Effect of Heparin on Adaptation of Vein Grafts to Arterial Circulation

Ted R. Kohler, Thomas Kirkman, and Alexander W. Clowes

We studied the effect of heparin on wall thickening in balloon-injured carotid arteries and vein grafts in rabbits. Heparin (0.3 mg/kg of body weight/hour) reduced intimal cross-sectional area in balloon-injured carotid arteries at 2 weeks (0.20±0.05 mm² vs. 0.05±0.02 mm², p=0.02). Autoradiography after a single pulse of tritiated thymidine revealed no labeling in the few intimal cells present in heparin-treated animals, whereas control smooth muscle cells (SMC) had a 10% labeling index. Heparin did not affect medial proliferation, suggesting that the decrease in intimal thickening was largely due to inhibition of SMC migration into the intima. Heparin caused a slight reduction in intimal cross-sectional area at 2 weeks in vein grafts (0.17±0.03 mm² vs. 0.09±0.02 mm², p=0.03) but no significant reduction in wall thickness at any other time and no reduction in SMC proliferation rate (thymidine labeling index). DNA content per surface area or dry weight was the same in control and heparin-treated vein grafts at 4 weeks, implying that SMC content and the amount of matrix made by individual SMC was not affected. These data suggest that either SMC in veins are less susceptible to heparin than SMC in arteries, or the mechanism of thickening is substantially different. Heparin may not block all forms of SMC proliferation and may only be a weak inhibitor in processes that primarily are in response to changes in pressure. (Arteriosclerosis 9:523–528, July/August 1989)

Vein grafts inserted into the arterial circulation thicken due to proliferation of smooth muscle cells (SMC) and deposition of elastin and collagen matrix. The mechanisms that control this response might be similar to those that cause SMC to proliferate in developing atherosclerotic plaque and in injured arteries. The vein-graft wall responds both to the injury that occurs at the time of grafting and to the increase in wall tension. In arteries, both injury and acute rise in blood pressure cause SMC to proliferate and the vessel wall to thicken. In arteries injured by balloon catheter, this process involves both the media and the intima; while in hypertensive vessels, the process is limited to the media. Hence, SMC migration from the media to the intima seems to be an important feature of the injury response but not the response to an elevation in blood pressure.

In rats, heparin is a specific inhibitor of SMC migration in vitro and in vivo. It inhibits intimal thickening after carotid artery balloon injury if started before SMC enter the S-phase of the cell cycle. Heparin also inhibits pulmonary medial wall thickening due to hypoxemia in experimentally produced pulmonary hypertension in mice and prevents fibrinoid vascular lesions in the kidney of spontaneously hypertensive rats. The purpose of the current investigation was to determine if heparin inhibits SMC proliferation in a well-characterized, experimental vein-graft model in rabbits. The effect of heparin on rabbit carotid balloon injury was studied to document its inhibition of SMC growth in this species. Our results indicate that although heparin inhibits intimal thickening in rabbit carotid arteries, it has only a minimal effect on SMC proliferation and wall thickening in vein grafts. These data suggest either a fundamental difference in the process of vessel-wall thickening in vein grafts compared to injured arteries, or that SMC from arteries are more sensitive to heparin inhibition than are SMC from veins.

