Re-endothelialization of microvascular carotid end-to-side anastomosis in the rat

A scanning electron microscopic study

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A carotid end-to-side anastomosis was performed on 25 male and female Wistar rats (mean weight 197.8 gm). The animals were sacrificed at time intervals varying from 0 to 21 days after the operation. The anastomosis was exposed, the aorta cannulated, and the animals perfused with a 2.5% buffered glutaraldehyde solution at a constant pressure of 80 mm Hg. The anastomoses were removed for scanning electron microscopic (SEM) and light microscopic studies. The SEM results indicate that after the acute platelet-fibrin reaction in the first 48 hours, the suture line itself becomes re-endothelialized after 4 days. On the stitches, however, a cellular population consisting of leukocytes transforming into flattened cells was seen after 2 days. The morphology of these cells and their role in the regeneration of endothelium is discussed. This study presents evidence supporting a blood-borne genesis of endothelial cells in vivo.

KEY WORDS □ microvascular anastomosis □ endothelium □ scanning electron microscopy □ blood-borne re-endothelialization

A great deal of research has been carried out on the reaction of the vascular wall to trauma. Most of the investigations were inspired by the desire to elucidate the pathophysiology of the development of atherosclerotic lesions. Many mechanical and chemical experimental models have been used, and a comparison is not easy. Further study has recently been stimulated by the development of microsurgical vascular techniques.

Three distinct methods have been used in these studies. Light microscopy (LM) allows the study of the profile of the vessel wall and a maximum working magnification of ×1200. Transmission electron microscopy (TEM) is also a profile method with a maximum magnification of ×10⁶. Scanning electron microscopy (SEM) is a surface method with a magnification between ×25,000 and ×50,000. In the 1930's, an LM method for the surface study of the endothelium was developed, mainly by German workers, and has been used more recently by Poole, et al.,²⁰ and Collatz Christensen and Garbarsch.⁷ This method, called the "Häutchen technique," enables one to study the surface of endothelium by stripping off the most superficial layer of the vascular wall, embedded in nitrocellulose.

Scanning electron microscopy is very useful for the study of cellular changes that occur at the surface of the vessel wall in cases of trauma and repair of the endothelium. This method was introduced in 1969 by the pioneers, Shimamoto, et al.,³⁸ for the study of the endothelium.

It is of the utmost importance to employ standard methods for preparation and fixation of the specimens in order to obtain uniform and reliable results,⁶,¹⁸ and to avoid artifacts, formerly described as normal or pathological characteristics of the tissue, such as intercellular bridges, pronounced longitudinal folds, artificial blebs, and craters in normal endothelium.²⁰ Gregorius and Rand⁰ described the most intense reaction to trauma in microvascular anastomosis occurring at some distance from the suture line and at places where vascular clamps were applied. Rosenbaum and Sundt,⁹ examined microvascular anastomoses in the rat, and studied the acute throm-
botic reaction at the site of the anastomosis. Both papers concentrate purely on the reaction of the vascular wall in the first hours after surgical trauma.

The aim of this study is to examine the process of re-endothelialization itself.

Materials and Methods

Twenty-five male and female Wistar rats (mean weight 197.8 gm) were anesthetized with Nembutal (pentobarbital) intraperitoneally. They were placed in the supine position with the head supported. Under a Zeiss diploscope a horizontal incision was made just above the manubrium sterni. Subcutaneous tissue and pretracheal muscles were bluntly dissected and held apart by wound retractors made from paper clips. A tracheotomy was performed at the lower end of the thyroid gland and the animal was intubated with a small polyethylene tube. Both the common carotid arteries were exposed as far as possible beyond the bifurcation of the internal and external carotid artery. During this procedure the tissue was frequently irrigated with a 1% lidocaine-saline solution in order to avoid vascular spasm and unwanted carotid sinus and vagal reflexes. The left carotid artery was tied off just proximal to the bifurcation. A Scoville clamp was applied at the proximal end of the vessel. The vessel was cut tangentially just below the bifurcation and rinsed with a heparin-saline solution. Two Scoville clamps, 1 cm apart, were applied to the right common carotid artery. With ultrafine microscissors a small oval slit, approximately 1.5 mm in length, was made in the vessel wall, and the vessel was flushed through with the heparin-saline solution.

