Virchows Arch. A Path. Anat. and Histol. 370, 193—205 (1976)

The Endothelial Surface of Growing Coronary Collateral Arteries. Intimal Margination and Diapedesis of Monocytes

A Combined SEM and TEM Study

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Received May 14, 1975

Summary. Slowly progressing coronary artery stenosis leading to complete occlusion within about 3 weeks was produced in dogs. Within this time collateral vessels had enlarged sufficiently to prevent myocardial infarction. Early, intermediate, and late (1 year after occlusion) stages of collateral development were studied with the scanning and transmission electron microscope. Early after coronary occlusion the number of endothelial cells per unit inner vascular surface had markedly increased and longitudinal bulges appeared in growing collaterals as opposed to the completely flat inner surface of small normal coronary arteries. The surface of many endothelial cells appeared rough and large numbers of monocytes adhered to the inner vascular surface. The endothelial cells formed three types of patterns: streams, whorls, and nonoriented mosaics suggesting different types of flow—jets, eddies, and low-shear flow, respectively. The existence of nonlaminar flow patterns could well be explained by the extremely tortuous course of collaterals and by segmental caliber changes (microstenoses) resulting from irregularities of the internal elastic lamina.

Later stages showed a tendency toward normal endothelial cell density, flattening of bulges, and absence of microstenoses. A completely normal inner surface was, however, never observed in midzone segments although the observation period extended up to 1 year after coronary occlusion.

Introduction

The heart of most mammals including man reacts to slowly progressing coronary artery stenosis by the growth of small pre-existing collateral vessels (W. Schaper et al., 1971b). This process diminishes infarct size (W. Schaper, 1971a) or prevents the occurrence of infarcts. The alterations of these vessels during growth and maturation have been studied with the light and electron microscope and they consist of intimal, medial, and adventitial changes (J. Schaper et al., 1972). The processes at the interface between the flowing blood and the vascular endothelium appear to be of special importance but the limitations of the transmission electron microscope are obvious at this region because only an extremely small sample of tissue can be viewed in cross section. The scanning electron microscope offers the advantage of a stereoscopic view over a relatively large surface area at relatively high magnifications. For these reasons growing collaterals of dogs submitted to chronic coronary artery occlusion were investigated with the scanning (SEM) and transmission electron microscope (TEM).

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Apart from the special problem under investigation we thought it interesting to compare the spontaneous endothelial changes observed in collaterals with those described by others in injured aortic endothelium of experimental animals (Björkerud and Bondjers, 1971; Collatz Christensen and Garbasch, 1973; Weber et al., 1973; Collatz Christensen, 1974; Weber et al., 1974).

Methods

Eight mongrel dogs of both sexes and with an average body weight of 19 kg were anesthetized with subcutaneous fentanyl (0.1 mg/kg) and with intravenous sodium pentobarbital (15 mg/kg). The chest was opened under sterile conditions during intermittent pressure respiration and Ameroid constrictors of appropriate size (Litvak et al., 1957) were slipped over the left circumflex and the right coronary artery. The chest was then closed in layers and the animals were allowed to recover.

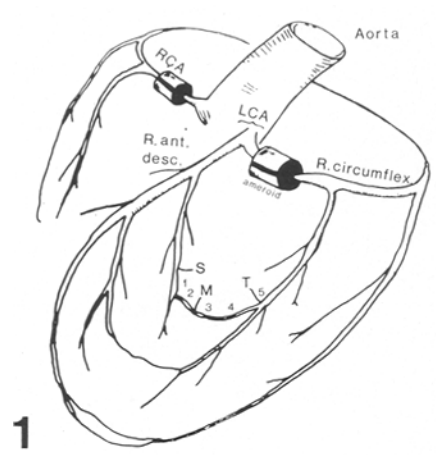
The animals were divided into three groups, i.e., early after coronary occlusion (about 3 weeks after operation, 3 dogs), intermediate (8 weeks after operation, 3 dogs), and late (1 year after operation, 2 dogs). There were no special control dogs because normal unaffected coronary arteries from each heart under investigation were felt to be a safer means of differentiating true transformation processes from other changes in the vascular wall. Thus, each animal from each group provided its own control tissue.

