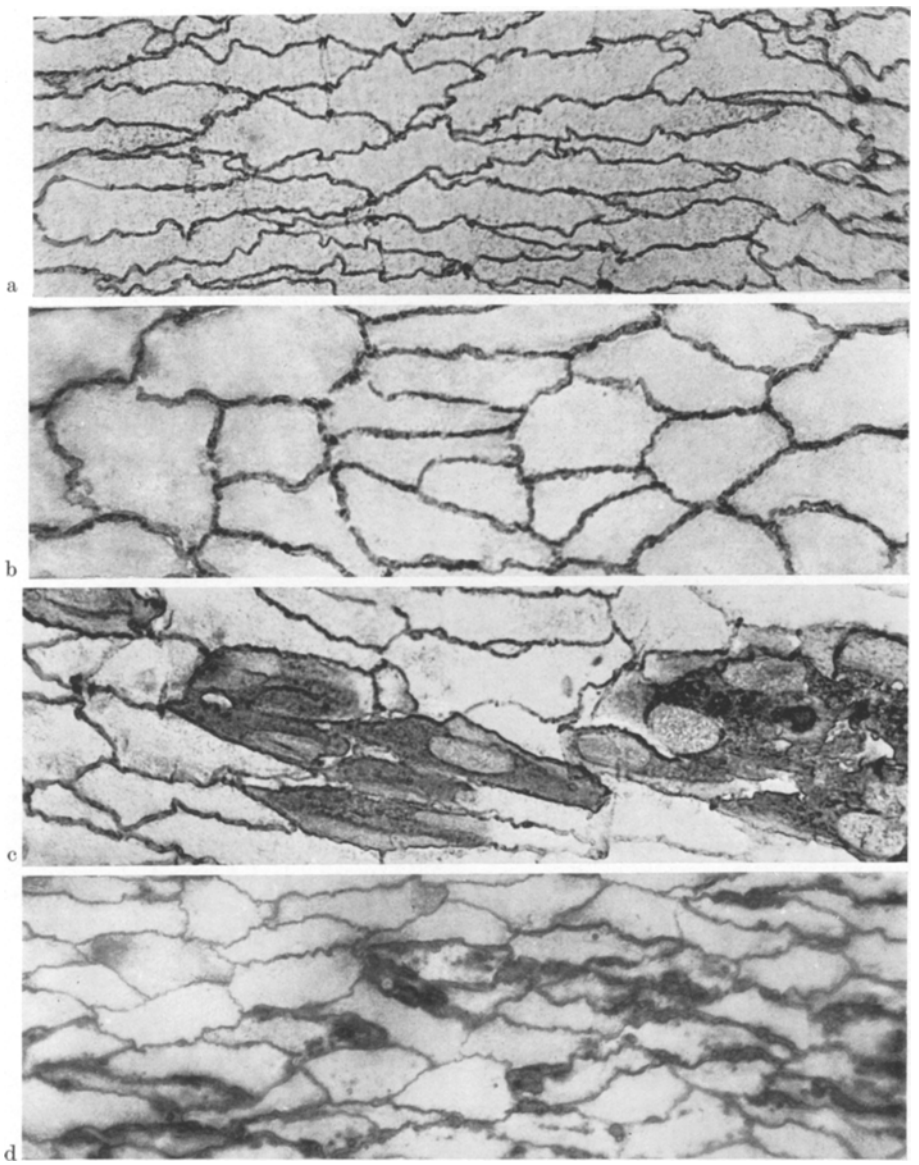


Fig. 1 a—d. Surface light microscopy of silver-stained rabbit thoracic aorta. a: i.v. injections of physiologic saline. b–d: i.v. injections of noradrenaline. Zeiss photomicroscope. a) Normal endothelial cells bounded by nodulated silverlines. A fine dustlike shadowing from precipitated silver is visible on the surfaces of the cells. × 640. Original magnification. × 160. b) Hexagonal cells left and right, polarized cells centrally, coarse uniform silverlines. × 640. Original magnification. × 160. c) Changed endothelial cells intensively stained by silver, the nuclear zones are clear and relatively unstained. × 640. Original magnification. × 160. d) Miscellaneous changes. Broadened silverlines with gaps and various uneven nodular accumulations of silver. × 400. Original magnification. × 100