The end-to-side anastomosis between the left and the right common carotid arteries was made with interrupted stitches of 10-0 Ethilon suture. The front wall was sutured in the usual way. The vessel was then rotated gently around the axis of the right common carotid artery by means of a fourth Scoville clamp. Care was taken to avoid subjecting the donor vessel to too much tension. Recently we have adopted a modified technique: the posterior wall of the anastomosis is sutured continuously from within, and the anterior wall with interrupted stitches from the outside. After completion of the shunt the animal was intubated orally; the tracheotomy and the wound were closed. Three animals died during or shortly after the operation, probably as a result of respiratory insufficiency due to a pre-existing tracheobronchitis (during the operation tracheal suction was frequently applied), or depression of respiration caused by the Nembutal.

At intervals varying from immediately to 21 days after the operation the animals were anesthetized again, and the shunts were exposed and inspected for obstruction. Only one shunt had been blocked. In 17 animals the shunts were prepared as follows for SEM study. The abdominal aorta was exposed and cannulated with a sialography catheter. The animals were perfused in toto with Ringer’s solution at a constant pressure of 80 mm Hg. The jugular veins were opened and excess solution allowed to drain out. Thereafter they were perfused with a 2.5% solution of buffered glutaraldehyde. The shunts were tied off while full, and were put in a 2.5% buffered glutaraldehyde solution. After 4 hours the tied ends of the shunts were cut away and the specimens opened with microscissors. The specimens were then fixed on a piece of cork with small needles, and replaced in the solution.

Four animals were treated in the same manner, but perfused with a 10% formalin solution for LM control study. The SEM preparations were fixed for 24 hours in a 2.5% buffered glutaraldehyde solution, rinsed in a 0.1 M phosphate buffer, and dehydrated in rinsing concentrations of acetone. The tissue was dried by the critical-point drying method, covered with a molecular layer of gold, and examined with a Cambridge Stereoscan S 4.*

Results

Results Immediately After Operation

The suture line and stitches are covered with a thin layer of coagulum, consisting of flocy threads of fibrin, in which cellular elements have been trapped (Fig. 1 c). The architecture seems to be random and no flow direction is discernible. Heavy cellular particles lie in the shelter of the stitches or in the craters made by the needle passing through the vascular wall. Rounded or coarsely lobulated thrombocytes with a few offshoots are seen.

Results 24 Hours After Operation

The platelet-fibrin reaction has markedly increased 24 hours postoperatively. The stitches are covered with a thick layer of coagulum, and the whole gives the impression of a snowdrift (Fig. 1 d). The fibrin threads are arranged in the flow direction of the blood. Platelets have long offshoots, lying mainly in the direction of the flow. The edges of the craters made by the needle are clearly marked. Leukocytes are caught in the meshwork of the platelet-fibrin coagulum, or lie in small groups in the craters. A few scattered red blood cells are seen.

Results 2 Days After Operation

The turbulent snowdrift effect has decreased by 2 days. The coagulum has stabilized, and is more massive and less reticulated than before. Some stitches are not covered by thrombus. On these the first signs of cell populations appear: platelets without

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offshoots and leukocytes. The cells have lost their spherical appearance and seem to be flattened. Their coarse microvilli have become finer and are limited to the central part of the cell.

Results 3 Days After Operation

The cell populations on the stitches have increased by 3 days and are more varied (Fig. 2 a). Transient forms from leukocytes are seen, which cling with small pseudopodia to the sutures and to flattened covering cells (Fig. 2 b and c). The pseudopodia increase in number, spread out, and flatten as the cytoplasm flows centripetally over the surface of the thread. The microvilli are no longer coarse lobules, but delicate dots (Fig. 2 d). At the base of some stitches an advancing re-endothelialization from the normal vascular wall is observed.

