Inhibition of Experimental Neointimal Hyperplasia and Thrombosis Depends on the Type of Vascular Injury and the Site of Drug Administration

Campbell Rogers, MD; Morris J. Karnovsky, MB, BCh, DSc; Elazer R. Edelman, MD, PhD

Background. Heparin inhibits vascular smooth muscle cell proliferation in tissue culture and limits neointimal hyperplasia after experimental arterial injury but has been ineffective in reducing clinical restenosis. We examined how this discrepancy might reflect suboptimal drug-tissue interactions and/or differences in the vascular response to injury.

Methods and Results. Intravenous infusion was compared with local administration of heparin to injured rabbit iliac arteries either from drug-impregnated polymeric controlled release matrices in the perivascular space or from drug-releasing endovascular stents. Occlusive thrombosis, seen in 42% of control stent-bearing arteries, and partial thrombosis were virtually eliminated by heparin delivery from any route. Intimal area 14 days after balloon withdrawal denudation alone was reduced to an equal extent by continuous systemic heparin or by perivascular heparin for the first 3 days. In contrast, endovascular stents produced more exuberant neointimal hyperplasia, the inhibition of which required continuous rather than only early heparin administration. Neither perivascular delivery limited to the first 3 days nor stent-based delivery reduced neointimal hyperplasia as effectively.

Conclusions. The antiproliferative and antithrombotic effects of heparin differ markedly, depending on the type of arterial injury and the mode of drug administration. Different forms of injury may require different therapies, and complications of arterial intervention such as excessive neointimal hyperplasia and thrombosis may demand alternate therapeutic regimens. Duration, dose, and site of delivery rather than frank resistance to therapy may explain why experimentally effective antiproliferative and antithrombotic agents fail clinically. (Circulation. 1993;88:1215-1221.)

KEY WORDS • restenosis • stents • heparin

R evascularization of obstructive atherosclerotic vessels induces thrombosis and neointimal hyperplasia, which in turn cause recurrent luminal narrowing. This process is so severe as to require additional intervention in 30% to 40% of coronary arteries after balloon angioplasty and in over 60% of aortocoronary saphenous vein bypass grafts within 5 years after surgery.1-3 The lesions in these diseases are composed primarily of vascular smooth muscle cells (SMC) and extracellular matrix, and efforts to limit neointimal hyperplasia have focused on a diverse set of compounds that inhibit SMC growth and thrombosis in tissue culture and in animal models of acute arterial injury.4-10 With most compounds, however, translation of experimental success to clinical use aimed at inhibiting restenosis has failed.11-21

Heparin, independent of anticoagulant properties, inhibits SMC proliferation in tissue culture22 as well as in denuded rat carotid arteries4 whether administered systemically or, in much lower doses, to the adventitial surface.23 In contrast, heparin delivered to the luminal aspect of experimentally damaged arteries has reduced neither neointimal hyperplasia nor thrombosis,14-20 and clinical trials using systemic heparin after balloon angioplasty have failed to reduce clinical or angiographic restenosis.14,27,28 Although thrombosis has been proposed as a direct contributor to intimal hyperplasia after experimental endovascular stent placement,29 elimination of thrombosis by heparin has not reduced the rate of restenosis after coronary stent placement.30,31

We now report that the means of drug administration, site of drug delivery, and extent of arterial injury all contribute to the biological response to heparin. Neointimal hyperplasia and thrombosis were followed after single acute injury (balloon withdrawal denudation) or more chronic and severe injury (balloon withdrawal followed by endovascular stent placement) in rabbit iliac arteries. Arteries were treated with heparin delivered via local intra-arterial, local perivascular, or systemic intravenous routes. Although all forms of heparin treatment reduced stent thrombosis and limited neointimal hyperplasia after balloon withdrawal injury, only continuous perivascular or intravenous drug delivery produced effective inhibition of neointimal hyperplasia.
after the more chronic and severe injury of endovascular stents. These results may help explain the numerous clinical failures of experimentally effective regimens aimed at limiting restenosis.

Methods

Animal Care and Surgical Procedure

Twenty-five New Zealand White rabbits (Millbrook Farm Breeding Labs, Amherst, Mass) of either sex, weighing 3.5 to 4 kg, were housed in individual mesh cages and maintained on rabbit chow and water. Beginning 1 day before surgery, aspirin (0.07 mg/mL, Sigma, St Louis, Mo) was added to drinking water for an approximate daily dose of 5 mg/kg.