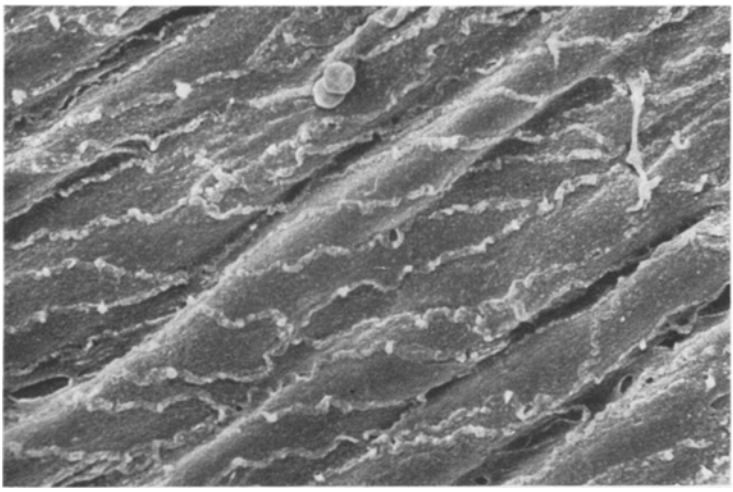
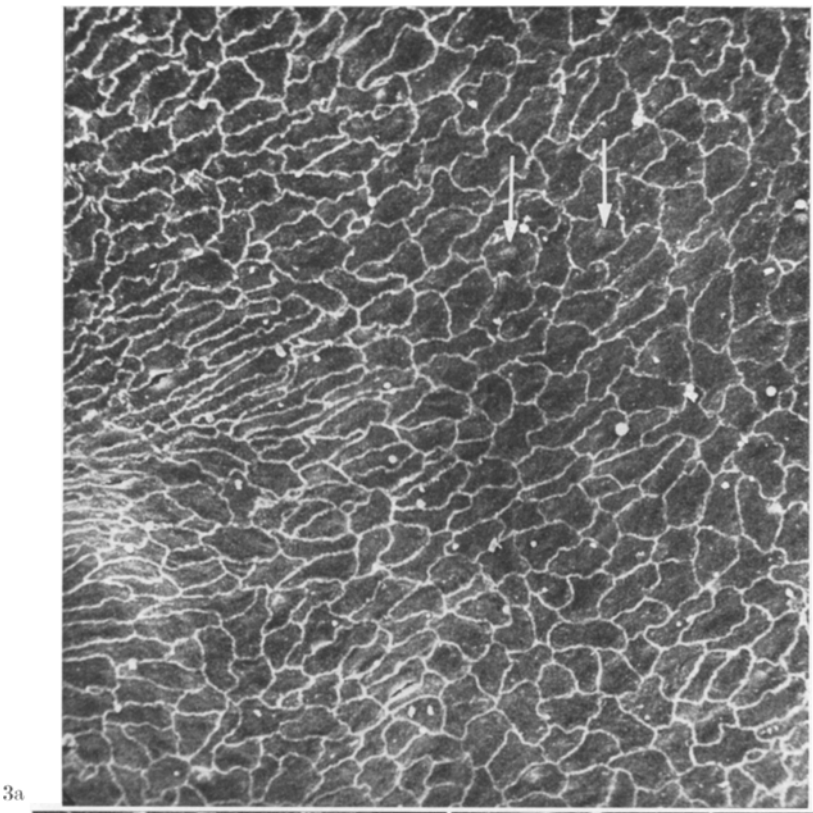


