Endogenous heparin activity is decreased in peripheral arterial occlusive disease

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Background: Naturally occurring heparin-like activity in the form of endogenous heparin and heparin sulfate proteoglycans has been shown in normal human plasma. Exogenous low-dose heparin improves pain-free walking distance and maximum walking distance in peripheral arterial occlusive disease (PAOD). Is reduced endogenous heparin activity responsible for some of the problems found in PAOD? This study compared heparin-like activity in patients with PAOD with that in healthy subjects and explored its relationship to disease severity.

Methods: In part 1, native and heparinase-modified thromboelastography was performed on peripheral venous blood samples in three groups of patients to measure heparin-like anticoagulant activity. Group 1: 15 control subjects (median age, 60 years; range, 49-74 years; ankle-brachial pressure index [ABPI] > 0.9); group 2: 14 patients with intermittent claudication (median age, 66 years; range, 56-80; ABPI, 0.69 [SD, 0.09]); group 3: 14 patients with rest pain (median age, 67.5 years; range, 54-84 years; ABPI, 0.45 [SD, 0.08]). In part 2, heparin equivalent to that in normal plasma was added to blood samples from 15 patients with short-distance claudication (n = 4) or rest pain (n = 11), and baseline (without heparinase) thromboelastography was performed to exclude lack of antithrombin as a cause of diminished heparin-like activity.

Results: In part 1, all patients with PAOD had a significant increase in coagulability compared with controls. Heparinase-modified thromboelastography in controls showed a significant decrease in the latent period between placing the sample in the analyser, where it is recalcified, to the initial fibrin formation (ΔR time; P = .002) compared with native TEG, confirming endogenous heparin-like activity. Using ΔR time as a measure of heparin-like activity, a significant reduction was found in patients with claudication (0.33 minutes; 95% confidence interval [CI], 0.004-0.65; P = .02) and in those with rest pain (0.25 minutes; 95% CI, 0.02 to 0.52; P = .02) compared with that in controls (0.78 minutes; 95% CI, 0.39-1.16). The ΔR time also correlated with the ABPI (r = 0.35, P = .02), suggesting declining heparin-like activity with increasing ischemia. In part 2, exogenous heparin restored the thromboelastography in PAOD patients to normal, suggesting that lack of endogenous heparin-like compounds rather than reduced antithrombin levels was responsible for changes in coagulation.

Conclusion: Patients with PAOD have reduced endogenous heparin-like activity that correlates with disease severity. (J Vasc Surg 2008;47:1033-8.)

Clinical Relevance: Studies have confirmed the presence of natural heparin-like anticoagulant activity in the form of endogenous heparin and heparan sulfate proteoglycans in normal human plasma. We know that supplementation with low-dose exogenous heparin improves pain-free walking distance and maximum walking distance in peripheral arterial occlusive disease. Could some of the pathology of peripheral arterial occlusive disease relate to an underlying lack of endogenous heparin-like activity?

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Endogenous heparin produced by connective tissue-type mast cells and heparan sulfate proteoglycan (HSPG), a vessel wall glycosaminoglycan from the vascular endothelial cells, has been shown in normal human plasma. Exogenous low-dose heparin improves pain-free walking distance and maximum walking distance in peripheral arterial occlusive disease (PAOD). Is reduced endogenous heparin activity responsible for some of the problems found in PAOD? This study compared heparin-like activity in patients with PAOD with that in healthy subjects and explored its relationship to disease severity.

Methods: In part 1, native and heparinase-modified thromboelastography was performed on peripheral venous blood samples in three groups of patients to measure heparin-like anticoagulant activity. Group 1: 15 control subjects (median age, 60 years; range, 49-74 years; ankle-brachial pressure index [ABPI] > 0.9); group 2: 14 patients with intermittent claudication (median age, 66 years; range, 56-80; ABPI, 0.69 [SD, 0.09]); group 3: 14 patients with rest pain (median age, 67.5 years; range, 54-84 years; ABPI, 0.45 [SD, 0.08]). In part 2, heparin equivalent to that in normal plasma was added to blood samples from 15 patients with short-distance claudication (n = 4) or rest pain (n = 11), and baseline (without heparinase) thromboelastography was performed to exclude lack of antithrombin as a cause of diminished heparin-like activity.