In order to obtain early stages of vascular growth coronary arteriography was carried out in general anesthesia once a week from the second week on. Angiography was selectively carried out for each coronary artery with Urografin 76% (Schering), 5–6 ml injected by hand. Each animal of the first group underwent this procedure twice or three times at weekly intervals. Since no endothelial alterations were observed in the control vessels of these animals, this procedure was considered to be more useful than possibly injurious because the selection of animals with actually occluded arteries had been rendered possible.

Coronary angiography was not carried out in the intermediate and late groups because the Ameroid constrictors are known to produce arterial occlusion in 18 ± 3 days (W. Schaper, 1971a).

When, upon coronary angiography, constrictor-equipped arteries were found occluded, or after 8 weeks, or 1 year p.o., the chest was opened and the heart was perfused via a wide-bore catheter (aorta clamped over catheter) with a 20° C oxygenated Tyrode's solution for 3 min. The perfusate was delivered by an occlusive roller pump at a rate of 300 ml/min which is known to produce a perfusion pressure of 60–80 mm Hg. The still beating heart was then perfused with distilled (Fahimi and Drochmans, 1965) 3% glutaraldehyde in 0.1 M cacodylate buffer at pH 7.4 for about 8 min.

According to our experience in light and electron microscopy this perfusion pressure and time are sufficient to open the coronary arteries and to prevent them from collapsing after perfusion has been stopped. The elastic membrane remains unfolded and intimal foldings of the vessel wall are avoided (Schaper, 1971a). Contractions stopped during the first minute of fixative perfusion. Easy drainage of blood and perfusates was ensured by incision of the right atrium. The heart was then removed from the thorax and all visible collateral blood vessels were cut out on blocks of myocardium according to Longland's scheme (Longland, 1953) dividing collateral vessels into stem, midzone, and re-entry (Fig. 1). In the dog's heart, the collaterals are easily recognizable with the naked eye because most of them are situated on the epicardial surface (W. Schaper, 1971a). They vary in diameter from 300 μ at early stages of growth up to 1 mm or even larger. Side branches of the unaffected anterior descending artery corresponding in size to the collateral arteries served as control tissue. The tissue



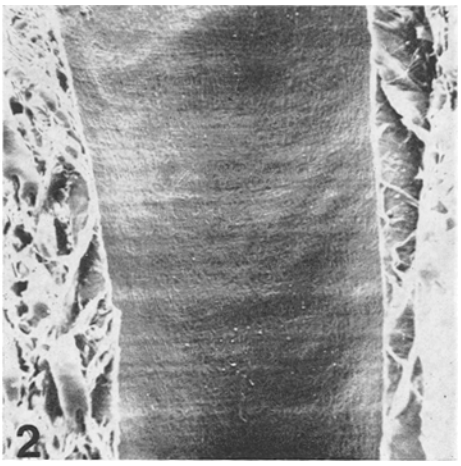


Fig. 1. Illustration of the position of Ameroid constrictors around left circumflex artery and right artery. A collateral vessel between left anterior descending artery and circumflex artery schematically presents the 3 different sections of vessel according to Longland (1953). *S* stem, *M* midzone, *T* reentry. Numbers indicate segments investigated. Control vessels taken from side branches of unaffected vessels

Figs. 2–10. SEM- and TEM-micrographs from 3 week-collaterals, midzone pieces

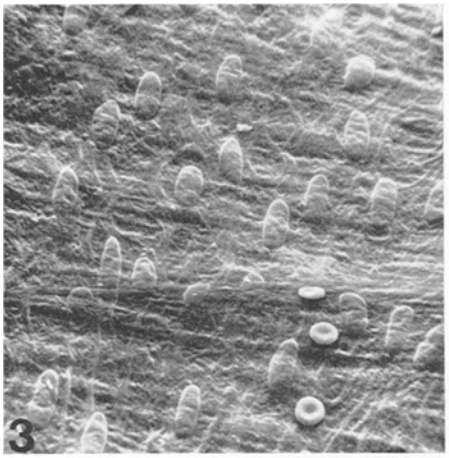
Fig. 2. Normal small coronary artery characterized by very smooth inner vascular surface and straight course over its entire length. ×100

