Endothelial Regeneration in Hypertensive and Genetically Hypercholesterolemic Rats

Margaret Forney Prescott and Klaus R. Müller

Endothelial regeneration after a narrow, superficial aortic injury was studied in rats with chronic Goldblatt hypertension, genetic hypercholesterolemia, or a combination of hypertension and genetic hypercholesterolemia. In all groups, endothelial continuity was restored within 24 to 36 hours by a combination of endothelial migration and proliferation. A line of increased endothelial density covering the previous wound was seen through 16 weeks after injury. Intimal thickening after injury did not occur in any of the groups. These results indicate that hypertension and hypercholesterolemia neither delay endothelial regeneration nor cause intimal thickening after a small injury in the rat. (Arteriosclerosis 3:206–214, May/June 1983)

Hypertension and hypercholesterolemia are known to be risk factors for the development of atherosclerosis. Yet, little is known about the mechanisms by which these risk factors affect the vascular wall on the cellular level. In animal models morphologic changes have reportedly occurred in both hypertension and hypercholesterolemia. In addition to the possibility that hypertension and hypercholesterolemia could cause direct injury to the vascular wall, some evidence also indicates that these conditions may interfere with the repair of vascular injury. For example, it has been reported that in vitro endothelial cell migration is inhibited by hypercholesterolemic sera. After a one-time removal of large areas of endothelium by balloon catheterization or air drying, it was shown that hypertension, but not hypercholesterolemia, increased the extent of intimal thickening. After multiple balloon injuries, however, spontaneously hypertensive rats fed a high fat diet had enhanced intimal thickening compared to similar animals fed chow. Since such extensive injuries are unlikely to occur in vivo, a new experimental model was introduced to produce a small, superficial intimal defect only a few endothelial cells wide. This small injury produced no intimal thickening in normal animals. The present work investigates whether chronic hypertension, genetic hypercholesterolemia, or both combined affect the response to such a narrow, superficial intimal injury by altering endothelial regeneration with subsequent development of local intimal thickening.

We used the RICO strain of genetically hypercholesterolemic rats, thus avoiding the artificial nature of hypercholesterolemia produced by the administration of a cholesterol-enriched diet. As previously reported, in the RICO rat the hypercholesterolemia is established by the day after weaning and increases progressively thereafter. Increased serum cholesterol is due to elevated concentrations of both LDL and HDL cholesterol.

Methods

Experimental Protocol

Male Rattus norvegicus control rats (Tif:RAIf, a Sprague-Dawley derived strain) and genetically hypercholesterolemic RICO rats (selectively bred for hypercholesterolemia from the control strain) were obtained from the Tierfarm AG, Sisseln, Switzerland. Four groups of 37 to 53 animals, wounded at 22 weeks of age, included:

Group 1. Untreated control Tif:RAIf rats
Group 2. Tif:RAIf rats with chronic Goldblatt hypertension
Group 3. RICO rats
Group 4. RICO rats with chronic Goldblatt hypertension.
Rats were housed five per cage and fed ordinary rat chow (NAFAG AG, chow number 890) and water ad libitum. Hypertension was produced by the single clip Goldblatt technique performed at 4 weeks of age.\textsuperscript{15} Systolic blood pressure was measured at 2-week intervals using the tail cuff method\textsuperscript{16} (W & W Electronic, Basel, Switzerland). Rats were considered chronically hypertensive if their blood pressure remained above 155 mm Hg for at least 14 weeks. After overnight fasting, orbital blood samples were collected before sacrifice. Sera were harvested immediately and stored at \(-20°C\). Concentrations of total serum cholesterol were determined enzymatically.\textsuperscript{17}

Endothelial wounding was performed at 22 weeks of age using a 0.15 mm stainless steel wire inserted into a sheath made from the tubing of a 2-F Fogarty catheter. The cut end of the wire was smoothed by filing. Rats were anesthetized with ether, and the catheter was inserted into the right femoral artery. The wire remained within the catheter as it was advanced up the aorta to the level of the diaphragm; it was then gently pushed 2.8 cm out of the catheter, producing a longitudinal injury. The wire was rotated 180°, and both the wire and the catheter were gently pulled back down the aorta, creating a second line of longitudinal injury. The wire and catheter were removed, and the femoral artery was sutured. Aortas were excluded if the distance between the two lines was less than 5 mm.