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tissue plasminogen activator (tPA), a mechanism independent of AT. In addition to its antihemostatic activity, heparin increases vessel wall permeability, suppresses the proliferation of vascular smooth muscle cells, suppresses osteoblast formation, and activates osteoclasts.

The heparin-AT complex is the main inhibitor of coagulation enzymes, including thrombin (IIa) and factors Xa, Xia, and XIIa. Of these, thrombin and factor Xa are the most responsive to inhibition, and human thrombin is approximately 10-fold more sensitive than Xa to inhibition by heparin-AT. Heparin binds to the lysyl residues on AT and induces a conformational change, thereby converting AT from a slow to a very rapid inhibitor of clotting enzymes. Antithrombin remains bound to thrombin in the thrombin-antithrombin complex (TAT), but heparin dissociates to be reused. Reduced AT and increased TAT levels in serum are among the significant hemostatic alterations found in patients with advanced peripheral vascular disease. Abnormally low levels of AT found in PAOD could be responsible for reduced thrombin inhibition even in the presence of normal endogenous heparin levels.

Long-term intermittent heparin therapy in non-anticoagulant doses has been shown to reduce fibrinogen levels and improve claudication as well as maximum walking distances. We hypothesized that part of the heightened coagulation activity in atherosclerotic disease relates to the reduced production of endogenous heparin-like substances in these patients. We therefore set out to measure endogenous heparin-like activity in patients with PAOD and to identify its relationship to the severity of disease. We also had to exclude the possibility that any apparent reduction in heparin-like activity was due to low AT levels.

METHODS

The Ethics Committee of Oxford Radcliffe Hospitals NHS Trust approved this study and all the patients who participated provided informed consent.

Table I. Demographic and vascular risk factor details of study groups

<table>
<thead>
<tr>
<th>Groups</th>
<th>Part 1</th>
<th>Part 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>IC</td>
<td>Rest pain</td>
</tr>
<tr>
<td>Participants, No</td>
<td>15</td>
<td>14</td>
</tr>
<tr>
<td>Sex, No.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>Male</td>
<td>13</td>
<td>10</td>
</tr>
<tr>
<td>Age, median (range) y</td>
<td>60 (49-74)</td>
<td>66 (56-80)</td>
</tr>
<tr>
<td>ABPI (mean ± SD)</td>
<td>1.04 ± 0.11</td>
<td>0.69 ± 0.09</td>
</tr>
<tr>
<td>Antihypertensive therapy, No.</td>
<td>4</td>
<td>6</td>
</tr>
<tr>
<td>Diabetes mellitus, No.</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>Smoking (current), No.</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>Hyperlipidemia treatment, No.</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>Previous event</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MI, No.</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>CVA, No.</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>DVT, No.</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Antiplatelet therapy, No.</td>
<td>4</td>
<td>5</td>
</tr>
</tbody>
</table>

ABPI, Ankle-brachial pressure index; CVA, cerebrovascular accident; DVT, deep vein thrombosis; IC, intermittent claudication; MI, myocardial infarction.

Part 1. Sequential patients presenting to the vascular outpatient department with claudication or rest pain due to atherosclerotic disease were asked to participate, and 14 patients with intermittent claudication (IC) and 14 patients with rest pain were recruited. From amongst general surgical patients, 15 subjects aged >45 years with no symptoms of PAOD, an ankle-brachial pressure index (ABPI) >0.9, and no history of cardiovascular, cerebrovascular, or malignant disease were recruited. Control and patient group demographic and vascular risk factor details for parts 1 and 2 are given in Table I.

Part 2. Also recruited were 15 patients with symptoms of PAOD, including short-distance claudication (n = 4) and rest pain (n = 11), admitted for peripheral vascular reconstruction procedures. Patient demographic and vascular risk factor details are given in Table I.

No patient in any of the groups received any form of exogenous heparin or any other anticoagulation agent. Within the 2 weeks before study, aspirin was only antiplatelet agent used.

Baseline thromboelastography. Thromboelastography (TEG) is a rapid, sensitive, global test of coagulation using whole blood. It measures the viscoelastic properties of a blood clot and provides information about the combined function of all the factors involved in the process of clot formation and its subsequent dissolution. A small amount of whole blood (340 µL) is placed in a cuvette, and a pin suspended by a torsion wire is lowered into the sample. The torque experienced by the pin as the clot forms is recorded as a trace. Five parameters—R, K, angle (α), MA, and CI—are usually measured (Fig 1).