Methods

Carotid Injury

Twenty-five New Zealand White rabbits weighing between 2.5 and 4 kg each were subjected to common carotid balloon injury after anesthesia with intramuscular injection of xylazine (7 mg/kg) and ketamine hydrochloride (35 mg/kg). After intravenous administration of 1000 units of heparin, the right common carotid endothelium was stripped by passage of a 3F balloon embolectomy catheter introduced through an arteriotomy in an external branch. Under the same anesthetic, continuous intravenous infusion of either heparin (Sigma type II, 0.3 mg/kg of body weight/hour) in normal saline (13 animals) or saline alone (12 animals) was achieved with an osmotic infusion pump (Alzet, Alza Corporation, Palo Alto, CA) placed in the subcutaneous tissue over the back and connected to a Silastic catheter inserted into the jugular vein. Active pumping began at approximately 4 hours, and steady-state plasma levels of heparin were reached by approximately 8 hours after pump insertion. Pumps contained approximately 2 ml of solution delivered at a rate of 5 μl/
hour (all 2-week infusions) or 2.5 μl/hour (4-week saline infusions). Satisfactory infusion was assured by measuring the residual amount of fluid in the pump and demonstrating prolongation of the whole-blood clotting time in animals receiving heparin. Four-week heparin infusions were accomplished with 2-week infusion pumps that were replaced at 2 weeks under a second anesthetic. Animals were given a standard diet of rabbit chow. Their care complied with the “Principles of Laboratory Animal Care” and the “Guide for the Care and Use of Laboratory Animals” (NIH Publication No. 80-23, revised 1978).

Vein Grafts

In a separate group of 43 animals, interposition vein grafts were placed in the right common carotid artery with our previously described technique. After intravenous administration of 1000 units of heparin to anesthetized animals, the common carotid artery was divided between clamps, and a segment of external jugular vein was interposed in an end-to-end fashion using interrupted 7-0 polypropylene sutures (Surgilene, Davis and Geck, Danbury, CT). Topical papaverine was used to prevent vessel spasm. The vein was not distended or irrigated during the procedure. Animals received either continuous intravenous heparin or saline at the same dose and by the same method as described above.

Morphology

Animals were sacrificed, and the vessels were perfusion-fixed at 1, 2, or 4 weeks. Prior to sacrifice, the rabbits were anesthetized and intravenously given 25 mg/kg Evans blue in phosphate-buffered saline and 1000 units of heparin. After additional anesthesia of 5 mg/kg of intravenous acepromazine maleate, laparotomy and left thoracotomy were performed for placement of an inferior vena cava drainage catheter and a descending thoracic aorta perfusion catheter. After injection of an additional dose of acepromazine maleate, the animals were sacrificed by exsanguination with 2 to 3 liters of lactated Ringer’s solution. Perfusion fixation was accomplished by intravenous administration of 1000 units of heparin to anesthetized rabbits, CT). Topical papaverine was used to prevent vessel spasm. The vein was not distended or irrigated during the procedure. Animals received either continuous intravenous heparin or saline at the same dose and by the same method as described above.

Measurement of DNA Content

In a third group of 10 animals, vein grafts and infusion pumps containing either heparin or saline were placed as previously described. The animals were sacrificed by a drug overdose 4 weeks after graft placement. Five-millimeter lengths of left carotid artery and previously undisturbed external jugular vein and 4-mm diameter punch biopsies of vein graft were isolated from fresh tissue. Specimens were dried for 24 hours at 37°C, and dry weights were obtained. DNA content was measured by a modified diamino benzene acid fluorometric assay by using calf thymus DNA as the standard and expressed as μg DNA/mg dry weight or μg DNA/mm² surface area.

Statistics

Statistical comparisons between heparin and control values (one-tailed p values) were made with the Mann-Whitney (unpaired data) or Wilcoxon (paired data) non-parametric tests (Statistical Package for the Social Sciences). Differences were considered significant at a p value of 0.05 or less.

Results

Carotid Injury

There were no early technical failures; one control animal died at 6 days. The remaining animals were sacrificed at 1 week (four control and four heparin-treated), 2 weeks (four control and five heparin-treated), and 4 weeks (three control and four heparin-treated).

