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Light and scanning electron microscopic changes observed in experimental arterial forks of rabbits

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Summary. Experimental arterial forks were fashioned by anastomosing one common carotid artery to the contralateral vessel by microvascular surgery in rabbits. In one rabbit group the forks were examined histologically by the serial section technique from 5.5 to 25 months post-operatively. The second group was used for scanning electron microscopy of the arterial endothelial surface from 1 to 257 days post-operatively. Intimal proliferation was observed at the lateral angles histologically at sites comparable to those where intimal proliferation occurs spontaneously in human infants. In addition, disruption of the internal elastic lamina with minimal intimal proliferation occurred at the sides of the neo-apex, mostly in the transposed artery. These disruptions corresponded to predominantly transversely orientated tears of the internal elastic lamina from 8 days post-operatively in the scanning electron microscopic study. They were similar to the early atrophic changes preceding berry aneurysm formation in human cerebral arterial forks. The results indicate that both intimal pads (cushions) and elastic tears can be haemodynamically induced.

Keywords: arterial forks, atherosclerosis, aneurysms, mural atrophy, elastic tissue tears, intimal proliferation, scanning electron microscopy

Intimal proliferation, widely acknowledged as being an early stage of atherosclerosis, occurs in cerebral arterial forks of foetuses and neonates, not diffusely but as localized thickenings at specific anatomical sites (Stehbens 1960). This localization is consistent in man and also in the cerebral arteries of other animals examined (Stehbens 1972). Intimal thickenings extend, coalesce, and progress to overt atherosclerosis without any sharp line of demarcation (Stehbens 1963); in some forks, preaneurysmal areas of thinning or mural atrophy develop and ultimately proceed to berry aneurysm formation. Similar zones of mural thinning or atrophy can be induced experimentally in the common carotid artery of rabbits by altering vascular haemodynamics (Stehbens 1986) and experimental berry aneurysms of cerebral arteries have been produced by an imbalance of flow at cerebral arterial forks with or without hypertension and/or lathyrism (Hazama & Hashimoto 1987). Therefore experimental arterial forks were fashioned by microcvascular surgery in the neck of rabbits to examine by light and electron

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microscopy the changes induced in such forks by altered blood flow.

Materials and methods

In all, 33 rabbits aged 6–8 months, of mixed breed and both sexes, were used. Each animal was anaesthetized with intravenous sodium phenobarbitone and ether, and the neck opened ventrally in the midline under sterile, aseptic conditions. The two common carotid arteries were mobilized and dissected free of fat and loose areolar tissue. One common carotid artery was ligated proximally, clamped distally and sectioned obliquely just distal to the ligature. The open distal stump was flushed with sterile saline and its oblique end anastomosed to the medial side of the contralateral common carotid artery with continuous 8-0 monofilament nylon microvascular sutures to fashion an arterial fork (Fig. 1). The transposed carotid artery was passed ventral to the trachea in 12 rabbits (Group A) and the site of anastomosis was determined so as to avoid undue stretching of the transposed carotid artery. The transposed artery was passed dorsal to the trachea and oesophagus in 21 rabbits (Group B) to reduce the curvature imposed by the trachea. All animals were maintained on a stock diet of rabbit pellets and water *ad libitum* until sacrifice.

Group A rabbits, kept for varying periods of time (5.5, 5.5, 7.5, 11, 12, 15, 19, 20, 21.5, 22, 22, 25 months) post-operatively, were sacrificed by an overdose of intravenous sodium phenobarbitone preceded by intravenous heparin (1000 IU). The chest was opened and a catheter inserted into the ascending aorta via the left ventricle. A ligature was placed about the base of the aorta to hold the catheter in place whilst the carotid arteries were perfused with buffered formal saline for 20 min at a pressure of 100 mmHg. The anastomosed common carotid arteries were then carefully dissected, removed, and pinned with stainless steel pins onto a cork board or sheet of rubber at approximately the same angle of anastomosis. After further fixation the forks were embedded in paraffin and serial histological longitudinal sections were cut to produce **Y**-shaped sections according to the technique previously described (Stehbens 1960). Most sections were stained with Verhoeff's elastic stain and eosin and Mallory's phosphotungstic acid haematoxylin. Those remaining were stained with either haematoxylin and eosin, toluidine blue or periodic acid Schiff.