Fig. 2. Scanning electron micrcgraph of silver-stained rabbit thoracic aorta after repeated i.v. injections of physiologic saline. Control animal. The endothelial cells are bounded by nodulated silverlines, and lying on coarse parallel folds produced by the inner elastic lamella. Two blood cells (top), and various artifacts are present: one endothelial fold (top right), and several holes in the endothelial surface. × 800

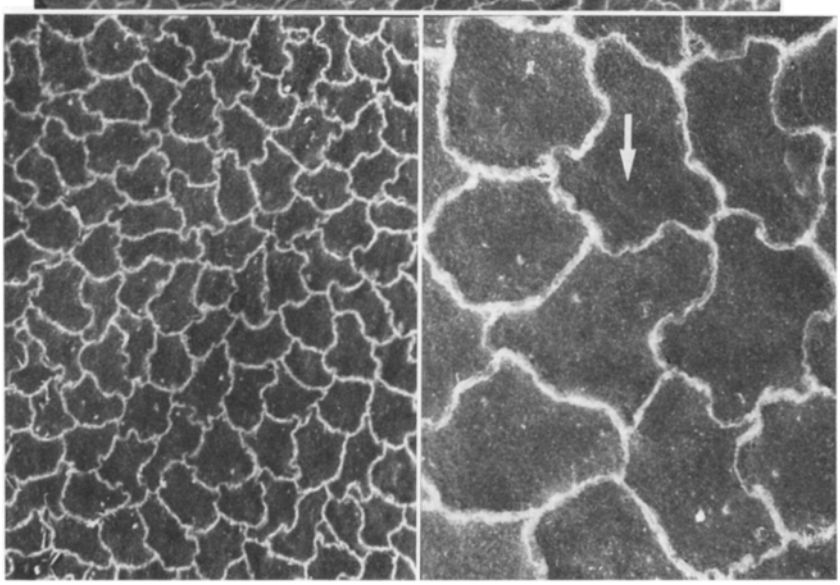
2. Hexagonal Patterns of Silverlines. Hexagonal patterns of silverlines (Figs. 1b and 3a–c) were prominent in areas of macroscopic changes, in the bottom of bulges, on ridges, and covering plaques. Accordingly it dominated in noradrenaline injected animals, but it also occurred on macroscopically normal looking plain surfaces, and was sporadically observed in the control group. By light microscopy (Fig. 1b) the cells were of equal size, the silverlines were uniform, coarse and straight, and a fine dustlike shadowing from precipitated silver covered the cell surfaces. By SEM ridges and depressions were evident (Fig. 3a), the silverlines were elevated (Fig. 3b and c), and the nuclei could present evident swelling (Fig. 3a and c). Bizarre jig-saw like patterns were frequent (Fig. 3b), and areas of mixed normal and hexagonal endothelium were prominent.

3. Change of Individual Endothelial Cells. This change was observed merely in the group of noradrenaline injected animals and occurred in areas covered by otherwise normal endothelium as well as in areas with hexagonal patterns of silverlines. The extension and degree of the lesion varied from solitary changed cells, sporadically occurring, to extensive areas with confluent groups of changed cells (Figs. 1c and 4a–c), intensively stained by silver. By surface light microscopy the cell bodies were intensively brown stained by silver, whereas a zone around the nucleus appeared clear and unstained (Fig. 1c). By SEM the cells were bright, the surface wrinkled to a varying degree with evident nuclear swelling (Fig. 4d and e), and the cell body generally appeared smaller and narrower than the cell bodies of neighbouring intact cells (Fig. 4d).