blocks, about 7 mm long, 3 mm thick, and 5 mm wide, were further fixed in 3% glutaraldehyde in 0.1 M cacodylate at 4° C for 12 h. Small tissue samples were then taken from the large block for TEM and processed routinely. The remaining blood vessels were carefully cut open lengthwise to expose the inner vascular surface. In order to standardize experimental conditions, we chose the part of the vessel situated on the myocardium for further treatment and investigation. After rinsing in 0.1 M cacodylate with 7.5% sucrose for 12 h the tissue was osmicated for 2 h. Since freeze-drying without substitution of water before quenching failed to give reproducible results, critical-point drying was used (for technical details see König et al., 1975). Critical-point drying was achieved using amyl acetate as intermediate fluid and carbon dioxide as final transitional fluid as described by Anderson (1951). Shrinkage of the tissues was about 15–20% but no artefacts or distortions due to shrinkage were evident. After drying the specimens were quickly put into the evaporation chamber to avoid possible distortion of the now hydrophile tissue by absorption of air moisture. A carbon-silver sandwich layer was chosen as evaporation layer. The samples were viewed in a S4-10 Cambridge SEM at 20 KV or exceptionally at 30 KV.

Results

The results are displayed pictorially in Figures 2–17.

Collateral arteries and normal small coronary arteries were differentiated according to their anatomical situation. Collaterals must, by definition, connect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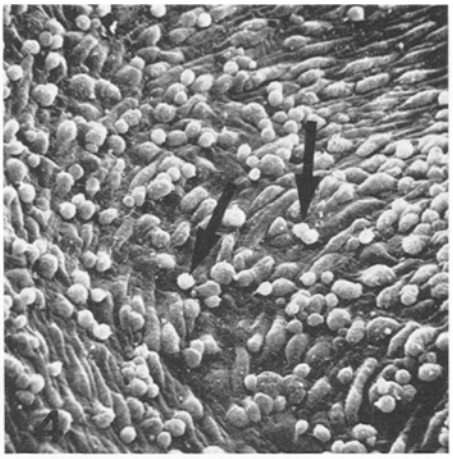
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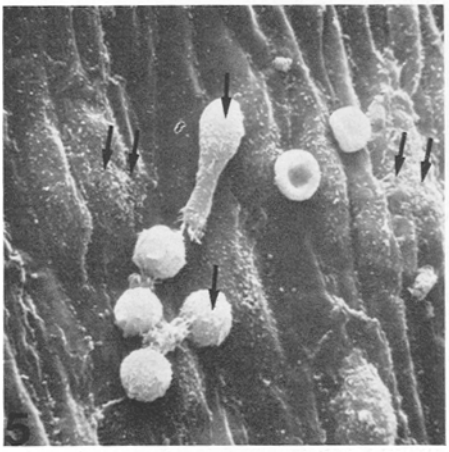
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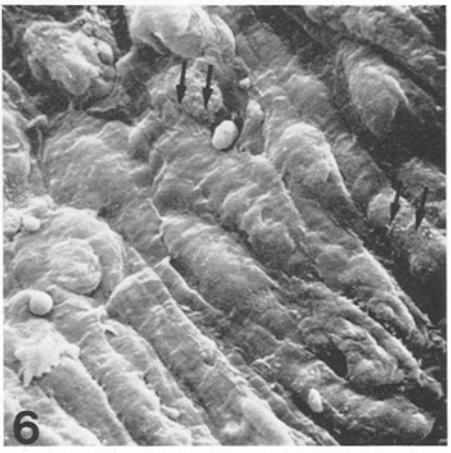
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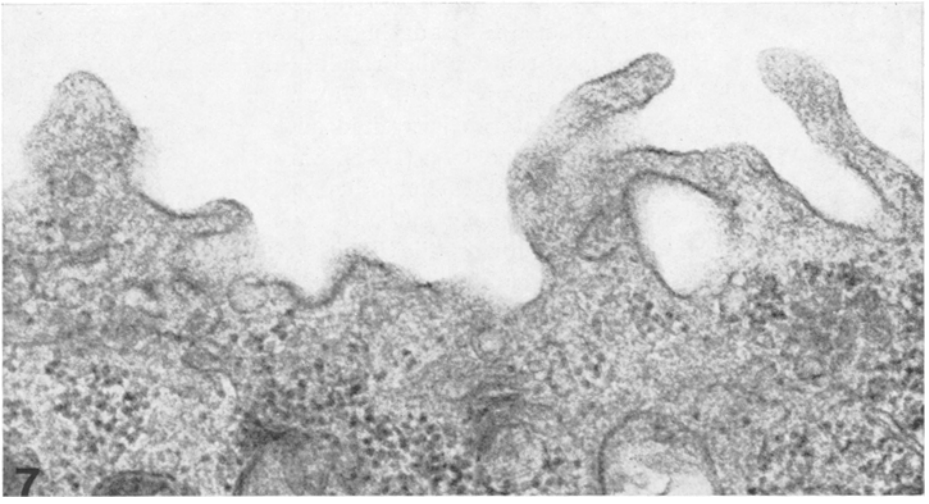
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two larger coronary arteries. Macroscopically, both types of vessels have the same appearance, except for the tortuosity seen in collaterals. They are 300–500 μ in internal diameter at early stages of growth. This diameter increases at later stages up to 1,000 μ and above.