Results 4 Days After Operation

The suture line itself appears to be completely re-endothelialized 4 days postoperatively (Fig. 3 a). The axis of the cell bodies and their nuclei is in the direction of the flow. At both sides of the suture line firm endothelium is seen as a transparent veil (Fig. 3 b).
FIG. 2. Scanning electron micrographs showing the cell population surrounding the stitches 3 days postoperatively.  

a: Stitch with a typical cell population. Clusters of leukocytes can be seen adherent to the thread and flattening out. × 350. 
b: A leukocyte “holding” the stitch with small pseudopodia. Notice the marked ear-lobed microvilli. × 3500. 
c: Two leukocytes (1) and an erythrocyte (c) can be seen. × 3500. 
d: Sample of cell population at a stitch, with leukocytes flattening out (asterisks). To the right a so-called “ant hill” cell (a) is seen. × 1400. 
e: Detail of the “ant hill” cell (a), showing the small dotted microvilli and enlarged cell body. Note that the cell boundaries are far beyond the center of the cell (asterisks). × 3500. 
f: Large “ant hill” cell covering most of the thread. A slight mechanical injury can be seen below. × 1400.
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Fig. 3. Scanning electron micrographs of endothelium covering the suture line on the 4th day. a: New endothelium has covered the suture line between the stitches. The cells are arranged in the direction of the blood stream. X 140. b: Detail of an area at the bottom of Fig. 3 a, just outside the picture. New endothelial cells are growing as a veil over a damaged area and can be discerned by their microvilli. X 700. c: In another area the new endothelial cells lie perpendicular to the folds in the internal elastic lamina. Their appearance is quite normal. X 700. d: Advancing endothelium at a remote place from the suture line over a non-thrombogenic blanket of platelets and fibrin.

The appearance of the cells is quite normal: fine microvilli lie over the nuclei and at the edges of the cell body. They cover the subendothelial folds in a transverse direction (Fig. 3 c), adapting to the new flow direction from donor to recipient vessel.

An increased number of leukocytes, flattened in form, is found in the cell populations on the stitches. The covering cells grow and enlarge their territory and almost resemble ant hills in shape (Fig. 2 e and f). The boundaries of the cell extend beyond the central zone, covered by numerous tiny microvilli as a sign of increased metabolism. The needle craters are smoother and their cell population is uniform: some of the erythrocytes are spherical leukocytes.

From then on, re-endothelialization of the stitches advances rapidly. “Ant hill” cells increase in number (Fig. 4 a) and flatten out (Fig. 4 b). Their boundaries grow together, and cover the thread almost completely (Fig. 4 c). Endothelium extends from the base and comes into contact with the differentiated blood-borne cells on the stitches (Fig. 4 d). This advancing process develops into complete endothelialization (Fig. 5 a).

Results 7 Days After Operation

The suture line and most of the stitches are covered by a substantial layer of endothelium at 7 days. At some distance from the suture line regeneration of endothelium has taken place. Here and there it has
FIG. 4. Scanning electron micrographs showing late stages of re-endothelialization of the stitches. 

a: Transitional forms of "ant hill" cells covering the thread almost completely. × 2600. 
b: Stitch with flattened "ant hill" cells. Note the complete re-endothelialization of the suture line. The edges of the needle craters are smoothed and covered by endothelium. Inactive leukocytes are sheltering in the craters. At lower right the base of the stitch junction with endothelium is seen, slightly damaged. × 650. 
c: Detail of the base of the stitch seen in Fig. 4 b. × 2600. 
d: Detail of the top of the stitch seen in Fig. 4 b, showing flattened cells with their boundaries approaching each other. The cell bodies seem to be connected by bridge-like structures. × 2600.

the appearance of the so-called "cobblestone endothelium" (Fig. 5 d). In some specimens marked foldings of the lamina elastica intima are seen with normal rhomboid endothelial cells, interconnected by bridges.