Four to seven animals from each group were sacrificed at intervals of 5 minutes, 18 hours, 24 hours, 36 hours, 48 hours, 96 hours, 15 days, 5 weeks, and 16 weeks after wounding. One hour before sacrifice each animal was given \(^{3}H\)-thymidine (intravenously, 36 hours, 48 hours, 96 hours, 15 days, 5 weeks, and 16 weeks after wounding). Animals were anesthetized with ether and perfused via the left ventricle at a pressure of 30 mm Hg below the systolic pressure measured for each individual animal. Solutions were infused in the following sequence: 0.1 M phosphate-buffered 1% glutaraldehyde-4% formaldehyde fixative (2 minutes), 0.25% aqueous AgNO\textsubscript{3} (1 minute), and fixative repeated (20 minutes). Aortas were carefully excised, cleaned of periadventitial fat, opened longitudinally, pinned on Teflon sheets, and stored in fixative.

After immersion fixation for at least 24 hours, aortas were processed for transmission electron microscopy (TEM), scanning electron microscopy (SEM), or whole mount autoradiography. For both SEM and autoradiography, pieces of midthoracic aortas were dehydrated through increasing concentrations of ethanol and critical-point-dried with CO\textsubscript{2}. Samples were then mounted, sputtered with gold and analyzed at 15 KV with a Cambridge S4–10 microscope. Endothelial cell density was determined by counting the number of cells per 100 \(\mu\) \(\times\) 100 \(\mu\) field on SEMs at x600 magnification. Samples for autoradiography were processed by the method of Reidy et al.\textsuperscript{18} Briefly, after critical-point drying, the tissue was dipped in nuclear track emulsion (NTB-2, Kodak), stored for 2 weeks at 4°C and developed. End face aortas were then analyzed by light microscopy.

Pieces of midthoracic aortas were processed for TEM by postfixation with 1% osmium tetroxide, en bloc stained with 0.1% aqueous uranyl acetate, dehydrated through graded alcohol and propylene oxide, and embedded in EM-Bed (EMS, Fort Washington, Pennsylvania). Selected areas of endothelial wounding previously located by SEM were gradually rehydrated and processed for TEM as previously described. Ultrathin sections were cut using a Reichert OMU2 microtome, stained with uranyl acetate and lead citrate, and examined with a Philips EM300 electron microscope at 80 KV.

**Results**

Weight, blood pressure, and total serum cholesterol levels are summarized in Table 1. The total serum cholesterol of the RICO rats was approximately twice that of the controls, irrespective of blood pressure. Goldblatt surgery produced a 50 to 77 mm Hg elevation of systolic blood pressure. As previously shown,\textsuperscript{14} RICO rats weigh less than their age-matched controls. All rats gained weight over the course of the study, but following Goldblatt surgery both the control and RICO groups gained weight more slowly than the nonoperated animals.

<table>
<thead>
<tr>
<th>Table 1. Blood Pressure, Plasma Cholesterol, and Weight at Time of Wounding (22 Weeks of Age)</th>
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<tr>
<td>Control (n = 53)</td>
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<tr>
<td>Blood pressure (mm Hg)</td>
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<tr>
<td>Total plasma cholesterol (mg/dl)</td>
</tr>
<tr>
<td>Body weight (g)</td>
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All values are means ± se. All parameters in all three test groups are significantly different (\(p < 0.01\)) compared with control rats.
The injury procedure produced two longitudinal lines of endothelial denudation. The line produced by pulling down the wire was found to be consistently six to eight endothelial cells wide; thus it was chosen as the wound to be analyzed. An incomplete monolayer of platelets could be seen adhering to the wounded area after 5 minutes (Figures 1 and 2). The internal elastic lamina remained intact, and there was no morphologically detectable damage to the underlying medial smooth muscle cells (Figure 2).