A. Normal Coronary Arteries

Figure 2 shows a typical small coronary artery in its entire length with its straight course and its smooth endothelial surface. The cytoplasm is flattened exhibiting fine prominent lines which might indicate the cellular borders. The oval nuclei are slightly prominent and they show alignment with the direction of flow (Fig. 3). Figures 2 and 3 are typical examples of several dozens of vessels and representative of hundreds of fields viewed.

B. Collateral Segments (Midzones) during Growth and Maturation

Midzone segments during growth are characterized by the greater density of endothelial cells per surface unit compared with that of normal coronary arteries of the same size (Fig. 4). Not only the number but also the individual cell size has apparently increased. As a result the inner vascular surface is no longer flat and smooth but it shows irregular bulges caused by the numerous enlarged endothelial cells (Figs. 5 and 6). Some of these cells have a rough surface (Fig. 5) which corresponds to the numerous small extensions in the TEM-micrograph of Figure 7.

Another characteristic feature of midzone segments during growth is the occurrence of numerous monocytes that appear attached squidlike to the endothelial cells (Figs. 4 and 5). Although the SEM-picture of this white cell can, at times, be confused with lymphocytes, TEM-pictures showing monocytelike cells attached to the endothelium and situated in the vascular wall allow identification of these cells (Figs. 9 and 10).

The most obvious characteristic of midzone segments is their irregular surface. One can distinguish between bulging protrusions caused by the high density of voluminous cells that are in perfect alignment with the bloodstream on one hand (Figs. 4 and 5) and caliber changes of the vessel that appear as transverse ridges and are mostly perpendicular to the stream axis (Figs. 11 and 12). Since

Fig. 3. Small normal coronary artery. Endothelium is flattened, oval endothelial nuclei oriented in longitudinal axis of vessel are clearly distinguished. ×650

Fig. 4. Increased number of endothelial cells bulging into vascular lumen. Note orientation of cells according to bloodstream. Monocytes (arrow) adhere to endothelial surface. ×120

Fig. 5. Endothelial cells protruding into lumen in longitudinal bulges. Nuclei not detectable due to greatly increased cell volume. Some cells show numerous small protrusions (double arrow), monocytes present (arrow). ×1,190

Fig. 6. Extremely irregular endothelial cells, some exhibit rough surface (double arrow). ×1,190

Fig. 7. TEM-micrograph of small protrusions of an endothelial cell comparable to those shown in Figs. 5 and 6. ×20,000

these ridges are apparently obstacles to flow they might cause aberrant flow patterns that leave their imprints on the orientation of the endothelial cells. The different patterns of endothelial cells as streams, whorls, and nonoriented mosaics suggest jets, eddies, and low-shear flow areas in growing collateral arteries.

The intermediate stage at 8 weeks p.o. (5 weeks and 3 days after occlusion) shows less dramatic changes as compared to the early stage (Figs. 13 and 14). The endothelial cell density is still above normal, monocyte attachment is only occasionally observed, and caliber changes (ridges) that may cause turbulence are rare. A tendency toward a smoother surface is perceivable but not yet accomplished. One year after coronary stenosis an almost but not completely normal endothelial surface has formed (Figs. 15 and 16).

