Inhibition of Endothelial Cell Regrowth

Cessation of Aortic Endothelial Cell Replication after Balloon Catheter Denudation

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Aortas of male rabbits (2.0 to 2.5 kg body weight) were denuded of endothelium with a 4F balloon catheter and killed 7, 14, 60, 90, and 180 days later. Aortic endothelial cell regrowth was determined using Evans blue dye and scanning electron microscopy. Early endothelial outgrowth was rapid, but by 14 days the regrowth slowed markedly so that no further increase of endothelial outgrowth was detected at other time periods. Another group of animals received $^3$H-thymidine 1 hour before death and were killed 4, 14, and 42 days later. The endothelial cell replication in the aortas of these animals was measured from H"{a}utchen preparations. Endothelial cell replication was observed 4 days after balloon injury but was markedly reduced by 14 and 42 days. Intimal smooth muscle cell replication, however, was observed at both these later times. These results demonstrate that in vivo endothelial cell replication stops long before the aorta is repopulated and suggest that some mechanism other than contact between endothelial cells can prevent endothelial cell replication.


The purpose of this study, therefore, was to determine how rapidly endothelial regrowth occurs in the rabbit and whether the cells adjoining the denuded zones are actually dividing.

Methods

Endothelial denudation of rabbit aorta was accomplished with the balloon catheter method using a 4F air-filled balloon as described by Baumgartner. New Zealand white rabbits weighing between 2.7 and 3.0 kg at the time of injury were anesthetized with an intramuscular dose of Innovar-Vet (0.35 mg/kg body weight), followed by intravenous sodium pentobarbital (0.1–0.2 g/kg). The right femoral artery was exposed, and a Fogarty embolectomy catheter (Edwards) was introduced. The tip of the catheter was inserted to the level of the aortic arch. The balloon was then inflated with room air at the lowest pressure that would totally inflate the balloon outside the animal, approximately 15 to 18 psi. The endothelium was abraded by pulling the balloon tip to the bifurcation where the balloon was deflated. This process was carried out three times for each rabbit before removal of the catheter. The femoral artery was ligated, the wound was closed with wound clips, and the animals were allowed to recover.

Animals were fixed by pressure perfusion at different time periods after injury: 1 hour, 7, 14, 60, 90, and 180 days. Three animals were in each group. To visualize the extent of reendothelialization, Evans
blue dye (5 ml of 1% solution in normal saline) was administered via the lateral ear vein 1 hour before fixation. Anesthesia was given as mentioned earlier, and animals were perfusion-fixed via left carotid artery at 100 mm Hg with fixative containing 2% glutaraldehyde and 1% paraformaldehyde in 0.1 M phosphate buffer at pH 7.3. Perfusion pressure was maintained for 45 minutes. Intraluminal pressure was determined as described by Reidy and Bowyer. Aortas were removed and stored in fixative until examination.

For the purpose of determining endothelial replication rate, another group of nine animals was injured as described above. Groups of three animals were killed by perfusion fixation at 4, 14, and 42 days after ballooning with a 4F catheter. At 1 hour before fixation, animals received tritiated thymidine, 6.7 Ci/m mole, 500 μCi/kg body weight (New England Nuclear, Boston, Massachusetts), via lateral ear vein. Animals were then anesthetized and fixed by perfusion at 100 mm Hg intraarterial pressure for 45 minutes with 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.3. After immersion fixation of at least 24 hours, aortas were prepared for autoradiography. Segments of thoracic aorta were processed for autoradiography using a modified Hautchen technique, which renders the endothelium as a monolayer for coating with nuclear track emulsion (NTB-2, Kodak). Other segments were examined by whole-mount autoradiography, as described below.

Segments of thoracic aorta from tritium-labeled animals to be used for whole-mount autoradiography were cleaned of adventitia and pinned out flat on Teflon strips as mentioned above. After dehydration through graded alcohols and critical point-drying from liquid CO₂, the tissue was dipped in nuclear track emulsion (NTB-2, Kodak). The coated tissue was allowed to dry and was stored in a light-tight box at 4°C for 2 weeks. Photographic development, identical to that for the Hautchen preparations, included 5 minutes in D-19 developer (Kodak), 30 seconds in a dilute acetic acid stop bath, and 5 minutes in Rapidfix (Kodak). After rinsing in tap water for 1 hour, tissue was air dried, mounted on scanning stubs, and sputter-coated for 4 minutes with gold-palladium before viewing at 15 kV in a JEOL 35C microscope.

Results

Morphology

Use of the 4F balloon technique produced complete removal of the endothelium from the aortic surface. This was demonstrated by Evans blue staining and by scanning and transmission electron microscopy, which show the freshly denuded surface to be covered by a monolayer of platelets (figures 1 and 2). At all time periods after removal of endothelium, endothelial regrowth as visualized by Evans blue was observed around the aortic ostia. The length of the white islands of regrowth surrounding six pairs of intercostals was measured in the direction of the long axis of the aorta. These values are shown in

Figure 1. En face view of thoracic aorta 1 hour after balloon denudation. No endothelial cells are present and the luminal surface is covered with platelets. × 3600.
Figure 2. Transmission electron micrograph of rabbit thoracic aorta 1 hour after denudation. A basement membrane is present (BM) on which rests a monolayer of platelets (P). × 15,000.