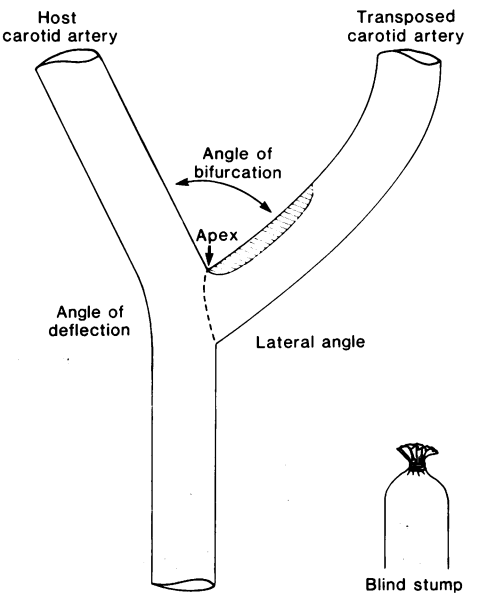

Fig. 1. Diagram of the experimental arterial fork. The interrupted line is the position of the suture line and the area where tears of the internal elastic lamina were most frequently found is indicated by oblique lines. The nomenclature used is as indicated. The face is the uppermost region of the fork in the centre of the **Y** and the back or dorsum is the corresponding region underneath the fork, usually representing the extension of the flow divider.

The rabbits of Group B were similarly sacrificed at varying intervals (1, 2, 2, 4, 5, 6, 6, 7, 7, 8, 8, 10, 12, 13, 14, 16, 23, 48, 131, 167, 257 days) post-operatively. Following a brief washout of blood with isotonic saline, the carotid arteries were fixed *in situ* by perfusion with half-strengh Karnovsky's fixative in cacodylate buffer at a pressure of

100 mmHg for 45 min. The trachea was reflected beforehand to avoid pressure flattening of the transposed artery. After perfusion, the fork was dissected and photographed *in situ*. The forks were then removed and immersion fixed in the same fixative for at least a further 12 h. Each arterial fork was bisected longitudinally, pinned out on a polythene sheet exposing the endothelial surface, and the location and orientation were recorded. The specimen was subsequently washed in several changes of cacodylate buffer, post-fixed in cacodylate buffered 1% osmium tetroxide, dehydrated through increasing strengths of ethanol, and critical point dried in carbon dioxide. Forks were cut into numbered segments in sequence with the location and presentation of each block recorded. They were coated with carbon followed by gold/palladium and the luminal surface was examined in a Cambridge Stereoscan 250 scanning electron microscope at 20 kV.

Results

Macroscopic

At the time of surgery it was observed that the host common carotid artery was somewhat deflected from its straight course at the site of anastomosis and the transposed common carotid artery ran a curved course around the ventral aspect of the trachea in Group A rabbits. At sacrifice the angles of bifurcation varied between 40° and 90°, the neo-apex being somewhat flattened, but in two specimens it appeared slightly everted suggesting incipient aneurysmal dilation. In wide-angled bifurcations the transposed common carotid artery curved anteriorly with varying abruptness as it coursed ventral to the trachea and the host carotid was angled to a varying degree at the site of anastomosis. An additional rabbit omitted from this group had been found to have organized thrombotic occlusion of the transposed artery.

In Group B rabbits, the bifurcation angles varied between 30° and 100° and the course of the transposed common carotid also exhibited variable curvature. The host artery was invariably deflected at the anastomotic site but the neo-apex was not everted.

Histology (Group A)

Common carotid arteries had all healed. Slight irregularity of contour of the anastomotic site was evident due to the sutures and fibrotic vascular scar tissue with foreign body giant cells about the sutures. Distinct musculo-elastic intimal thickening at the apex was minimal. In four specimens there was stretching or thinning of the anastomotic repair tissue in the apical region varying from flattening to a small evagination or bulge (Fig. 2) which accounted for the flattened appearance macroscopically. Some musculo-elastic intimal type proliferation present within two such pouches was similar to that seen in small micro-evaginations of the human cerebral arterial forks (Stehbens 1963). In another fork (22 months postoperatively) there was a slight mural evagination just distal to the neo-apex but in the host artery, with loss of elastic laminae, some intimal proliferation and thinning of the underlying media (Fig. 3) were observed. Another fork had a slight bulge at the proximal end of the suture line.