C. Reentrant Segments during Growth and Maturation

Reentrant segments are, according to Longland (Longland, 1953), short pieces of vessels that connect midzones with proximally occluded subbranches of the vascular system. Since this vascular segment was larger than the midzone before occlusion its rate of growth is probably only modest. Endothelial surface changes are therefore rather undramatic and they consist mainly of a moderate degree of cellular crowding, i.e., increased endothelial cell density (Fig. 17). These changes disappear as the vessel matures.

Discussion

The following findings will be discussed here:

1. Increase in surface density of endothelial cells, especially in midzone segments in the early and intermediate stages of growth causing longitudinal bulges of the inner surface which align with the bloodstream.
2. The attachment of a large number of monocytes on endothelial cells.
3. Caliber changes of the vessel with a ridgelike appearance which presumably result in disturbances of flow.

Alignment of Endothelial Cells and Population Density

Vascular injury, most probably caused by excessive tangential wall stress (W. Schaper, 1967b) is a typical transient stage in growing collaterals (J. Schaper

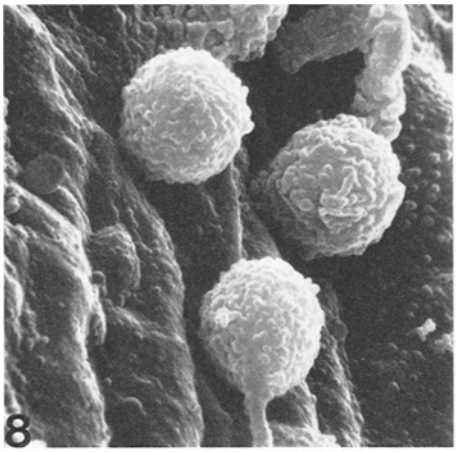
Fig. 8. Monocytes adhering to endothelial surface. ×2,940

Fig. 9. TEM-micrograph showing monocyte on vascular surface with pseudopod-like extension penetrating between 2 endothelial cells. ×4,900

Fig. 10. TEM-micrograph of monocyte lying between endothelium and inner elastic membrane. ×14,000

Fig. 11. Collateral vessel exhibiting numerous circumferential ridges. Note varying arrangement of endothelial cells. Upper part of vessel dilated and cells arranged transversally to direction of bloodstream suggesting turbulent flow (arrow). ×60