Results 10 Days After Operation

Not all the specimens are in the same phase. In most specimens, endothelial regeneration starts on the third day and is completed within 1 week. In some, after 10 days stitches are in full regeneration. In the same specimen one may find re-endothelialized stitches alternating with those populated by different cell forms 10 days after the operation. At some distance from the suture line, sheets of advancing endothelium can be seen growing over a blanket of platelets and fibrin (Fig. 3 d).

Results 14 Days After Operation

The last phases of re-endothelialization are seen at 14 days: lettuce-like leukocytes alongside regenerated endothelial cells. The larger needle craters are smoothly rounded, and their interior is covered by a continuous layer of endothelium. These usually re-
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FIG. 5. Scanning electron micrographs showing the completed endothelium. a: Endothelium covering a thread. It is not clear whether these cells are advancing growth from the endothelium of the vessel wall or from blood-borne elements. X 650. b: Endothelium 14 days after the operation. The stitch is completely covered by normal endothelium. X 700. c: Endothelium 3 weeks after the operation. X 70. d: Regenerated so-called "cobblestone" endothelium at a remote site from the suture line, 14 days after the operation. X 1400.

main as defects in the vascular wall. Endothelium at a distance from the suture line is completely regenerated and looks quite normal.

Results 3 Weeks After Operation

Apart from a single mechanical injury made during the preparation, re-endothelialization is complete at 3 weeks. Even the most erect stitches are overgrown (Fig. 5 c). The stitches can, on the whole, only be detected as small irregularities in the uniform layer of cells, forming a static picture of the flow properties of the blood.

Discussion

The endothelium forms a continuous layer and covers the inner surface of the entire vascular system: the heart, the arteries, veins, and lymph vessels. The cells, which are subjected to the rheological stress of the pulsating blood stream, have a high rate of metabolism. Their life span is relatively long, as determined by ³H-thymidine-labeling experiments. In 1 sq mm there are about 4000 to 5000 cells.

According to Maximow and Bloom, endothelial tissue is mesodermal in origin and is formed by flattening of mesenchymal cells. Under LM the cells
have the appearance of fibroblasts. Their longitudinal axis is oriented in the direction of the flow of the blood stream.14 The morphological characteristics of normal endothelium seen by SEM were recently described as follows:29,46 a continuous blanket of rhomboid cells, arranged in the direction of flow of the vessel, the nuclei slightly pronounced, covered by microvilli and the boundaries of the cells also marked by microvilli. In normal endothelium, occasional blebs and craters are found, these being signs of slight endothelial damage probably due to hemodynamic stress on the branching points of vessels.20,23

Reaction of the Vessel Wall to Trauma

The reaction of the vascular wall to trauma can be compared to that of the skin.3 Characteristic of this reaction is the phenomenon of subendothelial thickening. Our LM results did not justify the inclusion of this subject in this study; the reaction is, however, important in understanding the events that revolve around vascular trauma, and has been described in detail.3,5,10,16,22,29

Subendothelial thickening can only be seen in typical profile investigations as shown by LM and TEM. The SEM method of examination only reveals the events that take place on the surface; the first sign of injury is the appearance of blebs and craters.20,23,27 Other signs of endothelial damage as seen by the SEM method are a more pronounced nucleus, transverse folds on the cell body, an increase in the number of intercellular bridges, and the longitudinal folds of the internal elastic lamina in the vessel wall. It is extremely difficult to separate the artifacts of faulty preparation and fixation from the signs of slight injury in vivo; no consensus is found in this matter.27

In the case of gross injury, the intercellular connections are separated, and sloughing of cells can be seen.17