- R time is the latent period between placing the sample in the analyser, where it is recalcified, to the initial fibrin formation; it correlates with the time to initial fibrin formation and is used to estimate heparin like anticoagulant activity in a given blood sample. R time is prolonged by anticoagulants like heparin and coagulation
factor deficiencies and is shortened by hypercoagulable conditions.

- **K time** is a measure of time from the beginning of clot formation until a fixed level of clot firmness is reached (amplitude of 20 mm). This TEG parameter is shortened by increased fibrinogen level and to a lesser extent by increased platelet activity and is prolonged by agents that affect both.

- The **angle** is the angle between the horizontal line through the center of the TEG tracing and the line tangential to the developing “body” of the TEG tracing. The angle represents the acceleration of fibrin buildup and cross-linking (clot strengthening). Similar to K time, the angle increases with raised fibrinogen levels and to a lesser extent with platelet activity.

- **MA**, or maximum amplitude, is a direct function of the maximum dynamic properties of fibrin and platelet bonding by glycoprotein (GP) IIb/IIIa receptors; it represents the ultimate strength of the fibrin clot and is dependent on the number and function of platelets and their interaction with fibrin.

- **CI** (coagulation index) describes the patient’s overall coagulation status and is derived from the R, K, MA and angle of blood tracings \( CI = -0.245R + 0.0184K + 0.1655MA - 0.0241\alpha - 5.0220 \).20

**Heparinase-modified thromboelastography to measure heparin activity.** We measured the change in R time \( (\Delta R \text{ time}) \) between a baseline TEG tracing and that obtained from an aliquot of the same sample when exposed to a heparinase-modified TEG protocol. This \( \Delta R \) should be attributable to the contribution of heparin-like activity in the original sample from substances susceptible to heparinase breakdown. We used modified TEG cups containing heparinase-I, an enzyme obtained from *Flavobacterium heparinum*, which selectively breaks down the glycosaminoglycans, heparin, and heparan sulfate. Heparinase has been shown to neutralize heparin more effectively than protamine in blood samples obtained during cardiopulmonary bypass.21 This method has been shown to be both sensitive and specific for the detection of changes due to heparin activity compared with conventional tests of anti-Xa activity, activated partial thromboplastin time, and activated coagulation time.22 This technique is also used in cardiac surgery to check the extent of heparin reversal by protamine in postoperative patients23 and to detect increased endogenous heparin-like activity after orthotopic liver transplantation. We validated this method in our previous study involving patients with PAOD and heparinase-modified TEG.24

**Blood sampling.** Samples were collected from the antecubital vein without tourniquet use to avoid activation of the coagulation cascade. Samples were stored in citrated tubes for 1 to 2 hours and were analyzed after recalcification using both native and heparinase-modified TEG, a method that gives better reproducibility than immediate analysis of a fresh sample.25

**Statistical methods.** The TEG data within groups were analyzed using the Wilcoxon signed rank test. Significant differences in TEG data between groups were sought using a Mann-Whitney *U* test. The relationship between \( \Delta R \) time and ABPI was identified using the Spearman correlation test. A value of \( P < .05 \) considered significant (two-tailed).

**RESULTS**

**Part 1.** The baseline TEG variables showed a significant increase in the coagulability of blood samples from patients with symptoms of PAOD (intermittent claudication and rest pain) compared with the control group: R time, \( P = .001 \); K time, \( P = .003 \); angle, \( P = .003 \); MA, \( P = .004 \); and CI, \( P = .004 \) (Table II). We also found a significant relationship between the overall coagulability of blood and the severity measured by the ABPI of the disease in patients with PAOD \( (r = -0.395; P = .009; \text{Fig 2}) \).
In the control group, heparinase-modified TEG showed a significant decrease in the R time compared with the native (baseline) TEG, confirming the presence of heparinase-I–sensitive endogenous heparin-like activity. The same method revealed a significant reduction in R time in blood samples from patients with PAOD, confirming the presence of a detectable heparin-like activity in those patients. However, the R time (endogenous heparin-like activity) was significantly lower than in control subjects (0.78 minutes, 95% confidence interval [CI], 0.39-1.16 minutes; \( P < .020 \)) in both the claudication (0.33 minutes, 95% CI, 0.004-0.65 minutes; \( P = .020 \)) and the rest pain group (0.25 minutes, 95% CI, 0.02 to 0.52 minutes; \( P = .016 \); Fig 3). We found no significant difference in R time between claudication and rest pain groups.