At the proximal end of the anastomosis, intimal proliferation was more pronounced within the entrance of the transposed carotid than on the side of the host artery (Fig. 4) and was not centred on the anastomotic site as would be the case if the intimal proliferation was merely the response to surgical trauma. This proliferation was therefore suggestive of a lateral angle pad and the underlying internal elastic lamina exhibited thinning or an interruption. The thickenings varied. In one fork of 11 months and a bifurcation angle of 90°, the pad extended distally along the full length of the transposed carotid segment sectioned.

The host carotid artery was deflected to a varying degree opposite the anastomosis and

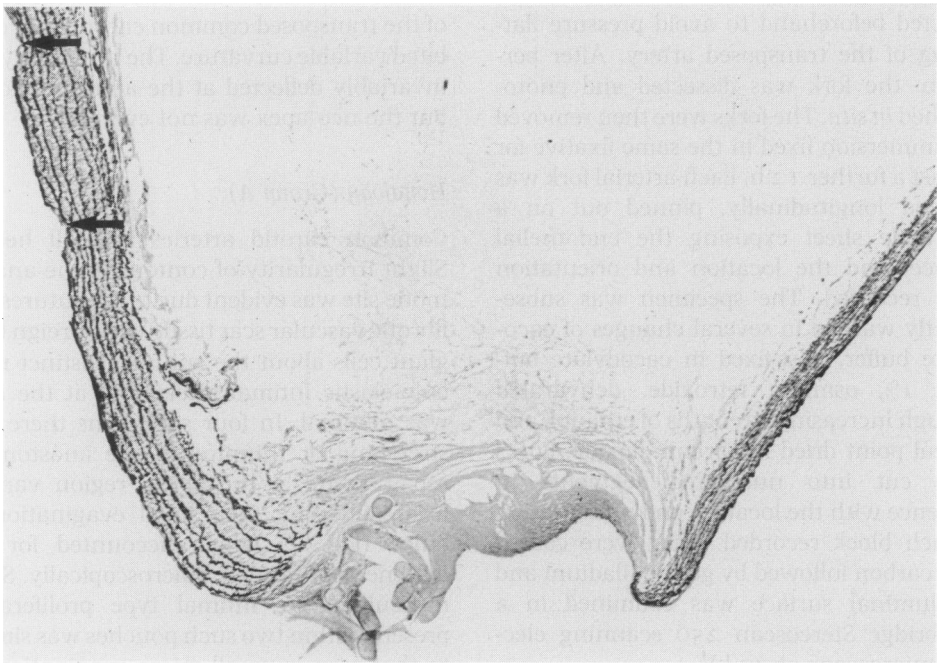

Fig. 2. Apical region of an experimental fork demonstrating a small evagination as the result of stretching of scar tissue. Suture material has caused an artefact to the left side of this region. The internal elastic lamina is intact along the wall of the branch to the right but is interrupted by tears (arrows) on the left side of the fork (15 months post-operatively, Verhoeff's elastic stain and eosin × 50).

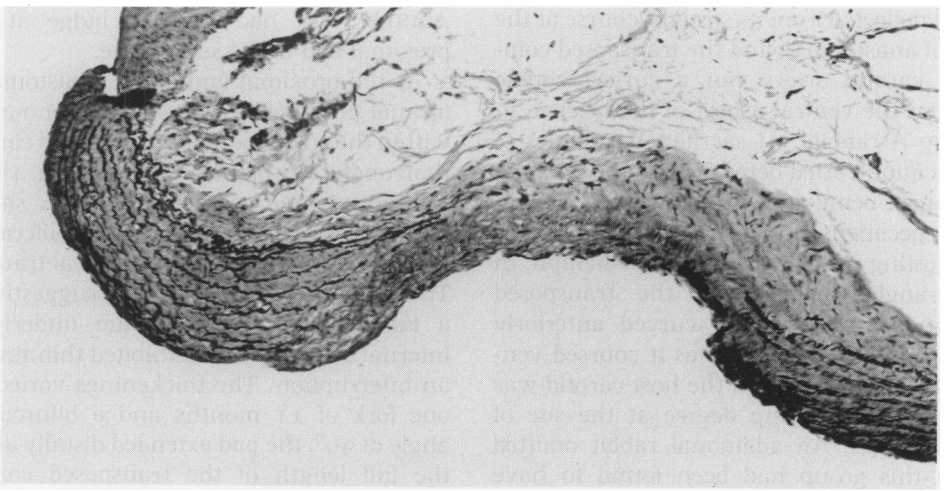

Fig. 3. Small evagination in wall of host artery just distal to the neo-apex (to the left). Note interrupted elastic laminae and disrupted architecture (7.5 months post-operatively, Verhoeff's elastic stain × 130).