Findings in the ballooninjured carotid animals sacrificed at 1 and 4 weeks and all vein-graft animals were given 0.5 mCi/kg intravenous tritiated thymidine (New England Nuclear, Incorporated, Boston, MA, 6.7 Ci/mM) 1 hour prior to sacrifice. Unstained, deparaffinized, 5-μm histologic cross-sections on glass slides were dipped in Kodak NTB-2 emulsion, stored at 4°C for 2 weeks, developed with Kodak D 19 developer, and stained with hematoxylin. Labeled nuclei were identified under oil-immersion light microscopy by their overlying silver grains (5 grains or more). The total number of nuclei in each area was calculated by multiplying the cross-sectional area by the number of nuclei per square millimeter (estimated by counting nuclei in several representative, defined areas using a light microscope with a reticle). The thymidine labeling index (percent) was calculated as the number of labeled nuclei divided by the total number of nuclei in the section and multiplied by 100.

Scanning Electron Microscopy

Specimens from the midportion of vein grafts were pinned out on Teflon sheets, dehydrated through graded alcohol solutions, dried by the critical-point method, mounted on studs, and sputter-coated with gold-palladium.
animals at 7 days were stained blue, indicating loss of endothelium. Areas of regenerating endothelium were noted as circular white patches at the orifice of small branches. These areas grew larger over time until they nearly encompassed the entire injured carotid at 4 weeks. White regions have previously been shown on scanning electron microscopy (SEM) to be covered by regenerating endothelium. On histologic cross-section, the intima became progressively thicker as cells accumulated; intimal cross-sectional area was maximal at 2 weeks (Table 1, Figure 1). The medial thickness increased only slightly. Heparin-treated carotid arteries had significantly less intimal and medial thickening than controls at 2 weeks. By 4 weeks, the differences between cross-sectional areas in heparin-treated and control arteries were no longer significant. In injured carotids from control animals, proliferation, as documented by thymidine autoradiography (thymidine-labeling indices, Table 2), was markedly elevated in the intima at 1 week and was still slightly elevated at 4 weeks (SMC thymidine index in normal rabbit carotid artery is <0.02). In heparin-treated animals, very few SMC were present in the intima at 1 week, and none of these were labeled, whereas the control animals had a significant intimal accumulation of SMC, with a 10% thymidine labeling index. At 4 weeks, the thymidine index was not significantly different in control and heparin-treated carotid arteries (Table 2). Proliferation was increased to a lesser extent in the media at 1 week and returned to baseline at 4 weeks. Whole-blood clotting time was significantly prolonged in animals receiving heparin (Table 3).

Vein Grafts

There was one intraoperative, technical failure, and there were three late deaths (two control, one heparin-treated), and four late graft thromboses (two control, two heparin-treated). The remaining animals were sacrificed at 1 week (five control and five heparin-treated), 2 weeks (four control and four heparin-treated), and 4 weeks (eight control and eight heparin-treated).

Vein grafts from control animals displayed progressive intimal hyperplasia, as previously described. In earlier experiments, we have demonstrated partial loss of endothelium along the grafts, but primarily at the sites of anastomoses in specimens examined shortly after surgery. On histologic cross-section, these grafts demonstrated areas of endothelial denudation and deposition of platelets, microthrombi, and leukocytes. The endothelial surface was fully restored by 2 weeks, and very few platelets or leukocytes were seen on SEM after this time. In the current study, cross-sectional areas increased over the 4-week period of observation (Table 4). Despite complete re-endothelialization, SMC proliferation, which was greatest at 1 week, continued at an increased rate in both the intima and media for 4 weeks (Table 5).

Heparin treatment resulted in a slight decrease in intimal and medial cross-sectional wall areas, which was only significant in the intima at 2 weeks (Table 4, Figure 2). SMC proliferation, as measured by thymidine labeling, was not inhibited by heparin (Table 5). Similarly, DNA content in vein grafts both per dry weight and per surface area at 4 weeks was not diminished by heparin and was significantly greater than that of unoperated veins (Table 6). The DNA content of normal jugular veins and carotid arteries was not affected by heparin (Table 6).