4. Miscellaneous Changes. Under this heading changes were registered, which did not lend themselves to a common classification and presentation by light



3a



3b 3c

Figs. 3—6. Scanning electron micrographs of silver-stained rabbit thoracic aorta after repeated i.v. injections of noradrenaline

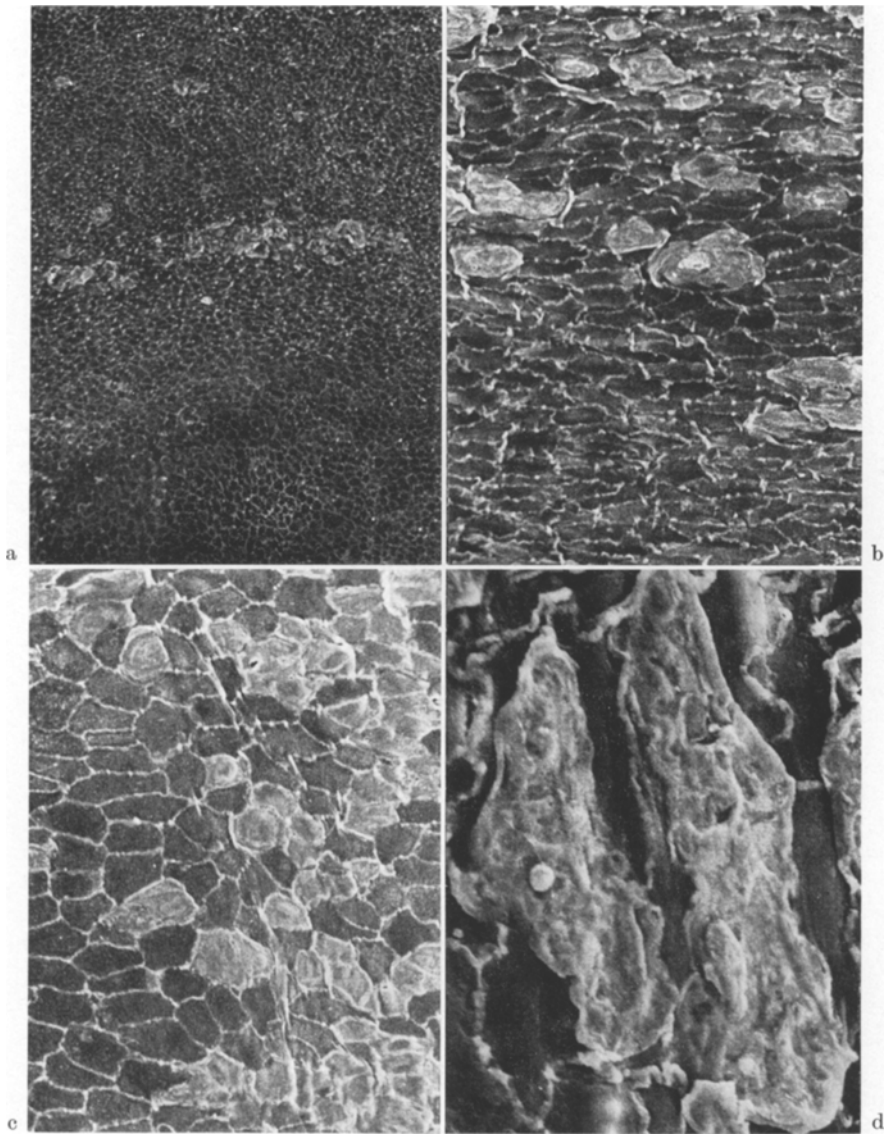


Fig. 4a—d

Fig. 4 a—e. Changed endothelial cells. a) Hexagonal endothelium with a zone of severely changed endothelial cells. × 80. b) Polarized endothelium with single changed cells. × 240. c) Hexagonal endothelium with clones of changed cells. × 240. d) Changed cells with nuclear swelling and wrinkling of the surface. × 2400. e) Changed hexagonal cell with evident nuclear swelling. × 2400. (See p. 40)

Fig. 3 a—c. Hexagonal cells. a) Uneven endothelial surface with several polarized cells and two round swollen nuclei (arrows). × 240. b) Bizarre jig-saw like pattern. × 240. c) Hexagonal cells with coarse uniform silverlines, rounded elevations of the surfaces, and one visible oval nucleus (arrow). × 800

