Reduction in Vascular Lesion Formation by Hirudin Secreted From Retrovirus-Transduced Confluent Endothelial Cells on Vascular Grafts in Baboons

Anders Lundell, MD, PhD; Andrew B. Kelly, DVM; Johanna Anderson, MS; Monique Marijanowski, PhD; Jeffrey J. Rade, MD; Stephen R. Hanson, PhD; Laurence A. Harker, MD

Background—The hypothesis that thrombin mediates the formation of neointimal vascular lesions at sites of mechanical vascular injury has been tested in baboons by measurement of the effects of hirudin delivered by retrovirus-transduced hirudin-secreting vascular endothelial cells (ECs) lining surgically implanted arterial vascular grafts (AVGs).

Methods and Results—The antithrombotic efficacy of baboon ECs transduced with cDNA encoding hirudin was assessed in vitro and in vivo on thrombogenic segments in chronically exteriorized femoral arteriovenous (AV) shunts. Bilateral brachial AVGs lined with hirudin-transduced versus nonhirudin control ECs at confluent density were surgically implanted, and vascular lesion formations at distal graft-vessel anastomoses were compared after 30 days. Hirudin-transduced ECs secreted 20±6 ng·10⁶ cells⁻¹·24 h⁻¹ (range, 14 to 24 ng·10⁶ cells⁻¹·24 h⁻¹) hirudin in supernatants of static cultures. Hirudin-secreting ECs on segments of collagen-coated graft interposed in chronic AV shunts decreased the accumulation of neointimal lesion formation at distal graft-vessel anastomoses, ie, 1.02 mm² (range, 0.88 to 1.95 mm²) versus 1.82 mm² (range, 0.88 to 2.56 mm²) in contralateral AVGs bearing nonhirudin control ECs (P<0.01).

Conclusions—Viral vector–directed secretion of hirudin from ECs lining implanted AVGs significantly reduces the formation of thrombus and neointimal vascular lesions. (Circulation. 1999;100:2018-2024.)

Key Words: lesion ▪ anticoagulants ▪ viruses ▪ grafting

Mechanical vascular injury produced by surgical implantation of arterial vascular grafts (AVGs), endarterectomy, angioplasty, or endovascular stenting induces the formation of neointimal vascular lesions that may cause ischemia. For example, ≈50% of the surgically implanted arteriovenous (AV) vascular grafts used for angioaccess in chronic dialysis patients occlude within 12 months of placement, resulting in AVG revision or replacement. Neither antplatelet therapy nor anticoagulation with heparin or coumarin decreases neointimal vascular lesion formation or its complications. Denuding vascular injury initiates tissue factor–dependent thrombin production, platelet recruitment, platelet secretion of storage-granule platelet-derived growth factor (PDGF), fibrin formation, accumulation of mononuclear blood leukocytes, and subsequent vascular lesion formation. Several lines of evidence indicate that thrombin initiates the molecular and cellular interactions leading to the formation of neointimal vascular lesions at sites of vascular injury by activating thrombin receptors (TRs) on platelets and other blood and vascular wall cells. Thrombin activates ≥2 separate but structurally related G protein–coupled, protease-activated receptors (PAR-1 and PAR-3), at least in mice. The experimental strategy developed for testing the thrombin hypothesis of neointimal lesion formation involves the local generation of hirudin at antithrombotic concentrations at sites of AVG-vessel anastomoses by lining AVGs with endothelial cells (ECs) that have been retrovirally transduced with cDNA encoding hirudin and measuring the extent of neointimal lesion formation 30 days after graft implantation.

Methods

Experimental Design and Animals Studied

The role of thrombin in the formation of neointimal vascular lesions that develop after mechanical vascular injury was investigated in...
baboons by comparing the effects of confluent hirudin-transduced ECs versus nonhirudin control ECs lining implanted AVGs. Initially, experiments were carried out to verify that (1) retroviral vectors containing genes encoding hirudin were stably transduced into cultured baboon vascular ECs and that gene product appeared in culture supernatant; (2) hirudin secreted by transduced ECs interrupted thrombus formation in vivo; (3) confluent EC-lined expanded polytetrafluoroethylene (ePTFE) AVGs were produced in vitro by attaching cultured baboon ECs on collagen-fibronectin–coated AVG segments, and the attached ECs were retained for 7 or 30 days; and (4) AVG neointimal vascular lesions developed at vessel-graft anastomoses after EC-lined AVGs were implanted. Subsequently, cultured baboon vascular ECs stably transduced with a retroviral construct containing a cDNA encoding hirudin were attached at confluent density to AVG flow surfaces and then surgically implanted between brachial arteries and veins. Each animal received a hirudin-secreting and a contralateral nonhirudin control. After 30 days, AVGs were harvested for histochemical and morphometric analyses of vascular lesions forming at proximal and distal vessel-graft anastomoses.