Discussion

The purpose of this series of experiments was to determine if heparin treatment might inhibit the process of wall thickening in vein grafts inserted into the arterial circulation. In a previous set of experiments, we showed that vein-graft thickening, like arterial-wall thickening, is the consequence of SMC hyperplasia and the synthesis
endothelial cell proliferation and inhibiting both pericytes. This effect is independent of thrombin inactivation.11 In fractions of heparin also inhibit SMC growth indicate that heparin treatment produces a net increase in matrix but that the stimulus to enter the cell cycle occurs very early, several rounds of proliferation immediately or not at all.10 The growth fraction does not increase SMC in the injured carotid synchronously respond by perhaps at the time of injury.9 Between 20% and 40% of SMC themselves in these two models.

Arterial Response to Injury

After balloon injury, rat carotid SMC start synthesizing DNA between 24 and 72 hours, and proliferation reaches a peak between 2 and 4 days.6 SMC begin to migrate from the media across the internal elastic lamina to form a new intima. Proliferation continues for several rounds and stops spontaneously after 1 week. Studies using ornithine decarboxylase expression as an early G, marker suggest that the stimulus to enter the cell cycle occurs very early, perhaps at the time of injury.9 Between 20% and 40% of SMC in the injured carotid synchronously respond by entering the cycle.10 The growth fraction does not increase after the initial event, suggesting that cells commit to several rounds of proliferation immediately or not at all.

Heparin inhibits both proliferation and migration of SMC in vivo and in vitro.2 The fact that nonanticoagulant fractions of heparin also inhibit SMC growth indicate that this effect is independent of thrombin inactivation.11 In vitro heparin has cell-specific effects, enhancing capillary endothelial cell proliferation and inhibiting both pericyte and SMC proliferation.12 Since heparin administration can be delayed for several hours after the mitogenic stimulus, it appears that heparin is only required in late G, for maximal effect.3,9 In the rat model of arterial injury, heparin treatment produces a net increase in matrix but alters the relative types that are accumulated in the intima; in the presence of heparin, elastin and collagen deposition is decreased and proteoglycans are increased.13 Because of these changes in the matrix, suppression of intimal thickening is marked at 2 weeks and is lost at 4 weeks in the injured rat carotid even though at this late time SMC mass is still decreased.14

Our results with heparin treatment in balloon-injured, rabbit carotid arteries were similar to those in the rat model. Significant reduction in intimal cross-sectional area occurred at 1 and 2 weeks. In the heparin-treated carotids at 1 week, there were very few intimal cells, none of which were labeled. In contrast, significant intimal thickening had occurred by this time in the control carotids, and 10% of these intimal SMC were labeled by the single pulse of tritiated thymidine. At late times, as in the rat model, differences in intimal area were absent. This may be due to increased accumulation of matrix in the heparin-treated rabbits. These data indicate that the response of rabbit artery to balloon injury is similar to that found in the rat and that both are influenced by the addition of heparin. Since medial proliferation was not affected by heparin, it appears that the decrease in intimal thickening was largely due to inhibition of SMC migration into the intima.

Vein-Graft Response to Arterial Circulation

Our previous studies with the rabbit carotid vein-graft model have demonstrated that wall thickening is the consequence of an increase in the mass of SMC and matrix.1 Damage to the wall at the time of grafting consists of patchy loss of endothelium with deposition of platelets, microthrombi, and leukocytes. The denuded surface is completely covered with endothelial cells by 2 weeks, but endothelial proliferation continues at an increased rate until 12 weeks after injury. SMC have been identified as the predominant cell type in the thickened wall by immunohistochemistry and transmission electron microscopy. SMC proliferation is maximal at 1 week and continues at a high rate well after the endothelial surface has been re-established. SMC thymidine labeling returns to near normal levels by 12 weeks. Graft wall thickness and perimeter increase steadily until 12 weeks. Rapid proliferation and accumulation of SMC is responsible for this thickening during the first 4 weeks. After this time, conti-

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Table 1. Compartment Areas, Injured Carotids