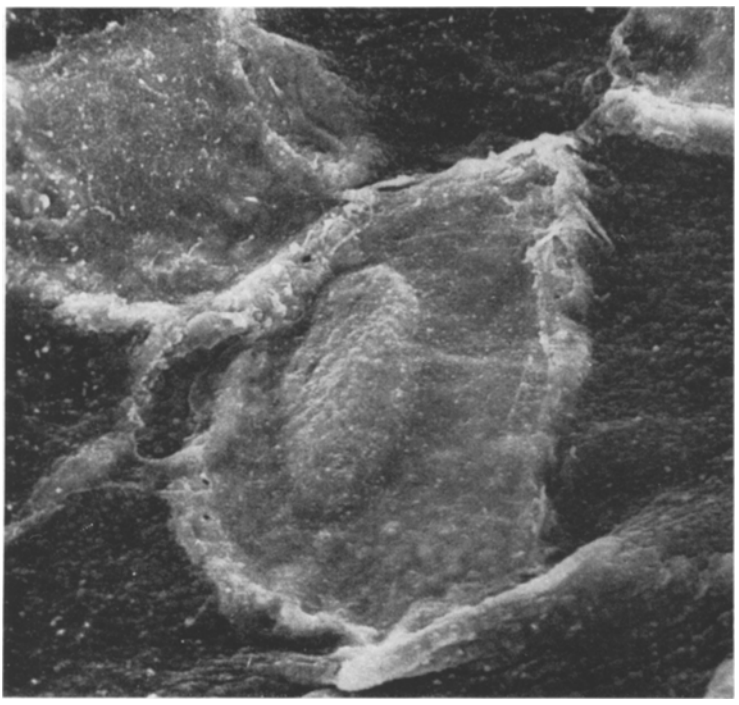


Fig. 4e

microscopy and SEM. Moreover no real difference existed between the light microscopical observations obtained from the control group and from the experimental group, whereas by SEM miscellaneous changes merely were observed in the group of experimental animals.

By light microscopy accumulations of endothelial cells presenting broadened silverlines with gaps and various uneven nodular accumulations of silver (Fig. 1d) were observed. No corresponding change was recognized by SEM, and it occurred with equal frequency in both experimental groups.

By SEM bizarre starshaped elements appeared on normal or hexagonal patterns of silverlines. They were mainly found at the corners of neighbouring endothelial cells, and tended to form ringshaped figures (Fig. 5a and b). Other free elements were cupshaped, and some resembled ghosts of erythrocytes (Fig. 6a and b). It is possible that some refracting elements on the surfaces of endothelial cells observed by light microscopy do reflect these changes.

Surfaces with nuclear swelling and unstainable by silver by SEM sporadically occurred in a few animals (Fig. 6a and b), and thrombotic elements, otherwise rarely observed, sometimes occurred on these surfaces (Fig. 6a) as a monolayer of fibrin and blood elements. Leucocytes and free mesenchymal cells were rarely observed by SEM. Equivalent findings by light microscopy may be represented by refracting free elements mentioned above.

