Low-molecular-weight heparins are heparin subfractions obtained by cleaving the original molecules into smaller components, with the aim of preserving the antithrombotic effects while avoiding the bleeding problems caused by unfractionated heparin. They range in molecular weight from 4,000 to 9,000 d, compared with more than 10,000 d for unfractionated heparin. The antithrombin effect of heparin depends on the molecular weight of the compound. At least 16–20 monosaccharides per molecule are necessary for the full expression of antithrombin activity, as the interaction of antithrombin
III with thrombin requires longer saccharide chains in the heparin molecule. Low-molecular-weight heparins retain the ability of unfractionated heparin to bind antithrombin III and to inactivate factor Xa but have greatly reduced interaction with thrombin and may thereby avoid the hemorrhagic effects of unfractionated heparin. The importance of the anti-factor Xa effect to the "in vivo" antithrombotic effect is the subject of ongoing controversy.4-8

In vitro, low-molecular-weight heparin subfractions induce less platelet aggregation than does unfractionated heparin.9 Experimental studies have shown that low-molecular-weight heparins effectively inhibit factor Xa in vivo and reduce thrombus formation in the low-shear, fibrin-rich venous system.7,8,10,11 They have also proven to be effective in clinical studies of the prevention and treatment of venous thrombosis.12-15 There is, however, much less information about these agents in the arterial circulation, where the thrombi are platelet rich and thrombin plays a central pathogenic role.16-18

The aim of this study was to evaluate the in vivo antithrombotic efficacy of a low-molecular-weight heparin (CY216) to reduce platelet and fibrinogen deposition and to prevent mural thrombus formation in an established porcine model of deep arterial injury. CY216 was compared with unfractionated heparin, with an anti-factor Xa effect similar to that of 200 units/kg CY216, and with placebo. Efficacy was related to the relative anti-factor Xa and antithrombin effects of these drugs.

Methods

Fifty-one normal pigs of the Babcock four-way cross stock (a mixture of Landrace, Yorkshire, Hampshire, and Duroc breeds), approximately 4 months old and weighing 36±1 kg (mean±SD; range, 28-46 kg), were randomized to one of six treatment groups: placebo (0.9% saline); low-molecular-weight heparin (CY216; Laboratoire Choay, Paris, France) at 100, 200, 400, or 500 Institute Choay units/kg; or unfractionated calcium heparin (Calciparin; Du Pont Pharmaceuticals, Wilmington, Del.) at 100 US Pharmacopeia (USP) units/kg (from porcine intestinal mucosa). These dosages are equivalent to 56,112, 224, 280, and 135 IU/kg, respectively. To rapidly reach nearly steady-state effects, the heparin preparations were given as an intravenous bolus (units/kg) followed immediately by a continuous intravenous infusion of the same dosage per hour that was administered via a Harvard pump (Harvard Apparatus, South Natick, Mass.) at a rate of 0.8 ml/min.

Experimental Protocol

The study conformed to the guidelines of the American Heart Association on research animal use and was approved by the institutional animal care and use committee. On the day before the procedure, autologous platelets were labeled with 300 μCi 111In-tropolone; the platelets were reinjected together with 250 μCi 125I-labeled human fibrinogen. On the study day, animals were sedated with 1,000 mg ketamine (Ketaset, Bristol Laboratories, Syracuse, N.Y.), and anesthesia was maintained with a titrated intravenous infusion (3-5 ml/min) of a mixture of 1,000 mg ketamine, 10 mg fentanyl citrate (U.S.P.C. Inc., Rockville, Md.), and 40 mg etomidate (Abbott Laboratories, North Chicago, Ill.) per liter of 5% dextrose. The pigs were intubated and mechanically ventilated with room air (Harvard respirator, Harvard Apparatus) until they were killed. The electrocardiogram and intra-arterial pressure were monitored continuously.

Before treatment, 14-gauge Angiocaths were placed in the left femoral vein for drug administration and in the right femoral artery for blood sampling. An 8-mm×3-cm polyethylene angioplasty catheter (Blue Max, Medi-tech, Watertown, Mass.) was advanced via the left femoral artery under fluoroscopic guidance to the left and then the right common carotid artery for balloon dilatation. Both common carotid arteries were dilated between the first and third cervical vertebrae by a standardized procedure adjusted for the size of the animal (five inflations for 30 seconds at 6-8 atm, with 60 seconds between inflations).16-18 Fifteen minutes after the last balloon inflation in the right common carotid artery, 120 ml of 0.5% Evans blue dye in 0.9% NaCl was injected into the ascending aorta to identify endothelial denudation with blue staining.18 The animals were killed by an overdose of pentobarbital. The carotid arteries were perfused in situ at physiological pressure with normal saline followed by 2% buffered glutaraldehyde; both carotid arteries were then harvested. After being cleaned of adventitia, the dilated portion was divided into two equal segments, and two similarly sized segments were taken immediately proximal and distal to the dilated area.