Twenty normal male baboons (Papio anubis) weighing 14 to 20 kg were used in these experiments, 4 to assess antithrombotic effects, 8 during early imaging and preliminary harvesting studies, and 8 undergoing the complete 30-day vascular harvesting protocol. All procedures were approved by the Institutional Animal Care and Use Committee in compliance with the National Institutes of Health guidelines (Guide for the Care and Use of Laboratory Animals, 1985), Public Health Service policy, the Animal Welfare Act, and related university policies. Baboons were observed to be disease-free for ≥3 months before entering the studies.

Endothelial Transduction and Secretion of Recombinant Hirudin

Jugular vein ECs obtained from juvenile male baboons by collagen digestion (collagenase type IV, Worthington Biochemical Corp) were transduced with a retroviral vector containing a cDNA encoding hirudin. The cells serving as controls underwent a similar transduction procedure without hirudin cDNA. The cDNA for hirudin variant-1 synthesized by Rade et al21 was transduced into hirudin. The cells serving as controls underwent a similar transduction procedure without hirudin cDNA. The cDNA for hirudin variant-1 synthesized by Rade et al21 was transduced into hirudin-transduced ECs and the other with non-hirudin control ECs by filling the sterilized collagen-coated AVGs with EC suspension and turning AVGs 90°/min for 30 minutes at 37°C to obtain even EC attachment. Subsequently, AVGs were mounted in a recirculating pump apparatus to maintain continuous perfusion of medium through the AVGs at 37°C for 2 to 4 hours with a flow rate of 15 mL/min, thereby establishing confluent ECs on segments of collagen-fibronectin–coated vascular grafts.

For AVG implantation surgery, animals received ketamine hydrochloride (20 mg/kg IM) for induction, 1% halothane by endotracheal tube for anesthetic maintenance, and buprenorphine (0.01 mg/kg every 8 hours as needed) for postoperative analgesia. Brachial arteries and veins were dissected free, and AVGs were positioned end-to-side as AV shunts. Before the vessels were clamped, heparin (100 U heparin sodium/kg IV; Upjohn Co) was injected. Anastomoses were completed with running sutures of 6-0 polypropylene (Ethicon Inc). Grafts were maintained with D-PBS at 37°C until blood flow through the AVGs was established. Surgical hemostasis was secured, and wounds were closed with running subcutaneous and intracutaneous Surgilene (Ethicon Inc). Thirty days later, AVGs were harvested under anesthesia in sterile conditions. The divided artery was flushed with 10 mL D-PBS through the graft, and AVGs with associated vascular anastomoses were removed. AVG-attached ECs were recovered by collagenase digestion from 3 pairs of AVGs and cultured in complete culture medium.

Morphological Evaluation

The graft and corresponding vascular anastomoses were fixed in 10% buffered formalin (Baxter, Inc) and kept at 4°C for 24 hours. The tissues were divided into 5-mm segments and embedded in paraffin, and 5-μm sections were cut. At least 7 randomly selected sections were prepared from each block per site, midtoe, midgraft, and midheel of each AVG. These sections were stained with Verhoeff–van Gieson’s elastin stain and analyzed with a Nikon Optiphot-2 microscope with a Hitachi HV-C 20 U color video camera connected to the microscope. Analysis was done with Image Pro Plus for Windows 1.3 software program (Media Cybernetics), and data were stored in a Dell PC. Analyses were made of the total area of the neointimal lesion and of the arterial media (mm²). The intimal/medial index was calculated as neointima area divided by media area.