Fig. 12. Enlarged area of foregoing picture. To left, endothelial cells form an uninterrupted streak suggesting linear flow (jet), to right there are whorls (eddies, arrow) and an area of low-shear flow (double arrow)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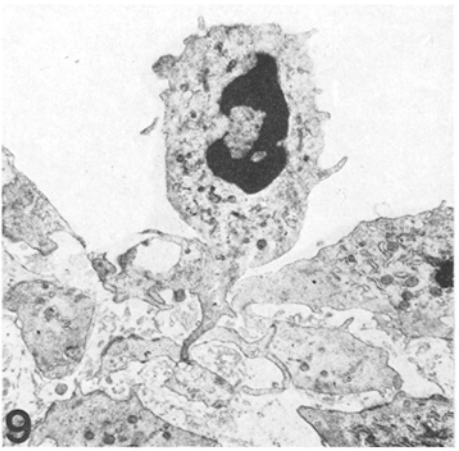
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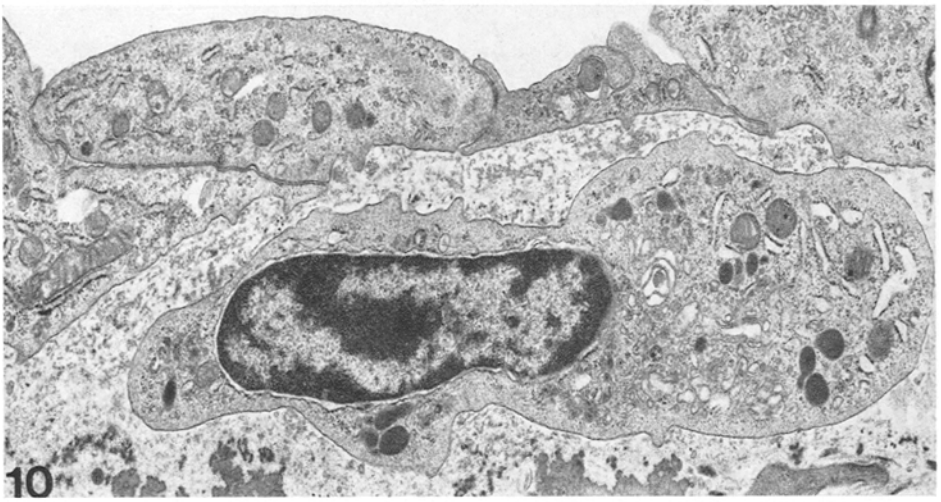
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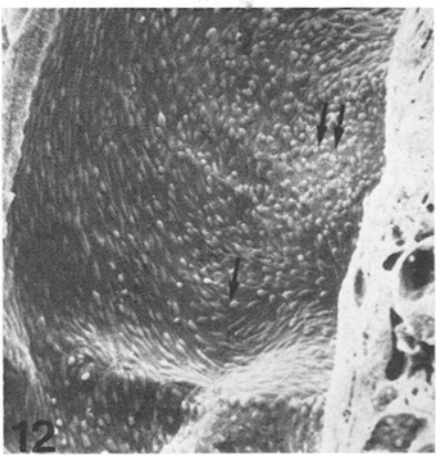
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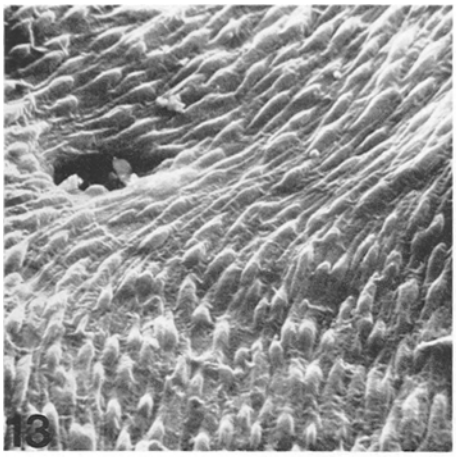
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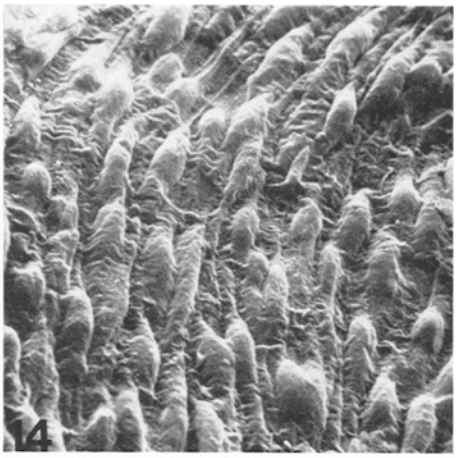
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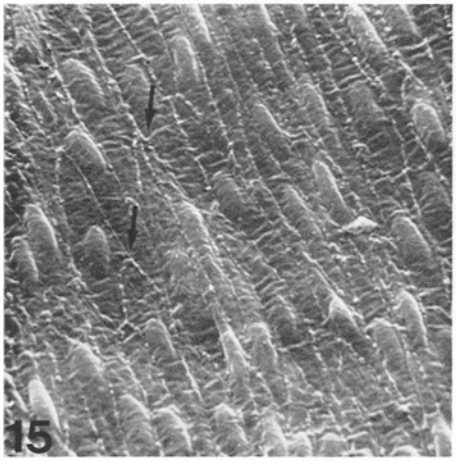
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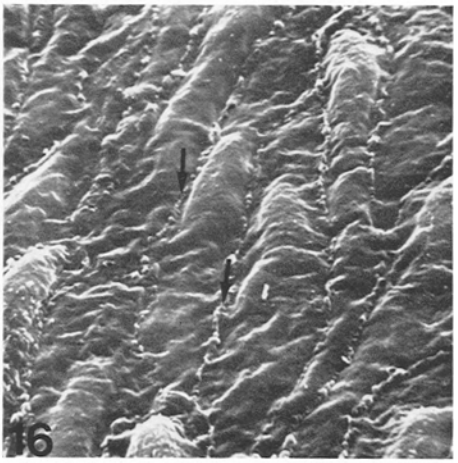
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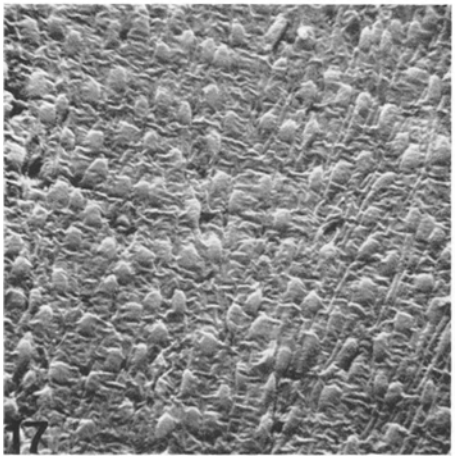
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et al., 1972). In our TEM-studies it was often noted that the stages of repair that finally result in a much larger vessel show many similarities with other models, i.e., mechanical trauma (Björkerud, 1969; Hoff and Gottlob, 1968), atherosclerosis (Thomas, 1963; Still, 1964), and experimental hypertension (Still, 1967; Wiener et al., 1969). The present SEM-results, however, show a very discriminating feature: flow alignment of endothelial cells is only observed in growing collaterals but not in the traumatized aorta (Björkerud and Bondjers, 1971; Collatz Christensen and Garbasch, 1973; Collatz Christensen, 1974). In a SEM-study, Asmussen and Kjeldsen (1975) described endothelial injury in human umbilical arteries caused by smoking. They observed cytoplasmic blebs on the endothelial surface which are quite different from the cytoplasmic protrusions resulting in a "rough" surface of the cell which we observed. Nelson et al. (1975) described the appearance of "small globular structures" on the endothelial surface of the carotid artery subjected to ischemia. In hypertensive rats, Still (1974) observed many small cytoplasmic protrusions of the endothelial cells which are more alike to those described in this study in growing arterial vessels.