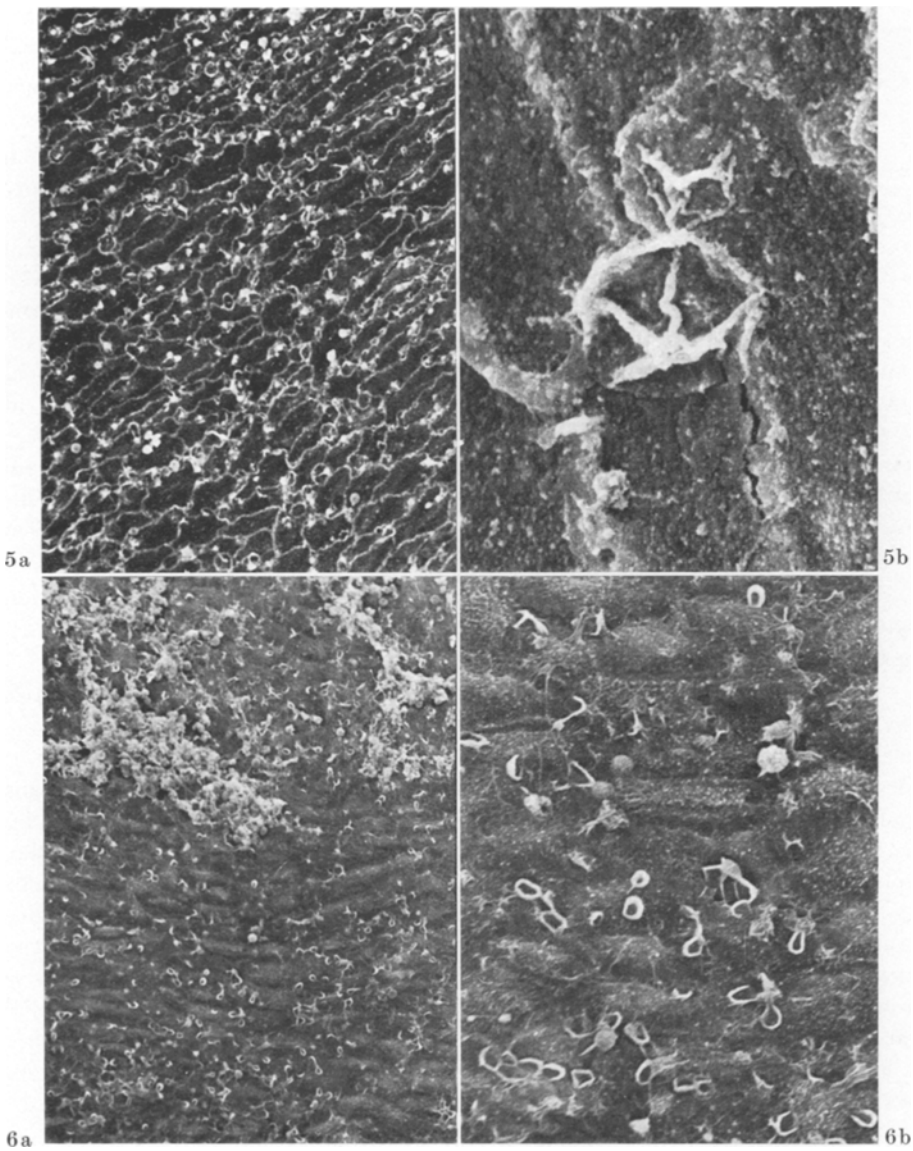


Fig. 5 a and b. Miscellaneous changes, starshaped free elements on the aortic intimal surface. a) Accumulation on corners of neighbouring cells. × 240. b) Ringshaped figures at the meeting of silverlines from four endothelial cells. × 2400

Fig. 6 a and b. Miscellaneous changes, swelling of surface cells unstained by silver. a) Thrombotic elements (top), and several free cells of blood. × 240. b) Bizarre free cellular elements of which some are deformed erythrocytes. × 800

Discussion

The observations in the present study represent the results of the integrated effects of all noradrenaline injections given throughout the period. The sequence of events during the experimental period leading to the end results is unknown.

The hexagonal patterns of silverlines were indistinguishable from the hexagonal patterns unveiled by SEM and surface light microscopy after the embolectomy catheter lesion. In the latter study the occurrence of hexagonal patterns were considered reparative, and further submitted to influences on polarization of cells from hemodynamic forces (Collatz Christensen and Garbarsch, 1973). The exact nature, however, is unclear. The most simple explanation is that the hexagonal endothelium merely reflects a mechanical dilatation of the aortic wall, causing a broadening of the pattern of silverlines. The occurrence of hexagonal endothelium on macroscopically uneven surfaces, on the bottom of bulges and at the tops of plaques and ridges rendered support to this concept. On the other hand hexagonal endothelium was observed also on macroscopically normal plain surfaces. Moreover mixed types of endothelium with polarized elongated cells intermingled with hexagonal polyhedral cells occurred both on plain and uneven surfaces.