<table>
<thead>
<tr>
<th>Time after Injury</th>
<th>Control</th>
<th>Heparin</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 week</td>
<td>0.04±0.01 (4)</td>
<td>0.01±0.01 (4)</td>
<td>0.04</td>
</tr>
<tr>
<td>2 weeks</td>
<td>0.20±0.05 (4)</td>
<td>0.05±0.02 (5)</td>
<td>0.01</td>
</tr>
<tr>
<td>4 weeks</td>
<td>0.14±0.01 (3)</td>
<td>0.11±0.04 (4)</td>
<td>NS</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Time after Injury</th>
<th>Control</th>
<th>Heparin</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 week</td>
<td>0.39±0.03 (4)</td>
<td>0.34±0.04 (4)</td>
<td>NS</td>
</tr>
<tr>
<td>2 weeks</td>
<td>0.50±0.05 (4)</td>
<td>0.30±0.04 (5)</td>
<td>0.01</td>
</tr>
<tr>
<td>4 weeks</td>
<td>0.41±0.01 (3)</td>
<td>0.38±0.03 (4)</td>
<td>NS</td>
</tr>
</tbody>
</table>

Values are means±SEM in mm². Numbers in parentheses are the number of animals in each group.

Table 2. Thymidine Labeling Index, Injured Carotids

<table>
<thead>
<tr>
<th>Time after Injury</th>
<th>Control</th>
<th>Heparin</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 week</td>
<td>10.0±5.8 (4)</td>
<td>*</td>
<td>NS</td>
</tr>
<tr>
<td>4 weeks</td>
<td>0.30±0.17 (3)</td>
<td>0.32±0.21 (4)</td>
<td>NS</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Time after Injury</th>
<th>Control</th>
<th>Heparin</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 week</td>
<td>2.1±0.8 (4)</td>
<td>5.4±1.8 (4)</td>
<td>NS</td>
</tr>
<tr>
<td>2 weeks</td>
<td>0.06±0.06 (4)</td>
<td>0.23±0.23 (4)</td>
<td>NS</td>
</tr>
</tbody>
</table>

Values are means±SEM in percent. The numbers in parentheses are the number of animals in each group. *There were very few intimal cells in the heparin group, and none were labeled.

Table 3. Whole-Blood Clotting Times

<table>
<thead>
<tr>
<th>Injury</th>
<th>Before heparin</th>
<th>24 hours after heparin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carotid injury</td>
<td>8.1±0.4</td>
<td>9.8±1.0</td>
</tr>
<tr>
<td>Vein grafts</td>
<td>4.2±0.3</td>
<td>13.1±2.0</td>
</tr>
</tbody>
</table>

The values are means±SEM in minutes. The numbers in parentheses are the number of animals in each group.

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Table 4. Compartment Areas, Vein Grafts

<table>
<thead>
<tr>
<th>Time after grafting</th>
<th>Intima</th>
<th>Media</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>Heparin</td>
<td>Control</td>
</tr>
<tr>
<td>1 week</td>
<td>0.13±0.04 (5)*</td>
<td>0.13±0.02 (5)</td>
</tr>
<tr>
<td>2 weeks</td>
<td>0.17±0.03 (4)</td>
<td>0.09±0.02 (5)</td>
</tr>
<tr>
<td>4 weeks</td>
<td>0.75±0.26 (8)</td>
<td>0.54±0.15 (8)</td>
</tr>
</tbody>
</table>

Values are means±SEM in mm². The numbers in parentheses are the number of animals in each group.