Figure 3; it can be seen that after an initial rapid outgrowth, endothelial repopulation of the aorta did not increase after 14 days. It is apparent that endothelial regrowth had essentially ceased by this time.

Endothelial Cell Replication

Four days after injury, large numbers of replicating cells were evident (figure 4A). Whole-mount autoradiograms viewed in the scanning electron microscope facilitated identification of replicating endothelial cells (figure 5). Labeled endothelial cells were found both at the edge of outgrowth and in already confluent regions. At 14 days after injury, the replication rate was reduced and labeled endothelial cells were largely confined to an oval-shaped band at the edge of outgrowth (figure 4B). Smooth muscle cell neointima was present in areas that had not reendothelialized. At 42 days, almost no labeled endothelial cells were observed either at the leading edge of the endothelium or elsewhere in the monolayer (figure 4C). Labeled smooth muscle cells, however, were observed at this time.

Discussion

One conclusion from this study is that there appears to be a marked species difference in the speed of regeneration between the rabbit and other animals. Data from our laboratory and from others show that in the rat there is essentially total endothelial regeneration within 28 to 42 days after ballooning. This has also been reported in both swine and primates. In the rabbit, however, there is relatively little endothelial regeneration, even after 12 weeks. Why regeneration stops is unclear. One possibility is that rabbit endothelial cells have a more limited replicative life span than cells of other species. In this model, regeneration ceased once the cells had used up their ability to replicate. There is evidence that human and bovine endothelial cells have a limited life span. At present there are no
Figure 4. Häutchen preparations of rabbit thoracic aorta showing labeled endothelial and smooth muscle cells. In each photograph, the area shown is immediately distal to an intercostal branch. Blood flow is from top to bottom. $\times$ 100. A. At 4 days after denudation, almost every endothelial cell is labeled. A region still denuded is visible in the bottom right corner. B. At 2 weeks after denudation, the endothelial cells are aligned with flow, whereas the luminal smooth muscle cells are arranged at right angles to flow. Only a few endothelial cells (E) are labeled, as are several smooth muscle cells (S). C. At 6 weeks after denudation, there is almost no endothelial regeneration and only one labeled endothelial cell is visible in this field (E). Several labeled smooth muscle cells are clearly visible (S).

Data for rabbit cells, although establishment of continuous cell lines has been reported. This may not be relevant to life span of nonestablished cells. We might speculate, therefore, that in vivo a sustained high endothelial cell turnover would eventually result in a break in the integrity of the endothelium. If so, this might explain the susceptibility of rabbits to atherosclerosis.

Figure 5. Autoradiographic preparation viewed by scanning electron microscopy of rabbit thoracic aorta 4 days after denudation. The flow divider of an intercostal branch is visible at the top of the field and many labeled endothelial cells are visible (arrows). $\times$ 110.
A possible explanation for the cessation of endothelial cell growth is that the pseudointimal cells, the luminal smooth muscle cells, cause inhibition of the endothelial growth. At about 14 days there is extensive intimal proliferation of medial smooth muscle cells into the intima, and it is around this time that endothelial cells stop growing. These smooth muscle cells might directly inhibit endothelial movement and growth by a mechanism akin to contact inhibition of movement and proliferation. Alternatively, the extracellular matrix made by these cells might inhibit endothelial cell movement and growth. Using a 4F balloon catheter in the same rabbit model, Wight et al. have shown marked changes in the glycosaminoglycan content of the subendothelium at the interface between regenerating endothelial cells and the remainder of the vessel. Similar changes have been described in the intima of advancing athero-sclerotic plaques. It is interesting to speculate that changes of this sort may interfere with the ability of endothelial cells to adhere and regenerate, ultimately leading to loss of integrity, exposure of the thrombogenic subendothelium, and the thrombosis characteristic of advanced atherosclerotic lesions.

A third possibility is that ballooning removes some component of the subendothelium required for continued growth. At present we have little knowledge beyond the morphology of the material removed by the catheter. Recent studies by Madri et al. suggest that there may be differences in the collagen composition of the intima at various levels in the vessel walls.

Another conclusion from this study relates to the relationship of our observations and those of Minick et al. using an in vivo system to studies using cultured cells. Studies by Vlodavsky et al. and Davies et al. in our laboratory have shown increased vesicular transport in replicating endothelial cells. Vlodavsky et al. have also shown increased endocytosis of low density lipoproteins in endothelial cells growing in culture from sparse density. These authors suggested that this increased rate of endocytosis correlates with an increased rate of transcellular transport and, therefore, with the increased accumulation of lipid seen beneath the “advancing edge” of reendothelialized regions in vivo. Aside from the difficulty of equating endocytosis with transcellular passage, the present data demonstrated that the endothelial cells in vivo do not show increased replication at the time of lesion formation.

References


Index Terms: endothelium • aortic denudation • balloon catheter • cell replication
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