It is probable that the endothelium takes part in cellular proliferation causing intimal thickening as well as augmentation of the intimal surface area recognized by Helin *et al.* (1970).

Cells morphologically indistinguishable from the hexagonal cells described in the present and previous works (Collatz Christensen and Garbarsch, 1973) have been studied by various methods of other authors. Fallon and Stehbens (1973) recognized bizarre patterns of polyhedral cells in the walls of artificial aneurisms and considered them as originally normal endothelium modified and damaged by hemodynamic forces. Caplan and Schwarz (1973) observed focally increased uptake of Evans Blue, increased (3H) thymidine labelling of endothelial cells and occurrence of less polarized cells in the aortae of normal young pigs at sites probably submitted to hemodynamic strains. These changes presumeably indicated increased endothelial regeneration resulting from hemodynamically induced injury. Accordingly, hemodynamic and proliferative changes of the endothelial surface may play a role in the changes observed in the present work. Further studies will have to be undertaken in order definitely to clarify the role of hexagonal endothelium.

Change of individual endothelial cells somehow resembled the "Kernaussparungen" in silver-stained damaged cells described light microscopically by Zinner and Gottlob (1961). According to these authors damage of endothelial cells rendered increased permeability to the plasmamembranes, whereas the nuclear membrane remained impermeable to silver; penetration of silver into the cells so stained the cell bodies brown, but left the nuclear regions clear and unstained. Gottlob and Zinner (1962) in their study of the repair of the intima after a "hard" mechanical trauma concluded that not only damaged cells, but also young unripe endothelial cells were submitted to the same disorder of increased cellular permeability as described above. Accordingly it is impossible by light microscopy to decide, if the changed cells in the present work are young

unripe endothelial cells, damaged cells or both. By SEM, however, the changed cells presented such varying degrees of shrinkage, folding of cellular surfaces, nuclear swelling and heaping up of cell bodies that a sequence of events finally leading to the expulsion of necrotic endothelial cells from the patterns of surface cells was strongly suggested. The presumed damage of individual endothelial cells seem to indicate a direct toxic effect of noradrenaline on endothelial cells parallel to the myotoxic effect from adrenaline described by Stief and Tokay (1935), Anitschkow (1914, 1933), Oester (1959) or from noradrenaline described by Friedman *et al.* (1955). The pathogenetic mechanism is obscure. Mechanical lesion from elevated blood pressure, augmentation of pulse amplitude and dilatation of the aortic wall may contribute to the damaging effects of noradrenaline (Lorenzen, 1963a). Hypoxia may be an additional factor, as Moss *et al.* (1969) proved reduced intramural oxygen availability from a direct metabolic effect on the smooth muscle cells of the vessel wall after noradrenaline i.v. Endothelial injury indicated by distinct calcification of the endothelial lining seemed to be an additional change of the aortic wall after systemic hypoxia (Garbarsch *et al.*, 1969), however, studies on the intimal surface anatomy were not undertaken. Degenerative lesions in the aortic media were related to changes in the plasmic lipid ratios after repeated injections of noradrenaline (Dury and Moss, 1954), and a similar mechanism may contribute to the damaging effect of noradrenaline on endothelial cells.

The spotty, multifocal distribution of changed endothelial cells is presently beyond explanation. It probably is the result of an interaction of two factors, the susceptibility of endothelial cells to injury, and the combined effect of several damaging factors. The susceptibility of cells may vary according to the life cyclus of cells, whereas the damaging factors may intensify locally from segmental dilatations or constrictions (Helin and Lorenzen, 1969), and from varying distribution of hemodynamic influences (Fallon and Stehbens, 1973; Caplan and Schwarz, 1973).

Miscellaneous changes occurred sporadically, lacked conformity between observations made by light microscopy and by SEM, and accordingly were difficult to interpret.