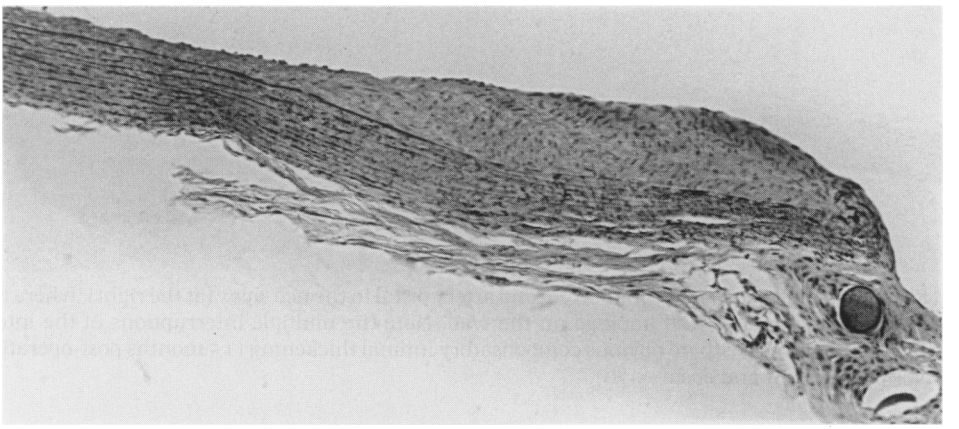

Fig. 4. intimal thickening extending from the proximal end of the suture line (at the right) is analogous to a naturally occurring lateral pad or cushion in location and appearance. The thickening extends into the transposed artery with thinning and interruption of the underlying internal elastic lamina (5.5 months post-operatively, Verhoeff's elastic stain and eosin × 110).

in eight forks there was very slight intimal thickening at this site, usually with one or two interruptions of the underlying internal elastic lamina, again suggestive of a lateral intimal pad or cushion (Stehbens 1960).

Most of the internal elastic lamina was intact in the host common carotid artery both proximally and distally, except for occasional isolated, symmetrical discontinuities at some distance from the anastomosis. These were considered to be due to the vascular clamps applied during surgery but, not always being present, were dependent presumably on the closing pressure of the clamps. An occasional small, inconsistent interruption observed adjacent to the suture line and thought to be an artefact was possibly due to inadvertent trauma caused by the microvascular needle. However, multiple interruptions of the internal elastic lamina were consistently located in the transposed carotid distal to the neo-apex where the flux of blood entering the newly fashioned branch would impinge (Figs 2 and 5) in all but one fork. The area of these interruptions varied from fork to fork, the maximum extent amounting to five diameters downstream of the neo-apex.

In one fork (7.5 months post-operatively) there were tears of the internal elastic lamina extending from the neo-apex distally (one diameter in length) in the host common carotid artery which was grossly angled, the proximal segment of the host artery being almost aligned with the transposed carotid.

In forks of long standing, very slight intimal proliferation with some fibrillary elastica was present about some elastic tears (Fig. 6) on the distal aspect of the transposed carotid. In some there appeared to be a depression in the wall or slight medial thinning in the floor of the tears (Fig. 5) even though by scanning electron microscopy, the margins of the elastic tears may have been masked.

In three wide-angled forks where the transposed common carotid underwent a distinct bend from a relatively transverse to an anterior course, multiple interruptions of the internal elastic lamina were observed on the outer or greater curvature with some intimal proliferation beyond the lesser curvature. It was considered these were not due to vascular clamps but analogous to the changes previously observed about experimental **U**-bends (Stehbens 1986). Other forks did not include such bends in the sections.