There are no data in the literature regarding SEM of small coronary arteries. The interpretation of surface changes of collateral vessels can therefore only be carried out in comparison to the unaffected blood vessels and to coronary arteries in other stages of development. Since rough-surfaced cells were only observed during the early stage of growth they are considered as a specific symptom for the growth process.

A very pertinent question is the inquiry into the causes of endothelial proliferation that was described earlier (W. Schaper et al., 1971b) on the basis of H^3-thymidine incorporation of endothelial nuclei. We, as well as others (Liebow, 1963; W. Schaper, 1971a), expressed earlier the belief that a combined action of physical and chemical forces leads to the proliferation of all cellular constituents of the vascular wall. The present studies with the scanning electron microscope show, however, a disproportionate increase of the number of endothelial cells per unit of inner surface. This observation suggests strongly that shear forces at the blood vessel wall interface act in the same way on endothelial cells as tangential wall stress acts on the smooth muscle cells of the tunica media. Since shear forces at the blood vessel wall interface are known to have risen high above normal values at early stages, the theory that relates flow velocity to diameter

Figs. 13 and 14. Views from 8-week collaterals, midzone pieces

Fig. 13. Still greatly increased number of endothelial cells arranged in direction of bloodstream. Monocytes absent. ×290

Fig. 14. Enlarged area of Fig. 11, showing increased volume and size of otherwise regular endothelial cells. ×580

Figs. 15 and 16. Views from 1-year collaterals (midzone pieces)

Fig. 15. Very regular pattern of almost flat endothelial cells; slight increase in volume and size, however, can still be observed. Cell borders prominent (arrow). ×580

Fig. 16. Although endothelial surface of this vessel is almost flat, cells are larger than in normal vessels. Small protrusions suggest cell borders (arrows). ×1,250

Fig. 17. Increased cell density and size in reentry segment of collateral at 3 weeks. ×290

(Thomae, 1895; Schoop, 1961)) appears applicable, at least for the tunica intima. At later stages when shear forces, flow velocity, and turbulence have declined because of a much greater arterial diameter, the initially increased number of endothelial cells covers a larger surface and population density normalizes again. Since nonlaminar flow is a prominent feature of early adaptive changes one is tempted to speculate that the later appearance of endothelial bulges and their alignment in the axis of flow might reduce turbulence by creating a laminar interface, as is common engineering practice in high flow fluid dynamics.