The light microscopical observations of broadened silverlines with gaps and uneven nodular accumulations of silver, and the occasional observation of rounded refracting elements on the endothelial surfaces resembled various endothelial changes following a "mild" mechanical trauma described by Gottlob and Zinner (1962). In the present work these changes occurred with almost equal frequency in the experimental and control group, and accordingly it remains doubtful, if they were induced by noradrenaline. Trauma of specimens, unavoidable by preparation may be responsible.

The starshaped free elements observed by SEM merely in the group of noradrenaline injected animals remained unidentified; their almost regular distribution at contiguous corners of endothelial cells was conspicuous. Other free elements probably represented cupshaped erythrocytes and ghosts from red cells. Surfaces with nuclear swelling unstainable by silver accompanied by moderate thrombotic elements seemed to indicate a severe local damage. Presently it is impossible to judge, if the aforementioned miscellaneous changes were produced

by the experimental procedure or if some of them represented artifacts from unknown causes.

Two basic questions remain unanswered: the chronology of morphological changes throughout the period of noradrenaline injections, and the question if any causal relation between the discrete appearance of damaged endothelial cells and the extensive occurrence of hexagonal endothelium do exist.

A comparison with the changes induced by the embolectomy catheter lesion (Collatz Christensen and Garbarsch, 1973) let suggest that only genuine endothelium, either normal, hexagonal or polarized mixed types covered the intimal surfaces in the series of noradrenaline injected animals. The foam like pattern of silverlines, evaluated as pseudoendothelium, probably formed from monocytes and/or mesenchymal cells was never observed in the noradrenaline experiment, but dominated in the cellular patterns after the embolectomy catheter lesion (Collatz Christensen and Garbarsch, 1973). Hence it may be deduced that the prerequisites of creation of pseudoendothelium, denuded surfaces with a scaffolding of uncovered tissue elements and fibrin did never exist during the period of noradrenaline injections.

A causal relation between damaged endothelial cells and hexagonal endothelium cannot be excluded. It presupposes, however, a very fast sequence of events leading from damage of endothelial cells to their expulsion from the patterns of surface cells. In case of a rapid exfoliation of damaged endothelial cells the hexagonal endothelium may represent a surface of endothelial cells compensating loss of cells by proliferation and/or mechanical expansion.

These questions basically remain unanswered in the present work. Further investigations and follow-up of the noradrenaline lesion by smaller doses of noradrenaline working over shorter periods of time are presently in work.

Comparison between the observations in the present work, and the results obtained by other authors is difficult on account of the large doses of noradrenaline used, and of the fact that no other authors used silverstaining for SEM. Shimamoto used small doses of noradrenaline as a one-shot lesion, and recognized within half an hour edematous reaction in the vascular wall (Sunaga *et al.*, 1969; Shimamoto *et al.*, 1971). None of the changes so induced were similar to the observations in the present work. Neither did SEM investigations of other non-mechanical experimental models unveil comparable lesions: carbon monoxide (Kjeldsen *et al.*, 1971), cholesterol feeding (Shimamoto *et al.*, 1971; Weber and Tosi, 1971a, 1971b; Weber *et al.*, 1973), i.v. injections of serumproteins (Chisolm *et al.*, 1972).

Conclusions. Noradrenaline in repetitive toxic doses induced changes in the surface anatomy of the rabbit thoracic aorta, i.e. extensive occurrence of hexagonal endothelium and discrete appearence of changed endothelial cells. The morphological changes induced in the earlier phases of lesion after one or a few noradrenaline injections are unknown, however, denuding of the intimal surface probably never existed. The hexagonal endothelium may simply be caused by mechanical dilatation of vascular wall, however, reparative processes and the effect of hemodynamic forces may be responsible. A causal relation between changed endothelial cells and hexagonal endothelium remains open to question. Further research including the study of the chronology of the

noradrenaline lesion and implication of other methods of investigation are presently in work.

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