Acute Posttraumatic Platelet Reaction

There is no uniform relationship between endothelial injury, platelet reaction, and the blood clotting mechanism.44 According to the classical concept of Duguid,19 there is an equilibrium between clotting and lysis at the inner surface of the vessel wall. In the case of endothelial damage, a platelet reaction is triggered mainly by the collagen fibers from the deeper layers of the vascular wall,41,42 by which an ultimate target area is formed by the basement membrane and the internal elastic lamina.5,39

Adhesion of the platelets to the fibers is followed by cohesion of the thrombocytes and thrombin, causing a compact coagulum to form.44 The platelet reaction consists of a platelet-release reaction. Thrombocytes degranulate, and metabolites such as adenosine diphosphate (ADP) and 5-hydroxytryptamine (5-HT) are released into the blood stream.2 This is probably a positive feedback mechanism, during which a great deal of energy is liberated and a stable low-energy state attained.41 At the surface of the thrombocyte, adenosine triphosphate (ATP) is broken down to ADP by the surface-bound ATPase, an active process necessary to maintain the platelets in a free form. Tissue injury causes release of ADP, the platelet wall-bound process is inverted and the platelet release reaction is triggered. During the degranulation the platelets become spherical in form with long offshoots,39 and a platelet-fibrin blanket is formed within a few hours, covering the injured vascular wall, a layer without thrombogenic properties.11

After 24 hours the platelet reaction stabilizes. This is exactly the same phenomenon as seen in our material in the first 24 to 48 hours.

Theories About Re-Endothelialization

The acute reaction of the vascular wall to trauma is followed by regeneration and repair. Re-endothelialization is an important part of this process. Although one recognizes the ontological mesenchymal origin of the endothelial cell, different theories are held about the actual source of new endothelial cells in vivo. Most experimental models bear on the re-endothelialization of vascular prostheses, rabbit-ear chambers, or experimental denudation of the vascular wall. Relatively few have any bearing on the repair after endarterectomy or microsurgical vessel anastomosis.11,16,17,20,25,29,36

Advancing of Cells from Defect Edges

Poole, et al.,33 who used the "Häutchten technique," described a platelet-fibrin blanket containing "monocytes," as the endothelium slowly advanced from the edges to the center of the defect.82 Discussing the different theories, they concluded that endothelium arose from endothelium, and smooth-muscle cells from smooth-muscle cells.82 Our Fig. 3 d has almost the same appearance as Fig. 3 in the article by Poole, et al.,82 both show endothelium advancing slowly over a blanket of platelets and fibrin. Perhaps this represents a two-stage process: 1) sloughing of damaged endothelium and the generation of a non-thrombogenic layer on the denuded vascular wall; and 2) regeneration of endothelium from the existing layer to cover the area. Florey, et al.,15 observed rapid re-endothelialization of Dacron grafts from the edges of the normal vessel wall. By means of "Häutchten" and TEM techniques they noted that the cells seemed to be endothelial, and that a basement membrane was formed, although incompletely. Nomura28 also explained his photographs as endothelial cells moving over a preformed layer of smooth-muscle cells. He, however, left open the possibility that the endothelial cells originate from smooth-muscle cells, as these share certain features with young endothelial cells such as fibers and dense bodies. Moseley, et al.,25 described the appearance of neo-endothelial islands at
Our study does not permit any conclusions about endothelium-like cells are described. Although no absolute proof of their identity can be given on purely morphological grounds alone, it cannot be concluded on its histological merits; to Mrs. Brenda Vollers and David Turner, M.B., Ch.B., F.F.A.R.C.S., D.A., for preparing the manuscript; to the Laboratory for Experimental Thoracic Surgery, State University, Utrecht (head G. A. Charbon, M.D.); to Prof. F. J. Keuning, M.D., for judging the paper and its histological merits; to Mrs. Brenda Vollers and David J. Turner, M.B., Ch.B., F.F.A.R.C.S., D.A., for preparing the English version of the paper; and to Miss B. Asselbergs, J. Turner, M.B., Ch.B., F.F.A.R.C.S., D.A., for retyping the manuscript.