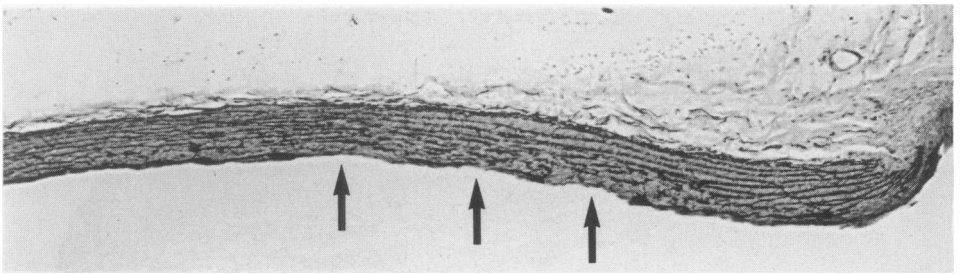

Fig. 5. Segment of the transposed common carotid artery distal to the neo-apex (at the right), where blood entering the new branch would impinge on the wall. Note the multiple interruptions of the internal elastic lamina (arrowed) with no obvious compensatory intimal thickening (15 months post-operatively, Verhoeff's elastic stain and eosin × 70).

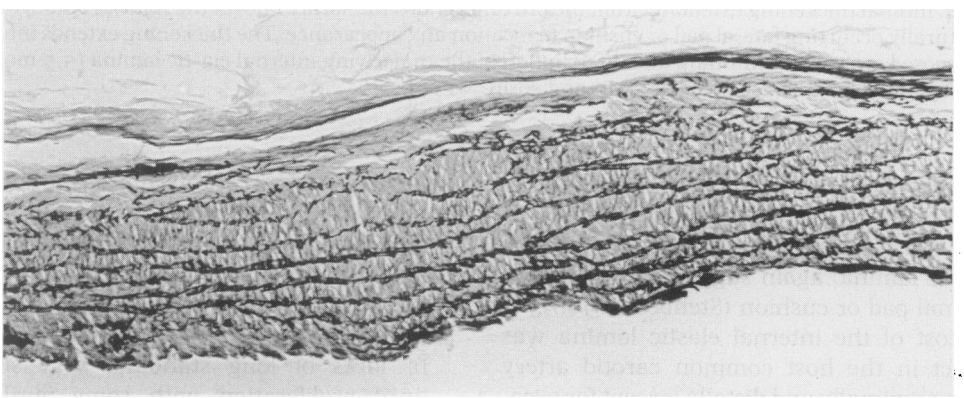

Fig. 6. Multiple tears in the internal elastic lamina with some intimal proliferation. Fine fibrillary elastica were present in these areas but are not recognizable as such in the photograph (15 months post-operatively, Verhoeff's elastic stain and eosin × 130).

Scanning electron microscopy (Group B)

In two animals (2 and 4 days post-operatively) thrombotic occlusion was observed at the anastomotic site. In a third animal (8 days post-operatively) thrombus, not thought to be occlusive, was present at the anastomotic site. In all remaining animals the anastomosis was patent with no obvious thrombus.

At distances far away from the forks both distally and proximally the normal luminal appearance was one of longitudinally orientated endothelial cells upon parallel corrugations of the underlying internal elastic lamina. The endothelial cells were elongated with tapering ends and indistinct cell margins. The site of maximum surgical trauma was about the anastomotic site, where the normal longitudinal pattern of the endothelial lining of the vessel was initially replaced by a thin layer of thrombus. Some minor endothelial injury extended for a short distance beyond this thrombus at the anastomotic site and elsewhere, especially at clamp sites where damage was generally restricted to the endothelial layer. Repair was rapid and endothelialization was complete within 2 weeks. Repair proceeded more slowly at the distal aspect of the anastomotic line, possibly due to suture line irregularity.

Apart from surgical trauma there were no

tears in the internal elastic lamina prior to 8 days post-operatively. At 8 days and in every animal thereafter tears were observed in the internal elastic lamina. Most were transversely orientated with relatively abrupt margins. In five animals, two of which were 6 and 7 days post-operative, there were two to five small interruptions of the internal elastic lamina in the host artery either proximal or distal to the anastomosis. These were situated at irregular locations and were considered to be of traumatic origin. In 11 rabbits tears were located on the distal surface of the proximal end of the transposed artery where the blood would be expected to impinge at the fork as observed by light microscopy (Fig. 1). In one rabbit (8 days post-operatively) the transversely orientated tears were observed just distal to the neo-apex but in the corresponding area of the host artery. The elastic tears were longer, more numerous and, with time, involved a larger area of the wall. Figure 7 is a photograph of a tear at 8 days with a thin layer of thrombus in the floor of the tear not covered by endothelium. Most tears however, even 8 days post-operatively, were covered by endothelium. At 2 weeks the floor of the tears was covered by small, densely-packed polyhedral endothelial cells (Fig. 8) consistent with recent endothelialization. With time these displayed prominent nuclei and progressively assumed a more elongated form to become orientated with the longitudinal axis in the direction of flow (Fig. 9). Endothelium over the residual remnants retained the large, elongated squamous morphology characteristic of rabbit arterial endothelium except close to the suture, where surgical trauma may have removed the endothelium.