Tissue Analysis

The numbers of 111In-labeled platelets and molecules of 125I-labeled fibrinogen/fibrin deposited on each arterial segment were quantified as previously described.19,20 Counting for 111In was performed on the day of surgery and for 125I, 2-3 weeks later after the 111In had decayed. The segments were cut open and photographed; computer-assessed planimetry was used to quantify the total area and the area of deep injury. Two rings from each arterial segment were stained with hematoxylin and eosin and van Gieson's stains. Deep arterial injury (defined as a tear through the internal elastic lamina) was advanced via the left common carotid artery, 120 ml of 0.5% Evans blue dye in 0.9% NaCl was injected into the ascending aorta to identify endothelial denudation with blue staining.18 The animals were killed by an overdose of pentobarbital. The carotid arteries were perfused in situ at physiological pressure with normal saline followed by 2% buffered glutaraldehyde; both carotid arteries were then harvested. After being cleaned of adventitia, the dilated portion was divided into two equal segments, and two similarly sized segments were taken immediately proximal and distal to the dilated area.

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Tissue Analysis

The numbers of 111In-labeled platelets and molecules of 125I-labeled fibrinogen/fibrin deposited on each arterial segment were quantified as previously described.19,20 Counting for 111In was performed on the day of surgery and for 125I, 2-3 weeks later after the 111In had decayed. The segments were cut open and photographed; computer-assessed planimetry was used to quantify the total area and the area of deep injury. Two rings from each arterial segment were stained with hematoxylin and eosin and van Gieson's stains. Deep arterial injury (defined as a tear through the internal elastic lamina into the media, correlated with dark Evans blue staining) or subendothelial injury (endothelial denudation with preservation of the internal elastic lamina; light blue staining) was documented by two independent observers. A twofold magnifying glass was used to examine the segments for the presence of mural thrombosis, which was performed without knowledge of the treatment group and before the arterial segment was counted for platelet and fibrinogen deposition.
Laboratory Tests

Before treatment, blood was drawn from the right femoral artery for platelet count, fibrinogen, hematocrit, antithrombin III, activated partial thromboplastin time (aPTT), thrombin time, and anti-factor Xa activity. Thrombin time, aPTT, and anti-factor Xa activity were also measured 10 minutes after starting the drug infusion, before each carotid dilation, and immediately before the animals were killed. Repeated samples were obtained for platelet count, fibrinogen, hematocrit, and antithrombin III at the time the animals were killed.

Platelet counts, hematocrit, and fibrinogen were determined as previously reported. Blood for drug levels, aPTT, thrombin time, and antithrombin III was mixed 9:1, vol/vol, with 3.8% trisodium citrate solution and centrifuged to obtain platelet-poor plasma. All samples were processed and assayed as reported previously. Heparin anti-factor Xa plasma activity was determined with the Stachrom Xa kit (American Bioproducts Co., Parsippany, N.J.). The anti-factor Xa activity was measured against the World Health Organization First International Standard for low-molecular-weight heparins (National Institute for Biological Standards and Control, Potters Bar, England). The conversion factors obtained were

\[
1 \text{ anti-factor Xa Institute Choay unit (CY216)} = 0.56 \text{ anti-factor Xa IU}
\]

\[
1 \text{ USP unit (Calciparin)} = 1.35 \text{ anti-factor Xa IU}
\]

Before drug administration, a basal bleeding time was measured in the ear by a standardized method. It was repeated in the other ear, 10 minutes after the initial bolus and the start of infusion of heparin or placebo.

Statistical Analysis

Analysis was done on a per-animal basis rather than by arterial segment because data from different segments in the same animal could not be assumed to be independent. Values are reported as mean±SEM. Group means for platelet and fibrinogen deposition were compared between treatment groups by analysis of covariance to adjust for the area of injury. Separate analysis was performed on segments with deep and superficial injury. Analysis of variance was used to test for a dosage effect on the aPTT, thrombin time, and anti-factor Xa activity. A Mantel-Haenszel \( \chi^2 \) test was used to test for a difference in the incidence of mural thrombus formation between treatment groups receiving low-molecular-weight heparin. Results were considered significant if \( p<0.05 \).