When we exposed the samples from the patients with PAOD to heparinase, we also observed a significant negative relationship between R time and the severity of disease by the ABPI (\( r = 0.350, P = .021 \)) demonstrating a fall in endogenous heparin-like activity as peripheral vascular disease advanced (Fig 4).

**Part 2.** The addition of heparin to the blood samples from patients with PAOD led to a significant increase in the R time of 5.84 minutes (95% CI, 4.98-6.69) vs 8.33 minutes (95% CI, 6.77-9.89, \( P = .002 \)) restoring coagulability to that of control subjects.

**DISCUSSION**

We have shown that heparinase-modified TEG can identify endogenous heparin activity in controls and patients with PAOD. We have also shown that PAOD is associated with reduced heparin-like activity in the blood and that the magnitude of reduction relates to the severity of atherosclerotic disease measured by the ABI. The alternative explanation, that the reduced heparin-like activity is due to lack of AT rather than lack of endogenous heparin-

### Table II. Baseline (native) thromboelastography data for control, claudication, and rest pain groups

<table>
<thead>
<tr>
<th>Native TEG parameter</th>
<th>Controls (95% CI)</th>
<th>IC (95% CI)</th>
<th>Rest pain (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>R time, min</td>
<td>9.40 (8.48-10.33)</td>
<td>7.50 (6.52-8.42)</td>
<td>5.55 (4.29-6.81)</td>
</tr>
<tr>
<td>K time, min</td>
<td>2.88 (2.52-3.23)</td>
<td>2.14 (1.61-2.66)</td>
<td>1.71 (1.29-2.13)</td>
</tr>
<tr>
<td>( \alpha ) Angle, deg</td>
<td>56.23 (53.51-58.95)</td>
<td>66.52 (60.64-72.39)</td>
<td>63.25 (58.74-67.75)</td>
</tr>
<tr>
<td>MA, mm</td>
<td>58.07 (55.30-60.83)</td>
<td>64.17 (59.04-69.30)</td>
<td>69.42 (62.34-73.50)</td>
</tr>
<tr>
<td>Coagulation index</td>
<td>1.06 (0.56-1.57)</td>
<td>2.21 (1.29-2.91)</td>
<td>2.32 (1.73-3.12)</td>
</tr>
</tbody>
</table>

CI, Confidence interval; IC, intermittent claudication; MA, maximum amplitude; TEG, thromboelastography.

\( ^a \)R time, latent period between placing the sample in the analyser, where it is recalcified, to the initial fibrin formation; K time, measure of time from the beginning of clot formation until a fixed level of clot firmness is reached (amplitude of 20 mm); \( \alpha \) angle, angle between the horizontal line through the center of the TEG tracing and the line tangential to the developing “body” of the TEG tracing; MA, a direct function of the maximum dynamic properties of fibrin and platelet bonding by glycoprotein IIb/IIIa receptors; coagulation index describes the patient’s overall coagulation status.

\( ^b \)P values refer to significance of difference between control group and claudication or rest pain group.

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**Fig 2.** Overall coagulability and peripheral ischemia.

**Fig 3.** Endogenous heparin-like activity in patients with peripheral arterial occlusive disease. \( \Delta R \) is the change in latent period between when the sample is recalcified and initial fibrin formation.
like compounds, has been excluded by restoration of R times to normal with a physiologic dose of exogenous heparin.

Tuman et al.19 used the heparinase-modified TEG method in 42 healthy volunteers and found no significant endogenous heparin-like activity in most of the samples analyzed 3 minutes after collection and citration, although they did find some activity in a small group that they ascribed to contamination. Ideally, blood samples should be analyzed ≤6 minutes after collection.26 This is often not practicable, and samples are usually citrated and analyzed after a delay.