With increasing post-operative time the tears became more numerous and the area of involvement more extensive. The margins of the tears in the internal elastic lamina became less distinct, seemingly due to slight intimal thickening as seen by light microscopy. They could still be recognized by scanning electron microscopy which also revealed the disruption of the regular corrugations of the luminal surface of the arteries. Some tears were joined by interconnecting longitudinal breaks in the internal elastic

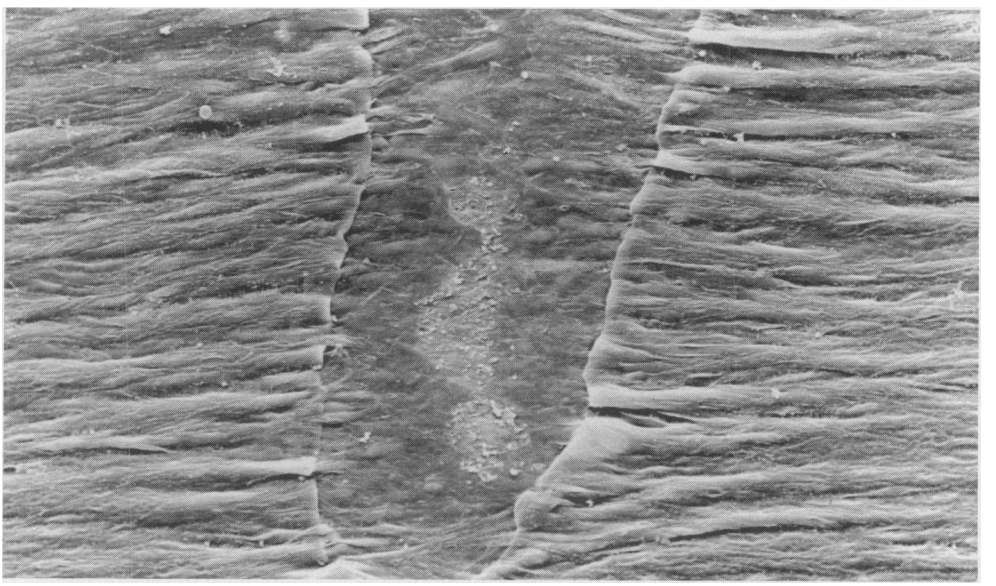

Fig. 7. Recent tear of the internal elastic lamina from a rabbit 8 days post-operatively with thrombus in the central denuded section of the floor of the tear. Note the sharp margins of the tear. Blood flow is from left to right (× 340).