Anesthesia was achieved with an intramuscular injection of ketamine (35 mg/kg, Aveco Co, Fort Dodge, Iowa), followed by intravenous sodium Nembutal (Abbott Laboratories, North Chicago, Ill) 4 mg/kg via a marginal ear vein. All animals received a single intravenous bolus of heparin (100 U/kg, Elkins-Sinn Inc, Cherry Hill, NJ) at the time of surgery. Animals were maintained on a warming blanket throughout surgery and recovery. Both femoral arteries were exposed and ligated, and catheters passed via arteriotomy. The endoaortic of the iliac arteries was denuded with a 3F balloon embolectomy catheter (Baxter Healthcare Corp, Edwards Division, Santa Ana, Calif), inflated in the abdominal aorta, and withdrawn three times to the femoral artery. Six animals received no further arterial manipulation (balloon group). In another group of 19 animals, a stainless steel slotted-tube stent, 7 mm in length, was inflated within each iliac artery (stent group). Each stent was mounted on a 3-mm angioplasty balloon (Advanced Cardiovascular Systems Inc, Santa Clara, Calif) and expanded with a steady 15-second inflation at 10 atm pressure. The arteries had approximate diameters of 2.5 mm, for a balloon stent to artery ratio of 1:2:1.

Heparin Administration

Ethylene-vinyl acetate copolymer (EVAc) matrices, 33% loaded with heparin (Choay heparin 1453, 12 000 to 18 000 DA, USP 160 U/mg, Paris), were prepared as previously described. Matrices (10×5×1 mm) were covered with either two or six coats of EVAc, and two 20-gauge or 27-gauge holes were bored at equal spacings into one matrix face. In this manner, heparin release was constrained to provide desired release kinetics. Heparin release was measured in vitro by incubating either heparin-impregnated EVAc matrices or stents with ionically bound heparin in lactated Ringer's solution at 37°C for 16 days. Aliquots of solution were sampled at regular intervals, and their heparin content was assayed using the metachromasia of Azure A (Fisher Scientific Co, Fairlawn, NJ) at 620 nm. Because heparin release from EVAc matrices was either highest after 2 days of incubation or was approaching steady-state release, (depending on the number of EVAc coats and the size of the holes), matrices were incubated at 37°C in lactated Ringer's solution for 48 hours before implantation in vivo.

In addition to the intraoperative bolus of heparin and daily oral aspirin, experimental groups received one of the following: no additional treatment (12 arteries in the stent group, 4 arteries in the balloon group); continuous left femoral intravenous infusion of heparin 0.3 mg·kg⁻¹·h⁻¹ via an osmotic pump (Alza Corp, Palo Alto, Calif) (4 arteries in the stent group, 4 arteries in the balloon group); controlled release of heparin from an EVAc matrix placed under the inguinal ligament and positioned in the perivascular space directly adjacent to the balloon-injured arteries (4) or stent-bearing arteries (10); or elution of heparin from an intra-arterial metal stent (12 arteries). Activated partial thromboplastin times (aPTT) were measured using a desktop analyzer (Ciba-Corning Diagnostics Corp, Oberlin, Ohio) at the time of procedure, 7 days later, and at the time the animals were killed.

Tissue Processing

Arteries were harvested 14 days after surgery. After a lethal intravenous injection of sodium Nembutal, inferior vena caval exsanguination, and 100 mm Hg pressure infusion of lactated Ringer's solution via left ventricular puncture, the iliac arteries were excised and fixed in Carnoy's solution (60% methanol, 30% chloroform, 10% glacial acetic acid).

Nonstented arterial segments were embedded in paraffin and cut in 6-μm cross sections. Stent-bearing arterial segments were isolated, oriented for proximal and distal ends, and embedded in K-Plast (Medim America Ltd, Wilmington, Del). Four to eight 5-μm arterial cross sections were then cut with a tungsten carbide knife from three sites along each stent: proximal end, middle, and distal end. This allowed integration of histological observations over the entire length of each stent, minimizing sampling error. Metal stent sections were not removed from the arteries before embedding. All sections were stained with Verhoeff's tissue elastin stain.

Intimal cross-sectional area was determined by means of computer-assisted digital planimetry. The extent of deep arterial injury caused by stent wires was quantified using the method of Schwartz et al. The antithrombotic efficacy of various heparin regimens was compared by contrasting both the frequency of complete stent thrombosis as well as the percent of the cross-sectional circumference of patent stent-bearing arteries covered with laminar thrombus (measured on histological sections with computer-aided planimetry).

Statistics

All data are presented as the mean±SE. Statistical analysis comparing treatment groups used a nonpaired t test. Values of P<.05 were considered significant.