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The Role of Smooth-Muscle Cell

In 1936, on the basis of LM evidence, Stchelkounoff suspected a relationship between smooth-muscle cells and endothelial cells. Many authors described an active thickening of the layer between the endothelium and the elastic internal lamina as a reaction to injury of the vessel wall. The ability of smooth-muscle cells to proliferate in atherosclerosis is well known. Wissler considered the arterial smooth-muscle cell to be a multipotential mesenchymal cell, able to form collagen, elastin, smooth-muscle fibers, and the basement membrane. Transient forms from smooth-muscle cells to endothelium or endothelium-like cells are described. Our study does not permit any conclusions about processes taking place in the deeper layers of the vascular wall.

The Role of Blood-Borne Cells

Discussion of the pedigree of the endothelial cell has a long history. It is very difficult to distinguish specific characteristics of different cells purely on LM grounds. In their experiments, Ghani and Tibbs used impermeable synthetic grafts. Stump, et al., constructed hubs in the axis of the blood stream, inaccessible to the sedentary cells of the vascular wall. In both papers, organization of the platelet-fibrin blanket is observed, together with advancing growth from the edges of the prosthesis. A covering consisting of three layers on a Dacron hub, floating freely in the blood stream, was noted by O'Neal, et al. The outer layer, as seen by TEM, definitely had all the characteristics of endothelium. In the pig, the middle layer was composed of cells that looked like fibroblasts; in the dog they resembled myointimal cells. The innermost layer in contact with the synthetic material consisted of a matrix of collagen fibers, fibroblasts, and multinucleated giant cells. Davies, et al., considered that hematogenic re-endothelialization was possible. In their denudation experiment, they observed a quick islet-like regrowth of endothelium. Cells resembling macrophages remain sheltered from the blood stream in the irregularities of the terrain. Gradually, these transform into endothelial cells. Although no absolute proof of their identity can be given on purely morphological grounds, transformation from monocytes via macrophages and fibroblasts to endothelial cells has previously been observed in diffusion chambers.

Baumgartner and Spaet observed adhesion of granulocytes to the denuded segment of the vessel wall 3 hours after the acute platelet reaction. After 1 day monocytes appeared, and after 4 days they observed a complete re-endothelialization. Labeling experiments with H-thymidine, in their opinion, supported the concept that mononuclear cells from the blood stream play a role in the process of endothelial regeneration.

This study provides an opportunity to observe a mode of re-endothelialization for structures lying out of the immediate reach of the vascular endothelium itself. It strongly suggests that the process of re-endothelialization is initiated by cells circulating in the blood stream, namely, white blood cells from the monocyte-macrophage type. That these cells are capable of flattening out and adopting the appearance of endothelial cells is shown by Figs. 2 and 4. Ongrowth by advancing endothelium at the base of the stiches and by blood-borne re-endothelialization occurs at the same time. It is theoretically possible that the new endothelial cells could originate from another location, floating freely in the blood stream, as maintained by Hauss, but this does not seem very likely.

Conclusions

Most authors agree about the possibility of regeneration of endothelium by advancing growth from the edges of the injured area. Opinions as to the source of the cells differ. It seems feasible that in larger vessels the ostia of the vasa vasorum contribute to this process. In deeper layers of the vascular wall the repair process is initiated soon after the trauma, resulting in subintimal thickening and a fragmentation and/or reduplication of the internal elastic lamina. Several authors have observed transitions from smooth-muscle cells to neo-intimal cells. However, on morphological grounds alone, it cannot be concluded for certain that these are true endothelial cells. This study does not allow any statement about this concept. The possibility of the origin of endothelial cells from blood-borne elements is also a tentative theory. The process of transformation from leukocytes, possibly monocytes, by way of macrophages and fibroblast-like cells to endothelial cells has been proposed, and this study presents evidence supporting this theory, as shown by SEM observations.

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