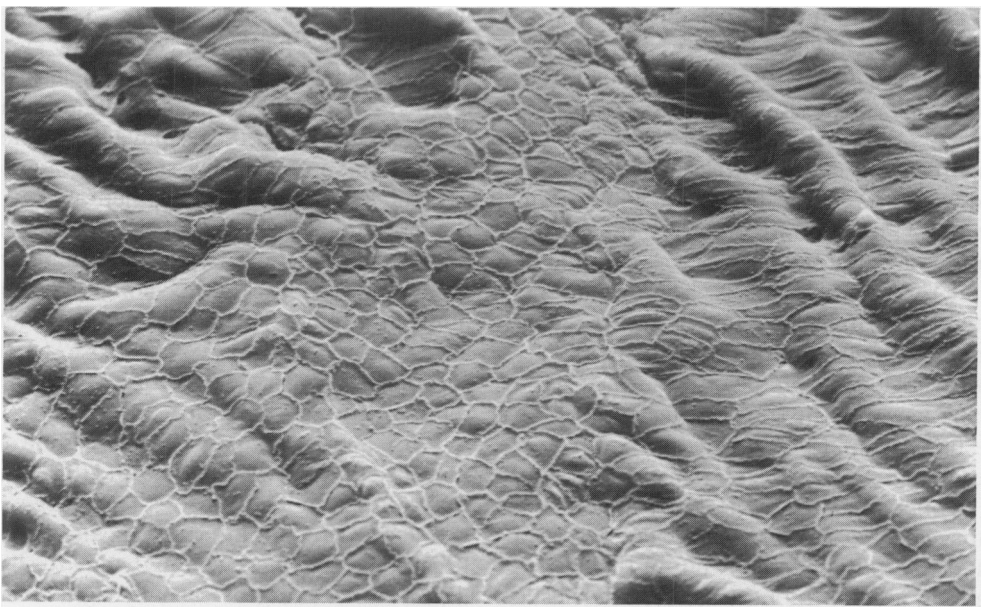

Fig. 8. Elastic tear which has been re-endothelialized in the transposed artery 12 days post-operatively. Note the numerous small polygonal endothelial cells with prominent boundaries and few microvilli on the surface. Endothelial cells over the elastic lamina are also small suggesting post-operative repair (×465).

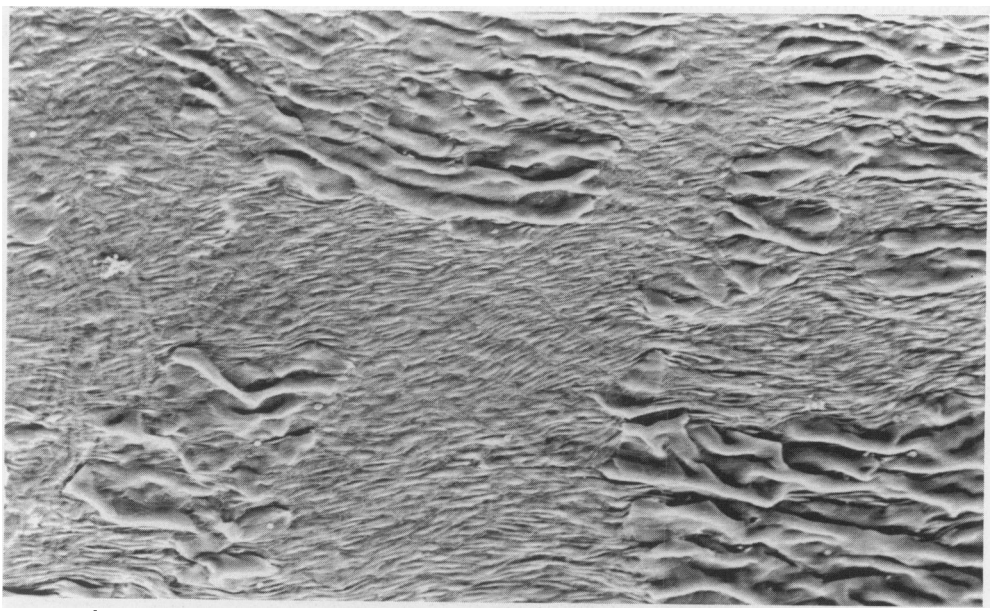

Fig. 9. Multiple tears of the internal elastic lamina. Longitudinal tears interconnect transversely orientated tears with loss of normal parallel corrugations of isolated remnants of the elastic lamina (131 days post-operatively). Note the numerous small, elongated endothelial cells with prominent nuclei aligned in the direction of blood flow (left to right). These endothelial cells are in contrast to those over the elastic lamina (×185).

lamina and resulted in sharply defined islands of elastic lamina with an irregular surface contour instead of parallel corrugations (Fig. 9).

Discussion

The proximity of the paired common carotid arteries makes them suitable for experimental production of an arterial fork. Limitations of these experimental forks are appreciated but no other technique is currently available and the rapid healing suggests that the technique is not excessively traumatic. The haemodynamic forces to which the new fork is subjected will be similar to those at naturally occurring forks but modified by the high area ratio. The long-term changes observed in these forks may also have applicability to arterial anastomoses in therapeutic surgery.

The microsurgical procedure involved in the production of an experimental fork is of longer duration, is technically more difficult, and is associated with more trauma than is the production of experimental arteriovenous fistulae or venous pouch aneurysms. In addition to technical difficulties the method requires much more exposure of the carotid arteries. Nevertheless, trauma was still mild as attested by scanning electron microscopy of the Group B animals.

Small micro-aneurysms observed in forks of Group A rabbits may have been due to stretching of the scar tissue which is known to occur at the junction of arteries with prosthetic implants. The frequency of the small evaginations distally however suggests that haemodynamic stresses participated since they occurred in animals only 11 months or more post-operatively. That one evagination occurred on the host side of the fork at 22 months post-operatively (Fig. 3) and not in anastomotic scar tissue, supports this concept.