Camenzind et al.25 investigated the effects of citrate storage on TEG and confirmed that TEG indicators were different in recalcified, citrated blood samples compared with native blood. The observed changes were progressive in samples during 0 to 30 minutes of storage but were stable thereafter, and the authors recommend analysis after a citrate storage period between 1 and 8 hours for reliable TEG results. We followed this method and performed native and heparinase-modified TEG after a citrate storage period of 1 to 2 hours for all samples in an effort to obtain reliable results. This may explain the difference between our results and those of Tuman et al.19

The reduction in heparin like activity is probably related to reduced production in the arterial wall. Studies of atherosclerotic human coronary arteries have shown a reduction in HSPG proportional to the increasing severity of atherosclerosis.27 Heparan sulfate content in the intima of human arteries, especially in atherosclerosis-prone regions such as coronary arteries, renal arteries, internal carotid artery at the level of the carotid sinus, abdominal aorta, and iliac arterial segments, has been shown to decrease with an increase in the severity of atherosclerotic lesions.28

Reduced “heparin-like” activity has obvious implications. Endogenous heparin and HSPGs found on the negatively charged29 luminal surfaces of the endothelial cells are essential for inhibition of coagulation at the endothelial surface30 and lipoprotein lipase function,31 both of which are defective in atherosclerotic vascular endothelium.32 Circulating endogenous heparin and heparan sulfates in the proteoglycan layer of the intima subjacent to the lumen bind with and activate antithrombin to form an effective anticoagulant complex on the endothelial surface. Endogenous heparin has been shown to decrease microthrombi formation at sites of endothelial injury.33

Heparin has a number of actions mediated independently of AT. It inhibits platelet activation after flow through vessels with a high and moderate shear stress.54 It also prevents endothelial cell dysfunction after ischemia by augmenting vasodilation mediated by endothelial-derived relaxing factor.35 These actions of heparin are of particular importance, because both high shear and ischemia-induced endothelial dysfunction38 are known to exist in patients with PAOD.

Studies of hemostatic factors and products of coagulation show a similar trend in both cardiac and PAOD patients, suggesting activation of coagulation. Once patients reach a stage where endogenous heparin-like activity is significantly reduced, then the obstructive effects of atherosclerosis on blood flow may be compounded by an increasing tendency to thrombosis within the downstream vessels. LMW heparins act by reducing fibrinogen level and whole blood viscosity, especially in narrowed segments (low flow) of the peripheral vessels. LMW heparin is antithrombotic and profibrinolytic by increasing anti-Xa levels and plasminogen activity33 and may therefore help in claudication by reducing the viscosity.

A variety of heparin-related products have also been shown to inhibit vascular smooth muscle cell proliferation and to alter the endothelial response after vascular injury. Exogenous heparin preparations suppress smooth muscle cell proliferation after injury to vascular endothelium, and LMW heparin has been shown to reduce neointimal hyperplasia in cultured human saphenous vein.39 Heparin in non-anticoagulant doses has been tried in patients to reduce stenosis after angioplasty, but it has been difficult to demonstrate a clear benefit.40 Inadequate levels of endogenous heparin may allow excessive intimal hyperplasia at points of trauma within the vascular system either owing to clinical intervention or in areas of turbulence within the circulation.

Studies in which small doses of subcutaneous LMW heparin (15,000 anti Xa U/d) were given to patients with PAOD, absolute claudication time and walking distance improved along with a significant increase in activated partial thromboplastin time that nevertheless remained within normal limits.17 A 3-month course of unfractionated heparin (12,500 IU once daily) has also been shown to improve pain-free walking distances to a greater extent than an antiplatelet agent, ticlopidine, alone16 in patients with PAOD. This study provides supporting evidence for the use of low-dose heparin in patients with PAOD. This may be particularly important at the time of prolonged immobility and surgical intervention.

CONCLUSION

Patients with advanced PAOD exhibit reduced endogenous heparin-like activity, which correlates negatively with
the severity of disease. This appears to be due to lack of endogenous heparin-like substances rather than impaired AT activity alone.

AUTHOR CONTRIBUTIONS

Conception and design: LH, AH
Analysis and interpretation: KS
Data collection: KS
Writing the article: KS
Critical revision of the article: AH, LH
Final approval of the article: LJ
Statistical analysis: KS
Obtained funding: LJ
Overall responsibility: LJ

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