Statistical Analysis

Data are presented as mean ± SD unless indicated otherwise. Significance was generally determined by Student’s t test with unpaired 2-tailed analysis, except for data not normally distributed, when nonparametric analyses were performed. Paired 2-tailed analysis was used to compare platelet and fibrin deposition on the grafts and in the

Thrombus accumulation on EC-seeded collagen-coated segments was quantified by measurement of the deposition of platelets and fibrin on the segment interposed in the AV shunt throughout 60 minutes. Autologous baboon platelets were labeled with 1 mCi 111In (111In-oxine) as previously described.23 Baboon fibrinogen was purified by β-alanine precipitation and labeled with 125I by the Iodine monochloride method as described previously.20

AVG Preparation, Implantation, and Harvesting

The AVGs were composed of thin-walled, 5-mm-ID, ringed, 10-cm-long segments of ePTFE (WL Gore and Associates). The graft segments were sterilized by autoclaving and were kept sterile thereafter. Before ECs were attached, the luminal surface was wetted with 95% ethanol and washed with 500 mL sterile water. The luminal AVG surfaces were coated with equine collagen (Horme) and fibronectin 20 μg/100 μL (Biomedical Technologies Inc) as described previously.22 Each graft was prewarmed to 37°C before cultured ECs were attached. At least 1.5×10⁶ cells were attached per AVG (final luminal area per AVG averaged ~12.5 cm²). One graft was covered with hirudin-transduced ECs and the other with non-hirudin control ECs by filling the sterilized collagen-coated AVGs with EC suspension and turning AVGs 90°/min for 30 minutes at 37°C to obtain even EC attachment. Subsequently, AVGs were mounted in a recirculating pump apparatus to maintain continuous perfusion of medium through the AVGs at 37°C for 2 to 4 hours with a flow rate of 15 mL/min, thereby establishing confluent ECs on segments of collagen-fibronectin–coated vascular grafts.

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Results

Hirudin Secretion by Transduced ECs In Vitro

Hirudin production by retroviral hirudin-transduced ECs was measured antigenically in conditioned media collected from static cultures and averaged 20±6 ng · 10^6 cells^-1 · 24 h^-1 (range, 14 to 24 ng · 10^6 cells^-1 · 24 h^-1; n=4). No antigenic hirudin or antithrombin activity was detected in the supernatant of nonhirudin control cultured ECs. These results verify stable expression of transduced gene product and are similar to the in vitro findings reported previously for hirudin-transduced cells in static culture.25

Effects of Hirudin-Transduced ECs on Thrombus Formation In Vivo

Platelet deposition on nonhirudin control EC-seeded segments averaged 0.82±0.24×10^6 platelets at 60 minutes, and platelet deposition on the segments bearing hirudin-secreting ECs was reduced to 0.52±0.17×10^6 platelets (Figure 1; P=0.03). Hirudin-secreting ECs also reduced platelet accumulation in propagated thrombotic tails extending downstream from the segments of vascular graft (0.59±0.17×10^6 platelets, versus 1.38±0.41×10^6 platelets in controls) (Figure 2; P=0.04). Decreases were also observed in fibrin deposition (Figures 1 and 2; P=0.08 in both cases). Thus, hirudin-transduced ECs produced local concentrations of hirudin that were antithrombotic in vivo.

Lasting EC Attachment to Collagen-Fibronectin-Coated Segments of Vascular Graft

Three approaches were used to evaluate EC coverage on AVG flow surfaces after surgical implantation: (1) in vivo serial imaging of 111In-labeled ECs on newly implanted AVGs; (2) scanning electron microscopy (SEM) of luminal AVG-ECs recovered after 7 and 30 days; and (3) measurement of hirudin secretion by ECs harvested from luminal surfaces of 30-day AVGs. After the implantation of AVGs bearing confluent 111In-labeled ECs in 8 baboons, graft-associated 111In-EC radioactivity was determined by daily quantitative imaging until 111In radioactivity fell below the level of detection, ie, ≤4 to 5 days. The 111In-EC radioactivity remained associated with implanted AVGs for ≥4 days. There was no significant reduction in the calculated number of 111In-labeled ECs attached to implanted AVGs after 96 hours, ie, 1.4±0.2×10^5 ECs/cm^-2 retained on newly implanted AVGs, compared with 1.3±0.3×10^5 ECs/cm^-2 on AVGs after 96 hours. This result is consistent with the previous report of durable confluent luminal surfaces for attached cultured ECs on collagen-fibronectin–coated graft segments exposed to arterial flows.24 These findings demonstrate that in this study, AVG-ECs resisted detachment during surgical manipulations and postoperative exposure to arterial shear rates.

SEM of hirudin-transduced and nonhirudin control AVG-ECs demonstrated confluent endothelium at both 7 (n=4 and n=3, respectively) and 30 (each n=3) days after surgery (Figure 3). Although these observations demonstrated confluent endothelium throughout the 30-day period of study, they do not exclude the possibility that a portion of attached transduced ECs may have been replaced by nontransduced endogenous vessel-derived ECs.