Appearance of Monocytes

During the phase of fast growth, when individual vessels enlarge by a factor of 2–8 times their original internal diameter (W. Schaper, 1971a) monocytes adhere to many endothelial cells. There is a preference of attachment points to regions of possible sluggish flow but this may only reflect the difficulties of monocytes to adhere in areas of fast flow (Schmid-Schoenbein, 1975). The interaction of white blood cells with vascular endothelium has been ascribed to inflammation at the microcirculatory level (Allison, 1955; Florey, 1970) and to changes in blood flow characteristics, i.e., changes in shear stress (Fry et al., 1968, 1969; Atherton and Born, 1972, 1973). Adhesion of monocytes to capillaries and arteriolar endothelial cells after microtrauma has been described by Florey (1970) and by Grant (1965). Björkerud and Bondjers (1971) reported on the appearance of monocytes on mechanically injured endothelium which were considered to transform into "pseudoendothelial" cells to rapidly cover intimal defects. Previous cytochemical studies from our group (Borgers et al., 1972; Borgers et al., 1973) have shown a similar enzyme pattern of monocytes, endothelial cells, and those cells that comprise the subintimal proliferation which is characteristic in mature stages of collateral growth.

Adhesion of monocytes to the endothelium in developing collaterals (present study) is only observed during the phase of rapid growth of the vessel. This fits well into the framework of current knowledge regarding monocyte physiology. On the cellular level, adhesion depends on the interfacial tension of the cell surface which is readily measurable by contact angle techniques (Van Oss and Gillmann, 1972a). The lower the electrostatic barrier of a cell the easier adhesion is established. From these experiments it is known that lymphocytes do not adhere because of their high surface tension which means that they are hydrophobic. Monocytes have a lower surface tension, they are less hydrophobic and they adhere easily. Van Oss et al. (1972b) also showed that monocytes are able to spontaneously displace water from the surface of other cells once molecular contact is made. The strong propensity of monocytes for forming pseudopods further facilitates adherence to other cells. Sticking of mononuclear cells on layers of growing fibroblasts and the production of migration inhibition factor (MIF) by cells during growth without an antigen present (Tubergen et al., 1972) are also well-known phenomena. MIF-release is dependent on activation of the cells to enter the generative cycle, particularly on cells in S-phase, whereas in contact-inhibited cells little or no MIF was found. It is proposed that margination of monocytes on endothelial cells, as seen in our study, is a growth-specific

process which is only apparent during the early stages of rapid vascular development. This process does not seem to be related to an inflammatory response in the vessel wall. Inflammatory reactions are observed occasionally during the early stage of growth, but this reaction is localized in the adventitial layer of the vessel which is separated from the endothelial lining by several layers of smooth muscle cells.

Turbulent Flow Pattern and Its Impact on the Endothelial Surface

The observed flow obstacles, i.e., sharp bends, tortuosity, and transverse ridges producing flow jets, eddies, and areas of low-shear flow are of different origin. The curvature of collaterals has been known for a long time, it stands out clearly in coronary angiograms in vivo and post mortem. This "meandering" of collaterals was explained (W. Schaper, 1971a) by the fact that these vessels, unlike normal coronary arteries, are situated in the main axis of cardiac contraction and relaxation. This causes rhythmic longitudinal tension finally resulting in a longer vessel.

Irregular transverse caliber changes that appear like "microstenoses" are regularly found in the early stages of the growth transformation of collaterals and they disappear with time. They are easily recognizable in negative moulds that are obtained when gelatin suspension of barium sulphate (used for angiographic purposes) are removed from these vessels. The origin of these microstenoses is probably related to the rupture of the internal elastic lamina, a common histologic finding during the early stage of collateral development (J. Schaper et al., 1972). Rupture of the elastic lamina in some areas of the vessel could produce aneurysmlike dilatation whereas microstenosis would occur at places where the elastic lamina still exists. Later on a new elastic membrane develops resulting in the disappearance of aneurysms and stenosis and a straighter course of the vessel.

The authors are indebted to Miss Adriane Rademakers, Mr. Paul van Even, and Mrs. B. Iacurti for their skilful technical assistance.

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