Results

Baseline characteristics of each of the treatment groups are shown in Table 1. The groups were well matched, apart from one CY216 group consisting of all male pigs. We have previously demonstrated no difference between male and female pigs in platelet or fibrinogen deposition in this animal model (J.H. Chesebro et al, unpublished data). Heart rate and blood pressure were stable throughout the procedure. There were no deaths or bleeding complications.

Platelet and Fibrinogen Deposition

Table 2 lists the number of segments with deep or subendothelial injury and the deposition of platelets and fibrinogen/fibrin for each type of injury by treatment group. There were no significant differences between different treatments aimed at reducing platelet deposition after deep arterial injury. Platelet deposition on areas of subendothelial injury was low and similar in all groups. Fibrinogen deposition was significantly reduced in animals that were given unfractionated heparin compared with placebo (\( p=0.01 \)). CY216 did not significantly reduce fibrinogen/fibrin deposition. On areas of subendothelial injury, fibrinogen deposition was very low and not reduced with any treatment.

Mural Thrombosis

In animals with one or more deeply injured arterial segments, macroscopic mural thrombosis was present in five of eight (63%) treated with placebo. CY216 produced a dose-dependent reduction in thrombus formation; at the highest dosage, only one of seven (14%) pigs had macroscopic thrombus, which was the same as in unfractionated-heparin–treated animals (Figure 1). When analyzed by arterial segments with deep injury rather than on a per-animal basis, there were thrombi in 74%, 45%, 30%, 14%, 5%, and 9% of the placebo, the 100, 200, 400, 500 units/kg CY216, and the 100 units/kg unfractionated-heparin groups, respectively (Table 2, \( p=0.02 \)). No arterial segment with subendothelial injury had mural thrombosis.
Laboratory Data

The anti-factor Xa activity for each drug dosage changed little during the procedure. The anti-factor Xa activity of unfractionated heparin was not significantly different from 200 units/kg CY216 at three of four time points and was significantly less than that at 400 or 500 units/kg CY216 (p<0.05, Figure 2a). The aPTT and thrombin time also were stable for each drug dosage throughout the procedure. Animals in the unfractionated-heparin group had the greatest prolongation of the aPTT (three times basal) and thrombin time (six to seven times basal), which was similar to those treated with 500 units/kg CY216 (Figures 2b and 2c).

Basal antithrombin III plasma concentration was normal in all groups and was unchanged at the end of the study (group means ranged from 87% to 98% thrombin inhibition). No drug effect was observed on the platelet count, fibrinogen level, or hematocrit.

Measurements of bleeding time are depicted in Figure 3. The bleeding time increased slightly and to a similar degree in many pigs. Only one pig (receiving 200 units/kg CY216) had a marked increase after drug administration. Unfractionated heparin was similar to CY216. There was no apparent dose-response effect of CY216 on the bleeding time.

Discussion

This study demonstrates that platelet thrombus formation after arterial injury by balloon dilatation is only moderately reduced by drugs with predominant anti-factor Xa activity. We evaluated dosages of low-molecular-weight heparin CY216 with a wide range of anti-factor Xa activity. Platelet deposition and the incidence of mural thrombosis were similar in pigs treated with 500 units/kg CY216 and 100 units/kg unfractionated heparin despite a significantly higher anti-factor Xa activity with the former. The anti-factor Xa activity of 100 units/kg unfractionated heparin was similar to that of 200 units/kg CY216; animals treated with 200 units/kg CY216 had greater platelet deposition and were more likely to have mural thrombosis (Figures 1 and 2 and Table 2), although these differences did not reach statistical significance. The prolongation of the aPTT was similar in animals treated with 500 units/kg CY216 and 100 units/kg unfractionated heparin. Therefore, it appears that the antithrombin activity of both types of heparin is the main determinant of reduced platelet thrombus deposition in deep arterial injury. This is consistent with previous observations of the same animal model. High dosages of unfractionated sodium heparin reduced platelet deposition and mural thrombosis in a dose-related manner.16-17-23

Pieters and Lindhout6 have demonstrated that the amount of factor Va rather than factor Xa is critical for the formation of thrombin. Small amounts of thrombin are sufficient to convert factor V to factor Va; factor Va, with factor Xa and Ca²⁺ on a phospholipid membrane, form the prothrombinase or activator complex, which accelerates thrombin generation up to 300,000 times that of factor Xa alone.24 Potent and specific thrombin inhibition, which blocks this positive-feedback loop, is extremely effective antithrombotic therapy. In this same animal model, recombinant hirudin