Results

Heparin Release Rates

Ethylene-vinyl acetate copolymer matrix slabs were constructed to release heparin with either rapid first order or more prolonged near-zero order kinetics (Fig 1). Matrix rectangles with two copolymer coats and two 20-gauge holes released heparin in a first-order manner with a peak rate of 50.6±4.6 μg/h after 60 hours' incubation. Because all matrices were incubated for 48 hours before in vivo implantation, this first matrix formulation provided peak heparin release 12 hours after insertion into experimental animals, with little
Fig 1. Graph shows heparin release rates in vitro in lactated Ringer's solution at 37°C from osmotic minipumps (closed squares) or polymeric heparin-impregnated matrices (open and closed circles). Matrices were constructed to release heparin primarily early during incubation (closed circles) or in a continuous fashion over 14 days (open circles). Each method was subsequently applied for heparin delivery to rabbit iliac arteries in vivo, pumps for intravenous infusion, and polymeric matrices for controlled perivascular delivery. Before in vivo use, pumps were incubated in lactated Ringer's solution at 37°C for 6 hours and matrices for 2 days.

release after the third in vivo day. More prolonged-release kinetics were obtained by applying six copolymer coats and constraining release to two smaller 27-gauge holes. This matrix formulation released heparin more gradually, reaching near-zero order kinetics (heparin release rates were between 13.4±1.4 and 15.2±0.6 μg/h) after the first 5 days. Again, because of 48 hours of preincubation before in vivo use, this second matrix formulation provided an increasing heparin release rate for the first 3 in vivo days, with steady-state release thereafter. Ionically bound heparin was released from metal stents at a rate of 1.0 μg/h for the first day, rapidly declining to a steady-state release rate of 0.3±0.1 μg/h for the ensuing days. Osmotic pumps delivered heparin at 300±6 μg·kg⁻¹·h⁻¹ (Fig 1), as determined by manufacturer's specifications and confirmed by determination of pump residual volumes at the completion of experiments.

Fig 2. Bar graph shows incidence of complete luminal thrombosis in rabbit iliac arteries 14 days after balloon withdrawal denudation followed by endovascular stent placement. Treated groups received heparin via intravenous infusion, from the stent itself, or from drug-impregnated matrices placed adjacent to the adventitia of stent-bearing arteries. Matrices were incubated in vitro for 2 days before placement and provided heparin release either early (i.e., primarily for the first 3 days after placement) or continuously over 14 days. Probability values reflect comparison with untreated controls.

Anticoagulation and Thrombosis

Activated partial thromboplastin times were measured in animals before surgery and at 7 and 14 days. Continuous intravenous heparin infusion prolonged the aPTT to at least two times control in each animal at 7 and 14 days. There was no prolongation of the aPTT by either perivascular or stent-released heparin in any animal at any time point.

Complete thrombosis at 14 days was observed in 42% of the arteries in animals receiving a single intravenous bolus of heparin at the time of stent placement and oral aspirin continuously (see Table and Fig 2). All forms of heparin administration reduced thrombosis in stent-bearing arteries. Only 8% of arteries implanted with stents releasing heparin demonstrated complete thrombosis (P<.004), whereas heparin delivered either intravenously or into the perivascular space completely eliminated occlusive thrombosis (see Table and Fig 2). As a measure of partial thrombosis, the percent of arterial circumference covered with laminar thrombus on histo-

<table>
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<tr>
<th>Heparin</th>
<th>Intima (mm²)</th>
<th>Injury score</th>
<th>Thrombosis</th>
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<tbody>
<tr>
<td></td>
<td>Balloon</td>
<td>Stent</td>
<td>Complete</td>
</tr>
<tr>
<td>None</td>
<td>0.26±0.03</td>
<td>1.15±0.11</td>
<td>0.56±0.07</td>
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<tr>
<td>Intravenous</td>
<td>0.12±0.03</td>
<td>0.41±0.05</td>
<td>0.54±0.02</td>
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<tr>
<td>(P&lt;.03)</td>
<td></td>
<td></td>
<td>(P&lt;.02)</td>
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<tr>
<td>Perivascular</td>
<td>0.11±0.03</td>
<td>0.82±0.08</td>
<td>0.50±0.08</td>
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<tr>
<td>(early)</td>
<td>(P&lt;.03)</td>
<td></td>
<td>(P&lt;.03)</td>
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<tr>
<td>Perivascular</td>
<td>0.52±0.08</td>
<td>0.55±0.14</td>
<td>0%</td>
</tr>
<tr>
<td>(continuous)</td>
<td>(P&lt;.006)</td>
<td></td>
<td>(P&lt;.04)</td>
</tr>
<tr>
<td>Stent-based</td>
<td>1.09±0.11</td>
<td>0.63±0.07</td>
<td>8%</td>
</tr>
<tr>
<td>(P=NS)</td>
<td></td>
<td></td>
<td>(P&lt;.004)</td>
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Injury was caused by balloon withdrawal denudation alone (balloon) or with accompanying endovascular stent placement (stent). Probability values reflect comparison with control animals receiving no postprocedure heparin.
logical cross section was calculated for each group. All modes of heparin delivery reduced partial mural thrombosis: stent-released heparin reduced partial thrombosis from 29±6% to 17±4%; intravenous heparin infusion eliminated partial thrombosis altogether (0±0%); and early or continuous perivascular heparin reduced partial thrombosis to 14±5% or 12±4%, respectively (P<.04 for each heparin-treated group compared with control, P=NS between heparin-treated groups; see Table).