Three pairs of 30-day EC-lined AVGs were harvested, and luminal ECs were recovered and established in culture with

![Figure 1. Effects of hirudin-secreting ECs on graft thrombosis.](image1)

![Figure 2. Effects of hirudin-secreting endothelial cells on propagated thrombotic tail.](image2)

![Figure 3. SEMs showing lasting confluent attachment of transduced and nontransduced vascular ECs.](image3)
medium selected for transduced cells. Supernatant conditioned media of these recovered ECs contained antigenic hirudin levels comparable to those observed in static cultures before AVG attachment, ie, $17 \pm 6$ ng $\cdot 10^6$ cells $^{-1} \cdot 24$ h $^{-1}$ (range, 9 to 25 ng $\cdot 10^6$ cells $^{-1} \cdot 24$ h $^{-1}$), compared with preattachment values averaging $20 \pm 6$ ng $\cdot 10^6$ cells $^{-1} \cdot 24$ h $^{-1}$ ($P>0.5$). These results document that at least a portion of transduced ECs attached on the luminal flow surfaces of implanted vascular grafts were retained throughout the 30-day period of study and continued to secrete hirudin.

Effects of Hirudin-Transduced ECs on the Formation of AVG Vascular Lesions

The 30-day AVG implants were all patent. AVGs bearing confluent hirudin-transduced ECs produced less neointimal lesion at the distal venous anastomoses than AVGs lined with nonhirudin control ECs, ie, 1.02 mm$^2$ (range, 0.11 to 1.95 mm$^2$) versus 1.82 mm$^2$ (range, 0.88 to 2.56 mm$^2$) (Table; Figure 4; $P<0.01$). No significant difference was observed in upstream proximal anastomoses, ie, 1.56 mm$^2$ (range, 0.33 to 1.8 mm$^2$) versus 1.99 mm$^2$ (range, 0.14 to 2.49 mm$^2$) ($P>0.1$; Table). However, lesion formation midway on AVG luminal surfaces was significantly less in the AVGs bearing hirudin-transduced ECs, ie, mean values of 1.28 versus 1.68 mm$^2$ in control AVGs ($P<0.05$). These data document that chronic hirudin secretion by transduced ECs reduced the amount of 30-day intimal proliferative lesion formation at downstream graft-vein anastomoses.

Discussion

This study in nonhuman primates demonstrates that local production of hirudin by transduced endothelial cells significantly reduces the formation of graft thrombus and intimal proliferative vascular lesions at sites of distal graft-vessel anastomoses. Because hirudin is highly specific for thrombin, these findings document the importance of thrombin in mediating the cellular responses in vascular lesions induced by mechanical vascular injury. This study also confirms the efficacy and safety advantages of local delivery of potent antagonists of hemostatic functions and the feasibility of effecting local beneficial responses by transferring genes encoding therapeutic molecules into vascular cells. These findings are concordant with the report that transient hirudin expression by adenovirus-transduced vascular cells in vivo decreases neointimal lesion formation in rats. These studies in nonhuman primates directly document local antithrombotic concentrations of hirudin and establish the relevance for primate vasculature, a significant concern regarding vascular studies in rodents.

The putative cellular processes contributing to intimal proliferative lesion formation that are inhibitable by hirudin include (1) direct stimulation of intimal migration and proliferation of vascular medial smooth muscle cells (SMCs)...

![Figure 4](http://circ.ahajournals.org/Downloadedfrom)