Table 2. Platelet and Fibrinogen/Fibrin Deposition by Treatment Group in Arterial Segments With Deep and Subendothelial Injury

<table>
<thead>
<tr>
<th>Dosage of low-molecular-weight heparin</th>
<th>UFH 100 U/kg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Placebo</td>
<td>100 U/kg</td>
</tr>
<tr>
<td>Pigs (n)</td>
<td>8</td>
</tr>
<tr>
<td>Deep arterial injury</td>
<td></td>
</tr>
<tr>
<td>Segments (No.)</td>
<td>19</td>
</tr>
<tr>
<td>Platelets (x10⁹/cm²)</td>
<td>42±28</td>
</tr>
<tr>
<td>Mural thrombi (percentage of segments)*</td>
<td>74</td>
</tr>
<tr>
<td>Fibrinogen (x10⁹/cm²)</td>
<td>35±8</td>
</tr>
<tr>
<td>Subendothelial injury</td>
<td></td>
</tr>
<tr>
<td>Segments (No.)</td>
<td>13</td>
</tr>
<tr>
<td>Platelets (x10⁹/cm²)</td>
<td>4±1</td>
</tr>
<tr>
<td>Fibrinogen (x10⁹/cm²)</td>
<td>10±3</td>
</tr>
</tbody>
</table>

Values are mean±SEM.
U, units.
*p=0.02 for differences between groups receiving low-molecular-weight heparin; †p=0.01 compared with placebo.
reduced platelet deposition to a single layer and totally prevented mural thrombosis at dosages that prolonged the aPTT two to three times the control level.16,17 The reduction in fibrinogen/fibrin deposition with fractionated and unfractionated heparin paralleled the changes in platelet deposition. Only unfractionated-heparin-treated animals had significantly lower fibrinogen/fibrin deposition than did those treated with placebo; a greater anti-factor Xa effect but a lesser antithrombin activity was associated with a lesser therapeutic effect. In this study, the dosage of unfractionated heparin that prolonged the aPTT three times basal reduced fibrinogen/fibrin deposition but did not significantly affect platelet deposition. In previous studies, unfractionated sodium heparin at a dosage that prolonged the aPTT to twice that of control also reduced fibrinogen/fibrin but not platelet deposition compared with placebo.17

There are limited data on low-molecular-weight heparin subtractions in other experimental models of arterial thrombosis. One heparin subtraction (PK10169) reduced thrombus formation in a canine model of coronary thrombosis induced by electrical injury.25 Another subtraction (CY222) reduced platelet deposition onto an arteriovenous shunt coated with collagen in the baboon.26 For a similar antithrombotic effect, unfractionated heparin caused greater prolongation of the coagulation time and bleeding time. In our study, there was neither an apparent difference between CY216 and unfractionated heparin nor a dose-response effect of CY216 in the prolongation of the bleeding time.

Low-molecular-weight heparin requires binding to antithrombin III as a cofactor to inhibit anti-factor Xa. This larger complex probably does not gain access to factor Xa bound to phospholipid membranes, as in the prothrombin activator (or prothrombinase) complex.27 Thus, the low efficacy of low-molecular-weight heparin
as an antithrombotic agent may not mean that factor Xa inhibition is ineffective in preventing arterial thrombosis because of limited access of the drug to membrane-bound factor Xa. Because factor Xa is generated more proximally in the coagulation cascade, effective factor Xa inhibition should reduce thrombin generation. Therefore, more specific and lower-molecular-weight inhibitors of factor Xa, such as antistasin from the Mexican leech28 and tick (Ornithodoros moubata) anticoagulant peptide,29 may better test this concept.

In conclusion, low-molecular-weight heparin CY216 was no more effective than unfractionated heparin in the porcine model of platelet-rich arterial thrombosis. At dosages that would usually be considered for clinical practice, CY216 did not significantly reduce platelet or fibrinogen/fibrin deposition in injured arteries. High dosages, producing antithrombin effects similar to those of unfractionated heparin, are needed for arterial thrombosis. These findings provide insight into the mechanisms of arterial thrombosis, lending further support for the central role of thrombin in this process.

Acknowledgments

We thank Institute Choay for supplying the low-molecular-weight heparin (CY216) and Marcia Simonson and Amy Pelot for their secretarial assistance.

References


Key Words • heparin • low-molecular-weight heparin • arterial thrombosis • thrombin • factor X
Antithrombotic efficacy of low-molecular-weight heparin in deep arterial injury.
M Heras, J H Chesebro, M W Webster, J S Mruk, D E Grill and V Fuster

doi: 10.1161/01.ATV.12.2.250
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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