Neointimal Hyperplasia

We compared heparin modulation of the arterial response to acute injury (balloon withdrawal with endothelial denudation) with the effect of heparin on the more chronic and severe injury imposed by the placement of metal stents within denuded arteries. Fourteen days after balloon injury alone, a highly cellular neointima had formed, separating the internal elastic lamina from the lumen. Intimal cross-sectional area was 0.26±0.03 mm² (see Table and Fig 3). Heparin after balloon injury was equally effective at inhibiting neointimal hyperplasia whether delivered via continuous intravenous infusion for 14 days or via controlled perivascular release from EVA-matrices providing local delivery of much lower doses primarily for the first 3 days (see Table and Fig 3; intimal areas 0.12±0.03 mm² or 0.11±0.03 mm², respectively; P<.03 for each compared with controls). The degree of inhibition of neointimal hyperplasia, expressed as the percent reduction in intravenously or locally treated groups compared with untreated controls, was 54% or 57%, respectively. Furthermore, balloon withdrawal alone resulted in no disruption of the internal elastic lamina.

The placement of an endovascular metal stent induced an intimal response more than fourfold greater than balloon withdrawal alone (see Table and Figs 4 and 5A; intimal area, 1.15±0.11 mm²). Heparin was delivered by way of intravascular osmotic pump infusion, intra-arterial heparin-bound stent implants, or heparin-impregnated polymeric matrices deployed in the perivascular space. Intimal area after intravenous deliv-
intravenous infusion or continuous local perivascular release reduced stent-induced neointimal hyperplasia by 64% and 54%, respectively.

In arteries subjected to a single superficial acute injury (balloon withdrawal denudation), deep mural injury was less common and neointimal hyperplasia was fourfold less extensive than after stent placement. Local perivascular delivery of heparin primarily over the first 3 days after single acute vascular injury was as effective as continuous intravenous heparin for 14 days (reducing neointimal area by 54% and 57%, respectively). These data confirm an earlier report that after rat carotid artery denudation, 3 or 7 days of intravenous heparin reduced SMC growth fraction, migration, and accumulation as effectively as more prolonged administration.34 In contrast, our data show that after endovascular stent placement, 3 days of local heparin delivery was markedly less effective than either continuous intravenous dosing or continuous local perivascular release at limiting neointimal growth.

Stent Thrombosis and Intimal Hyperplasia

Clinical coronary arterial stent placement has been complicated by both early thrombosis and subsequent neointimal hyperplasia leading to restenosis.30,31,35-37 Extremely potent antithrombotic regimens including antiplatelet, anticoagulant, and thrombolytic agents have been used to reduce the rate of clinically recognized acute thrombosis from 24% to 39%30,37 to less than 3%.31,36 Not unexpectedly, these regimens have been associated with a high incidence of vascular complications occurring in up to 16% of patients.36

A direct link has been proposed between thrombosis and neointimal hyperplasia induced by stents in swine coronary arteries,39 based on the observation that systemic administration of anticoagulant heparin reduced neointimal hyperplasia after stent placement in a similar model.38 In carotid or coronary arteries of swine treated with aspirin, however, heparin released from metal stents neither significantly reduced thrombosis nor limited intimal response, although the incidence of thrombosis in control animals was low.24,25 Moreover, although heparin in conjunction with other antiplatelet and anticoagulant drugs after human coronary stenting has virtually eliminated thrombosis, angiographic restenosis rates of 25% to 32% have persisted in clinical trials.30,31,36 Our data show that although heparin regimens that curtailed thrombosis such as continuous intravenous or perivascular delivery also greatly limited neointimal hyperplasia, other regimens such as stent-released heparin or early postprocedure perivascular heparin reduced both partial and complete thrombosis at 14 days but had little to no effect on neointimal hyperplasia. The relation between these two aspects of stent-induced arterial injury remains to be fully delineated.