The lack of consistency in location of occasional sporadic discontinuities of the internal elastic lamina and their frequent proximity to the suture site suggest that these were probably due to trauma at the time of surgery despite an occasional tear occurring spontaneously in rats (Osborne-Pellegrin *et al.* 1980). The elastic tears on the greater curvature of bends created in the transposed carotid artery of the two rabbits are similar in location to those previously reported in experimental **U**-bends fashioned in the common carotid arteries of rabbits (Greenhill & Stehbens 1985). It is likely that other animals also had such lesions but the bends were not included in the limbs of the experimental forks serially sectioned.

The consistent localization of the tears in the internal elastic lamina of the long-term experimental forks histologically and in the short-term experimental forks by scanning electron microscopy (only at, or after 8 days post-operatively) suggests they are not artefacts. The occurrence of tears in the host artery in a few specimens seems to depend on the degree of its angulation and the flow patterns at the fork. The similarity of the tears to those observed consistently at specific anatomical sites in the afferent arteries of carotid-jugular and femoral arteriovenous fistulae and in experimental **U**-bends (Greenhill & Stehbens 1983, 1985, 1987) supports the contention that blood flow is the primary factor in their occurrence. Their appearance (from 8 days post-operatively) is later than those previously produced in the carotid arteries (Greenhill & Stehbens 1983, 1985) and their onset is seemingly dependent on the haemodynamic stress involved in each model.

These elastic tears, the minimal intimal proliferation, the slight medial thinning associated with some tears and their location are all consistent with their being early atrophic lesions. They have been shown in previous studies to be early changes leading to berry aneurysm formation (Stehbens 1963, 1972, 1986; Greenhill & Stehbens 1985). They are also similar to the branching pattern of confluent tears observed in human arteries from infancy, seemingly at specific arterial sites (Meyer & Lind 1972; Meyer *et al.* 1980). These experimental forks

have both branches approximately equal in cross-sectional area to that of the stem. This gives a branching coefficient or area ratio of 2 which is considerably larger than in normally occurring forks (Stehbens 1974, 1975). Such a configuration might enhance the jet-like impact of the flux of blood at the site where the tears occurred consistently in these experiments and so this large area ratio may well be very important.

The infrequency of endothelial disruption with thrombus in these tears is similar to the findings of previous experimental models (Greenhill & Stehbens 1983, 1985, 1987). It seems likely that tears of the elastic lamina can occur without endothelial disruption, although it is also a distinct possibility the endothelium may regenerate or recover the denuded surface with remarkable speed. The increased endothelial cell density in the floor of the elastic tear was also observed in earlier vascular models of atrophic lesions. The early transition and reorientation of these small endothelial cells are seen in Fig. 9. It has been suggested that smooth muscle cells may simulate endothelial cells in regions of endothelial denudation (Reidy 1985) but in these experiments and previous studies well defined junctions occur with endothelium (Fig. 8) but not with muscle.

The original purpose of producing experimental forks was to determine the location of intimal proliferation that formed consequent upon the haemodynamic stresses so engendered. The intimal proliferation at the apex is less than that at the proximal end of the anastomosis and even then it is greater on the side of the transposed carotid, suggestive of lateral pad formation (Fig. 4). The sutures and associated scarring in the region of the face and dorsum of the fork interfere with visualization of any additional intimal thickening at those sites. On the side of the host artery opposite the anastomosis, the slight thickening was also suggestive of a lateral pad or cushion especially since that wall was angulated to a varying degree (angle of deflection). However, the presence of disruption of the underlying internal elastic lamina in both lateral pads is of interest since similar deficiencies occur in the pads (cushions) of human cerebral arteries at analogous sites (Stehbens 1960, 1963). There remains the possibility that these discontinuities may have been of traumatic origin but when located elsewhere they were not associated with the same degree of intimal proliferation. If not due to trauma then the discontinuities must also have been occasioned by haemodynamic stress though they are much later in appearance than those directly in the path of the flux of blood at the fork.

These experiments lend support to the concept that not only are intimal thickenings about the arterial forks of human and lower animals acquired but so also are the atrophic lesions of man and they are haemodynamically induced rather than being inherent or developmental features of the arterial wall.

Acknowledgements

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