In all groups, endothelial repair followed the same sequence of events with no significant differences in timing. By 18 hours, individual endothelial cells at the borders of the wound were seen migrating into the wounded area. The surface area of these cells was increased and they remained unlabeled by autoradiography (Figure 3). By 24 hours, endothelial cells came into contact with each other (Figure 4). In areas in which endothelial continuity had been completely restored, the endothelial cells were not yet oriented in the direction of blood flow (Figure 5 A). At this time, thymidine labeling had begun. Labeling occurred primarily at the sites of the original wound edges and was less frequent at the line of endothelial closure (Figure 5 B). By 36 hours, endothelial continuity was completely restored in all animals. The endothelial cells were aligned in the longitudinal di-
Figure 3. Aortic intima of a RICO Goldblatt rat 18 hours after injury. A. Scanning electron micrograph demonstrating endothelial cells at the edge of the wound migrating into the line of injury. The surface area of these cells is increased compared to endothelial cells remote from the wound. B. En face autoradiograph demonstrating that these migrating endothelial cells (arrows) are not thymidine-labeled. The wound area (at the bottom) shows faint transverse streaks.
Figure 4. Scanning electron micrograph of a control Goldblatt rat 24 hours after injury. In some places, opposing endothelial cells have made contact, while in other places, small gaps remain between spread endothelial cells.

Figure 5. Aortic intima of a control Goldblatt rat 24 hours after injury. A. Scanning electron micrograph showing that endothelial cell continuity has been restored. B. En face autoradiograph showing that most labeled cells occur at the edge of the original injury. Many endothelial cells covering the central portion of the wound are not labeled. Estimated borders of the original wound are indicated by arrows.

retraction and most were incorporating thymidine, including cells one to two rows beyond the original width of the wound (Figure 6). At this time, there was only slight endothelial bulging, whereas at 48 hours, bulging was pronounced in all cells covering the wound and individual cells were round (Figure 7 A). This shape presumably indicated that the cells were undergoing mitosis. Occasional cells were seen in the process of division (Figure 7 B). By 96 hours, there was a line of narrow cells (Figure 8) with a cell density approximately 2.1 times that of the nonwounded endothelium (Table 2).

A line of increased density remained through 16 weeks after injury (Figure 9), but by this time the cell density had decreased to only approximately 1.3 times that of the nonwounded areas (Table 2). The width of the line of altered endothelium remained the same as at previous time points. Thymidine-labeled endothelial cells were not seen along the previous wound line in the 15-day, 5-week, or 16-week postinjury groups.

In all groups, occasional multinucleated giant endothelial cells were observed along the line of increased density. Such cells were seen through 16 weeks after wounding (see Figure 9). The incidence of giant cells did not differ between groups.

In all groups studied, reendothelialization occurred without any intimal thickening (Figure 10).
**Figure 6.** Aortic intima of a RICO rat 36 hours after injury. **A.** Scanning electron micrograph demonstrating that endothelial cells covering the previous wound are narrow and arranged in the longitudinal direction, resembling a zipper. **B.** En face autoradiograph showing thymidine labeling of most endothelial cells along the wound line. Estimated borders of the original wound are indicated by arrows.

**Figure 7.** Aortic intima of a RICO rat 48 hours after injury. **A.** Scanning electron micrograph demonstrating boat-shaped endothelial cells closely packed together. Individual endothelial cells are round (arrow). **B.** Scanning electron micrograph of an endothelial cell undergoing division.
Figure 8. Scanning electron micrograph of a control rat 96 hours after injury. Endothelial cells covering the previous wound are narrower and more densely packed than the surrounding endothelium.

Figure 9. Scanning electron micrograph of a RICO rat 16 weeks after injury. Endothelial cells covering the previous wound are narrower and more densely packed than the surrounding endothelium, though less dense than at earlier time points. An endothelial giant cell can be seen along the previous wound line.

Figure 10. Transmission electron micrograph rehydrated from a SEM-visualized wound line in a control Goldblatt rat 5 weeks after injury. The endothelial cells remain densely packed with no intimal thickening over the previous wound line. There are no morphological alterations in the medial smooth muscle cells.
Table 2. Endothelial Cell Density per 10,000 μ²

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<th>Time after injury</th>
<th>EC density along wound</th>
<th>EC density in normal area</th>
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<tr>
<td>96 hours (n = 17)</td>
<td>70.3 ± 1.9</td>
<td>31.9 ± 0.8</td>
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<tr>
<td>5 weeks (n = 18)</td>
<td>50.9 ± 1.5*</td>
<td>30.1 ± 0.7</td>
</tr>
<tr>
<td>16 weeks (n = 20)</td>
<td>44.0 ± 1.1†</td>
<td>31.4 ± 0.7</td>
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* p < 0.001 vs 96 hours, Student’s t test.
† p < 0.01 vs 5 weeks, Student’s t test.
All values are means ± se. No significant differences were found between groups; thus, groups have been pooled.