Type of Vascular Injury Dictates Antiproliferative Effects of Heparin

Endothelial denudation in experimental animals has long been used as a model of vascular response to injury. Elucidation of the cellular responses and growth regulation after such injury has allowed identification of diverse compounds capable of inhibiting neointimal hyperplasia after arterial damage.4-6,10 Paradoxically, clinical investigations with these compounds after coronary interventions have failed to demonstrate beneficial reduction in restenosis.11-21 Our data show that two different models of experimental vascular injury within the same animal arterial system respond quite differently to an agent with known and well-characterized antiproliferative activity.

Experimental endovascular stent placement is associated with more severe arterial damage and includes a more prolonged phase of intimal SMC proliferation than is seen after balloon withdrawal injury alone.39,40 The number of proliferating SMC, identified by incorporation of bromodeoxyuridine or 3H-thymidine, is highest in the first week after either balloon injury or stent placement and virtually ceases within 14 days of balloon denudation but continues at high levels for greater than 28 days after stent placement.39-41 The fourfold increase in neointimal hyperplasia observed when endovascular stents were added to balloon-injured arteries may reflect a more extensive initial injury to the arterial wall33 or a more chronic stimulus for proliferation related to the presence of the indwelling stent itself.

Others have also reported that local luminal heparin delivery via perforated perfusion balloons or from metal stents does not inhibit neointimal hyperplasia after experimental vascular injury.24-26 Our data show that
local delivery of heparin to the adventitial surface but not to the luminal surface of stent-bearing arteries can reduce neointimal hyperplasia as effectively as systemic dosing. The failure of other methods of local heparin delivery may reflect differences in the biological activity of the heparin used, differences in the site of drug application (lumen vs adventitia), differences in the local concentrations of heparin achieved, or differences in the form of arterial injury (additional damage with a perfusion balloon vs single balloon injury vs primary stent deployment) rather than biological resistance to local heparin treatment. More chronic and severe arterial damage demands more prolonged administration of an antiproliferative agent to inhibit neointimal hyperplasia than does single denuding arterial injury.

The results in this study further highlight the divergence of human atherosclerosis and restenosis from animal models of acute arterial injury. The clinical failure of agents antiproliferative in some animal models may reflect differences in the extent of injury and/or differences between an otherwise normal, acutely injured blood vessel and an atherosclerotic vessel subjected to angioplasty or other manipulation. The transition from tissue culture and animal studies to human trials may be enhanced by further elucidation of the biological mechanisms that determine growth after severe or prolonged vascular insults.

Study Limitations

The site, duration, and amount of heparin delivered dictated the drug's modulation of thrombosis and neointimal hyperplasia after superficial acute or deeper chronic injury. We have not yet measured or localized the deposition of exogenous heparin within the arterial wall or correlated the distribution of drug with its biological effects. Measurement and localization of heparin in vivo are difficult because of heparin's high solubility and rapid degradation into smaller oligomers of varying biological activities. These issues are undergoing active investigation in our laboratory. The amount of heparin delivered from stents was small although adequate to reduce thrombosis: Stent-based heparin release provided only 2% of the dose of heparin provided by perivascular matrix-based heparin delivery. The delivery of higher doses for longer periods of time might produce different results.

Future Directions

The accurate transition from experimental models of vascular injury to clinical reduction in accelerated atheriopathies may be limited by differences in the biology of animal models and human disease, differential responses of various components of vascular repair, and dissimilarities between human atherosclerotic arteries and normal vessels of the laboratory animal. We believe that an additional explanation may lie in the methods used to deliver agents and in the failure of clinical trials to adequately extrapolate experimental tissue culture and animal data to human use, rendering the broad application of negative conclusions from such clinical trials unwarranted. The extent of experimental neointimal hyperplasia and the response to an antiproliferative agent, heparin, differs significantly between arteries subjected to a single acute injury and arteries subjected to more prolonged and severe injury. The latter model may be more akin to clinical coronary arterial instrumentation, characterized by deep intimal and medial disruption. Future clinical studies of experimentally effective antiproliferative therapies will need to address duration and degree of drug-tissue interaction in light of the duration and degree of the vascular response to injury before such compounds are deemed ineffective at limiting human restenosis.

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