Table 5. Thymidine Labeling Index, Vein Grafts

<table>
<thead>
<tr>
<th>Time after grafting</th>
<th>Intima</th>
<th>Media</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>Heparin</td>
<td>Control</td>
</tr>
<tr>
<td>1 week*</td>
<td>1.1±0.2 (4)</td>
<td>2.1±0.9 (5)</td>
</tr>
<tr>
<td>2 weeks</td>
<td>0.17±0.08 (7)</td>
<td>0.06±0.02 (7)</td>
</tr>
<tr>
<td>4 weeks</td>
<td>0.17±0.08 (7)</td>
<td>0.06±0.02 (7)</td>
</tr>
</tbody>
</table>

Values are means±SEM in percent. The numbers in parentheses are the number of animals in each group.

*Intimal thickening was inadequate for determining a labeling index at 1 week.

ued deposition of extracellular matrix, rather than SMC growth, causes further increase in wall area. The wall appears to stop thickening when the calculated wall stress, which was initially quite high due to the increased pressure in the thin-walled vessel, is reduced to normal arterial levels. This suggests that increased wall stress is an important determinant of vein-graft wall structure. Subsequent studies provided support for this concept by showing that an external support used to reduce wall stress reduced wall area. These data suggest that vein grafts have an initial response to injury similar to that of injured arteries and a more chronic, adaptive response to increased wall stress. The latter may be similar to the adaptation of arteries to hypertension.

A preliminary report suggested that heparin can inhibit the thickening response of rabbit carotid vein grafts. We found a slight reduction in intimal cross-sectional area at 2 weeks but no reduction in wall thickness at any other time and no reduction in SMC proliferation (thymidine labeling index) at 1, 2, or 4 weeks. The fact that the DNA content, both by surface area and by dry weight, was the same in control and heparin-treated vein grafts at 4 weeks implies that the SMC content and the ratio of SMC to matrix was not affected. Thus, at the latest time point, heparin had no discernible effect on either SMC growth or matrix production by SMC.

The lack of heparin effect on vein-graft remodeling could be due to inaccessibility of the heparin to the medial SMC. However, vein grafts have an early, patchy loss of endothelium that is repaired by 1 to 2 weeks. This makes the vein graft analogous to the carotid artery after balloon injury, where heparin is effective in limiting SMC proliferation. It is more likely that SMC in veins are less susceptible than SMC in arteries to the inhibitory effects of heparin or that the mechanism of thickening is substantially different in vein grafts than in balloon-injured carotid arteries. Migration of non-dividing SMC into the intima is an important component of thickening after balloon injury, and heparin's main effect in this model may be inhibition of this process. It is possible that SMC proliferation is

Figure 2. Light micrograph of vein grafts at 2 weeks. A. Control. B. Heparin treated. Arrowheads indicate internal elastic lamina. Hematoxylin and eosin, original magnification ×310
Table 6. DNA Content at 4 Weeks

<table>
<thead>
<tr>
<th>Area</th>
<th>Control</th>
<th>Heparin</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Graft</td>
<td>1.2±0.2 (5)</td>
<td>1.0±0.1 (4)</td>
<td>NS</td>
</tr>
<tr>
<td>Vein</td>
<td>0.06±0.01 (5)</td>
<td>0.08±0.02 (4)</td>
<td>NS</td>
</tr>
<tr>
<td>Artery</td>
<td>0.30±0.03 (5)</td>
<td>0.24±0.03 (4)</td>
<td>NS</td>
</tr>
</tbody>
</table>

Values are the means±SEM in micrograms. The numbers in parentheses are the number of animals in each group.

more important than migration in vein-graft adaptation and that heparin is less able to inhibit this response. Our data suggest that heparin may not be able to block all forms of SMC proliferation and may only be a weak inhibitor when SMC proliferate in response to increases in pressure.

Acknowledgments

The authors gratefully acknowledge the technical assistance of Thomas Opstad, Selina Certeza, and Stephanie Lara.

References


Index Terms: tritiated thymidine • smooth muscle cell • heparin • vein grafts • rabbit
Effect of heparin on adaptation of vein grafts to arterial circulation.
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doi: 10.1161/01.ATV.9.4.523

Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 1079-5642. Online ISSN: 1524-4636

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