There was no increase in the amount of extracellular material nor in the frequency of subendothelial cells when the intima along the previous wound line was compared with the intima of nonwounded areas. In addition, medial smooth muscle cells below the regenerated area did not show any morphological alterations.

Discussion

In this study the same rate and pattern of endothelial regeneration after a narrow longitudinal injury were found in all experimental groups. In contrast to an earlier report on a similar longitudinal injury, the initial event in our model was lateral endothelial cell migration, while endothelial cell replication along the entire wound line was only seen following wound closure. Ramsay et al. also found lateral endothelial spreading after a narrow longitudinal injury. Mallcak and Buck reported that endothelial migration preceded endothelial replication following freeze injury to the aorta. This is consistent with vitro studies showing that if an injury is small enough, endothelialization can occur through migration alone.

We found a zone of endothelial replication only one or two cells wider than the original wound line. In contrast, after balloon injury an increase in endothelial replication has been reported as far downstream as 100 cells from the wound edge. This opens the possibility that the signal for replication is transmitted differently in the longitudinal and circumferential directions and is dependent on the size of the injury.

Only after the endothelial continuum had been reestablished by a combination of endothelial migration and proliferation was there realignment of the endothelial cells in the longitudinal direction. Cell orientation has been shown to be due to both extracellular matrix orientation and the direction of blood flow. Increased endothelial cell density along the reendothelialized wound line is consistent with the observations of Reidy and Schwartz and Hirsch et al. Possibly this is species-specific, as a similar superficial wound in rabbits resulted in no increased endothelial density after 7 days. The cause of the decrease in cell density along the wound line which we observed between 96 hours and 16 weeks remains unclear. The fact that the endothelial cell density decreased while the width of the previous wound line remained constant, suggests that endothelial cells have been lost from the wound line with subsequent spreading of the remaining cells. It had previously been reported that a similar increase in the number of fibroblasts followed by a gradual decrease in cell density occurred in the healing of skin wounds.

Endothelial giant cells have been seen in normal arteries as well as in atheromatous lesions. Giant cells have also been reported in states with increased endothelial turnover, such as following cholesterol feeding or intravenous Escherichia Coli administration. The association of rapid endothelial proliferation and the occurrence of endothelial giant cells has also been reported following hemodynamic injury or endothelial removal. Poole et al. described the presence of giant endothelial cells 33 weeks after aortic abrasion. We documented the occurrence of such cells through 16 weeks after injury. We think that the morphologic change in these cells may be correlated with functional differences.

Our findings in control animals confirm the results of Reidy and Schwartz and Ramsey et al. who also found no intimal thickening following a narrow superficial injury to the aorta. However, we showed that hypertension, hypercholesterolemia, or both did not influence this healing process even though the blood pressure had been elevated for over 14 weeks and the cholesterol levels were approximately twice as high as in controls. The lipoprotein profile of the RICO rat, however, is a typical rat profile with an extremely low ratio of LDL to HDL and no cholesterol-rich B-VLDL as are present after cholesterol feeding of other species. Furthermore, the plasma cholesterol level of animals fed an atherosclerotic diet is usually at least four times higher than control levels, while the plasma cholesterol level of our genetically hypercholesterolemic rat is only twice as high.

Intimal thickening and smooth muscle cell accumulation appear to depend on the length of time the vessel remains denuded. As well as on the depth of injury to the vessel wall. Walker et al. reported intimal smooth muscle cell accumulation after a deep narrow injury, whereas an identical superficial narrow injury produced no intimal thickening.

In summary, our findings indicate that chronic hypertension and genetic hypercholesterolemia as well as a combination of the two neither delay endothelial regeneration nor cause intimal thickening after a narrow line of endothelial removal in the rat. In all groups, endothelial continuity was restored within 24 to 36 hours by a combination of endothelial migration and proliferation. Although these risk factors at the levels studied in our experiments apparently do not interfere with the endothelial response following a small denuding injury, they might interfere with other important functions of vascular wall cells. This is in agreement with recent suggestions that endothelial denudation is not a prerequisite for the development of atherosclerosis.
Acknowledgments

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