**Table.**

<table>
<thead>
<tr>
<th>Determination</th>
<th>Proximal AVG, Arterial Anastomoses</th>
<th>Distal AVG, Venous Anastomoses</th>
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<tbody>
<tr>
<td>Lesion area, mm$^2$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control non–hirudin-treated</td>
<td>1.99 (1.99; 0.14–2.49)</td>
<td>1.82 (1.73; 0.88–2.56)</td>
</tr>
<tr>
<td>Hirudin-treated</td>
<td>1.56 (1.33; 0.1–1.8)</td>
<td>1.02 (0.30; 0.11–1.95), $P&lt;0.01$</td>
</tr>
<tr>
<td>Lesion index</td>
<td></td>
<td></td>
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<tr>
<td>Control non–hirudin-treated</td>
<td>0.18 (0.14; 0–0.27)</td>
<td>0.23 (0.20; 0.11–0.26)</td>
</tr>
<tr>
<td>Hirudin-treated</td>
<td>0.20 (0.20; 0.03–0.36)</td>
<td>0.11 (0.07; 0.01–0.21), $P&lt;0.01$</td>
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Because the data were not normally distributed, results are presented as mean values with medians and ranges shown in parentheses, and statistical significance was estimated by nonparametric analysis.
(2) platelet activation, recruitment, and secretion of PDGF with consequent PDGF-dependent stimulation of medial SMC migration and proliferation; and (3) monocyte/macrophage activation, recruitment, and production of PDGF, with PDGF-dependent induction of SMC migration and proliferation. Because hirudin appears to prevent catalytic activation of TRs on platelets, leukocytes, vascular ECs, and SMCs with equivalent efficacy, the relative importance of each individual pathway cannot be differentiated from the present observations. Clearly, the levels of EC-secreted hirudin in the AVG outflow boundary layer were sufficient to inhibit TR-dependent platelet recruitment (Figures 1 and 2). The concentration of hirudin in the boundary layer has been estimated by use of computational fluid mechanics (axisymmetric steady Navier-Stokes and convective-diffusion equations solved by finite-element analysis program). The calculated levels of boundary-layer hirudin are 15 to 25 ng/mL, assuming the AVG to be 10 cm long and 4 mm in diameter, blood flow 100 mL/min, 1.5 × 10^6 attached ECs, and production in vitro of 20 ng · 10^6 cells·1 · 24 h^{-1}.

Because systemic concentrations of hirudin that prevent TR-dependent platelet recruitment and fibrin generation concurrently impair hemostatic function, we adopted the safety strategy of generating inhibitory concentrations of hirudin locally using AVG-attached hirudin-transduced ECs. These safety concerns regarding systemic hirudin therapy have been heightened by the recent controlled clinical trials evaluating the effects of systemic hirudin. In these trials, antithrombotic doses of hirudin produced abnormal bleeding compared with heparin, requiring that dosing be reduced to ensure hemostatic protection. The resultant antithrombotic outcomes were inconclusive. By short-term therapy with parental α_{IIbβ_{3}} integrin receptor antagonists produced striking benefits in acute coronary syndrome patients without life-threatening bleeding complications. These contrasting outcomes emphasize the importance of thrombin generation (and fibrin formation) in hemostatic protection.

The duration of hirudin therapy needed to reduce vascular lesion formation is not evident from the present study. If short-term interruption of platelet deposition and PDGF secretion is sufficient to decrease the formation of neointimal lesions in primates, requisite transient hirudin secretion can be achieved by use of adeno-associated viral vectors, as in the reports in rodents. However, if thrombin is generated for many days by macrophages expressing tissue factor, long-term inactivation of thrombin will be necessary. Accordingly, the present study was designed to generate local hirudin for at least 30 days. It seems likely that prolonged secretion of the foreign protein hirudin will eventually induce the formation of neutralizing anti-hirudin antibodies.

The design strategy of the present study required that hirudin-transduced AVG-ECs remain attached and continue to secrete hirudin throughout the 30-day study period. Persuasive evidence is provided that hirudin-transduced ECs remained attached for at least 4 days after EC-covered AVGs were implanted, that AVG-ECs remained confluent for 30 days, and that at least a portion of the AVG-ECs secreted hirudin 30 days after graft implantation. Other workers have not observed durable retention of attached cultured ECs on flow surfaces after exposure to flowing blood in vivo. Although the explanation for such disparate findings is not apparent, we attribute the persistent retention of attached ECs documented in the present study to (1) the dense, thickened composition of the collagen-fibronectin substrate to which the transduced ECs were attached; (2) extended media perfusion in vitro after initial EC attachment that promoted EC spreading and stable adherence before surgical implantation; and (3) strict maintenance of 37°C during all EC manipulations in vitro.

There are a number of clinically relevant implications of the present study. First, local antithrombin therapy has substantial efficacy and safety advantages over systemic therapy. Second, gene transfer of therapeutic molecules into vascular cells is a feasible strategy for modulating vascular responses. Third, intermediate abatement of intimal proliferative lesions is appropriate and useful. Because multiple complex pathways contribute to the formation of vascular proliferative lesions after mechanical vascular injury, elimination of a single pathway will only partially decrease lesion development. Complete prevention would require concurrent inhibition of all significant pathways, with the attendant risk of ensuing aneurysm and rupture. Because blood flow improves with the square of the increase in diameter, intermediate reduction in vascular lesion formation is generally clinically adequate.

We conclude that thrombin plays an important role in the formation of neointimal vascular lesions and that inactivating thrombin at sites of mechanical vascular injury is an effective means for reducing vascular lesion formation.

Acknowledgments

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References


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