Intimatan Prevents Arterial and Venous Thrombosis in a Canine Model of Deep Vessel Wall Injury

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ABSTRACT

Resistance of fibrin-bound thrombin to inactivation by the heparin/antithrombin III complex is considered a limitation in the use of heparin as an antithrombotic agent. Intimatan (dermatan sulfate) is a heparin cofactor II agonist that inhibits both free and bound forms of thrombin. The present study examines the hypothesis that Intimatan prevents thrombotic occlusion in response to vascular wall injury in a canine model of arterial/jugular vein thrombosis. The left carotid artery and right jugular vein served as vehicle-treated control vessels, whereas the right carotid artery and left jugular vein were subjected to electrolytic injury after administration of Intimatan (9 mg/kg bolus + 300 μg/kg/min infusion, i.v.) or dalteparin (Fragnmin) (400 IU/kg, s.c.). Intimatan significantly increased time to carotid artery (226.0 ± 14.0 min) and jugular vein (240.0 ± 0.0 min) thrombosis, compared with control vessels (carotid artery, 87.1 ± 7.9 min; jugular vein, 60.6 ± 7.4 min). Vessel patency was maintained in eight of eight jugular veins and seven of eight carotid arteries during treatment with Intimatan. Dalteparin significantly increased time to carotid artery thrombosis (122.1 ± 17.5 min) compared with control (64.3 ± 8.2 min), but did not change the time to thrombosis in the jugular vein. Only one carotid artery remained patent at the end of the dalteparin protocol. The two drugs produced minimal increases in bleeding times, and Intimatan increased the activated partial thromboplastin time above that observed with dalteparin. The results demonstrate that Intimatan is effective in preventing occlusive arterial and venous thrombosis in an experimental model of deep vascular wall injury.

Surface-bound thrombin is resistant to inhibition by the heparin/antithrombin III complex, thereby limiting the efficacy of heparin in arterial vessel wall disease (Hogg and Jackson, 1989; Weitz et al., 1990; Hogg et al., 1996; Becker et al., 1999). Bound thrombin which remains resilient at the site of injury after heparin treatment may then perpetuate thrombin generation via a feedback loop to promote rethrombosis (Kumar et al., 1994). In contrast to the heparin/antithrombin III complex which inhibits various serine proteases of the coagulation cascade, the activated form of heparin cofactor II is specific for thrombin and is also an efficient inhibitor of surface-bound thrombin (Liaw et al., 2001). As such, improved agonists of heparin cofactor II may present effective tools to elucidate more completely the role of thrombin in the promotion of vessel wall thrombosis and provide a novel therapeutic approach for the treatment of the thromboocclusive disorders. Heparin cofactor II activity is accelerated by dermatan sulfate (Tollefsen et al., 1983). In contrast to the heparin template-assisted assembly of the thrombin-antithrombin complex, the activation of heparin cofactor II occurs via an allosteric mechanism that involves a change in conformation associated with enhanced anti-thrombin activity (Liaw et al., 1999). Despite a favorable pharmacological profile, including less anticoagulant activity than heparin and an increased venous antithrombotic action (Desnoyers et al., 1989) the clinical use of dermatan sulfate has been limited by its low potency and solubility (Boneu et al., 1992).

Intimatan is an improved heparin cofactor II agonist that inhibits the bound conformation of thrombin and provides a sustained inhibition of vessel wall thrombogenicity in rabbit models of vascular injury (Buchanan and Brister, 2000; Buchanan et al., 2001). The present study examines whether Intimatan effectively prevents occlusive thrombus formation in response to vascular wall injury in a canine model of occlusive arterial/venous thrombosis. Experiments were designed to assess the efficacy of Intimatan to prevent or reduce the incidence of carotid artery and jugular vein thrombus formation in response to deep vessel wall injury. The results of the study demonstrate that Intimatan prevents both oc-
clusive arterial and venous thrombosis in the dog at doses that minimally affect the bleeding time.

Materials and Methods

Guidelines for the Use and Care of Experimental Animals

The procedures used in this study are in accordance with the guidelines of the University of Michigan University Committee on the Use and Care of Animals and conform to the standards in The Guide for Care and Use of Laboratory Animals (National Institutes of Health publication no. 86-23). Veterinary care was provided by the University of Michigan Unit for Laboratory Animal Medicine.

Reagents

Intimatan was supplied by Celsus Laboratories, Inc. (Cincinnati, OH) and dissolved in 0.9% sodium chloride solution for injection (saline). Dalteparin (Fragmin) was purchased from the University of Michigan Hospital Pharmacy as formulated for clinical use. All other reagents were purchased from Sigma-Aldrich (St. Louis, MO).

Model of Arterial and Venous Occlusion

Fifteen purpose-bred beagle dogs, weighing 9 to 13 kg, were anesthetized with sodium pentobarbital (30 mg/kg, i.v.), intubated, and ventilated with room air using a Harvard respirator (Harvard Apparatus, Holliston, MA), adjusted to deliver a tidal volume of 30 ml/kg at a frequency of 12 breaths/min. A catheter was inserted into the right femoral vein for drug administration. Blood pressure was recorded from the right femoral artery using a Stratham Transducer (Gould Inc., Cardiovascular Products, Oxnard, CA). A standard limb lead II electrocardiogram was recorded continuously to monitor heart rate. A carotid artery and jugular vein were isolated, and each was fitted with Transonic ultrasonic flow probes (Transonic Systems, Inc., Ithaca, NY) for continuous recording of blood flow. Recordings of blood pressure, lead II electrocardiogram, mean, and phasic blood pressure were obtained on a Grass model 7 polygraph recorder. Recordings of heart rate, blood pressure, carotid artery, and jugular vein thrombosis. Hemodynamic parameters including heart rate, blood pressure, carotid artery, and jugular vein blood flow were monitored throughout the protocol.

Experimental Protocol

For each experiment, the left carotid artery (LCA) and right jugular vein (RJV) served as control vessels, while the right carotid artery (RCA) and left jugular vein (LJV) served as drug-treated vessels. Animals were allowed 30 min to stabilize while the RCA and LJV were isolated and prepared for induction of electrolytic injury. Figure 1 illustrates the experimental protocol in detail. Before initiation of electrolytic injury in control vessels, animals were treated with either an intravenous “loading dose” and intravenous infusion of saline (Intimatan control) or a subcutaneous injection of saline (dalteparin control). Electrolytic injury was initiated in the LCA and RJV 10 min after the start of the infusion in the Intimatan group (9 mg/kg bolus + 300 µg/kg/min infusion, i.v.) and 2 h after the subcutaneous injection in the dalteparin group (400 IU/kg, s.c.). The optimal dose of Intimatan required to increase the time to thrombosis was determined in preliminary studies. The only parameter used to determine the optimal bolus and i.v. infusion for Intimatan was the inhibition of arterial and venous thrombosis. We began with an initial dose of 3 mg/kg followed by an infusion of 30 µg/kg/min. This dosing regimen was based upon preliminary data in the pig (unpub-

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**Fig. 1.** Diagrammatic representation of the experimental protocol used to investigate the effects of Intimatan and dalteparin on carotid artery and jugular vein thrombosis. Hemodynamic parameters including heart rate, blood pressure, carotid artery, and jugular vein blood flow were monitored throughout the protocol.

* Assessment of bleeding time, aPTT and platelet aggregation
lished data, Celsus Laboratories). Unfortunately, we did not observe inhibition of thrombosis with this dose. The dose was increased progressively in three separate experiments, and a final dosing regimen of 9 mg/kg bolus followed by an infusion of 300 μg/kg/min was established. The predetermined optimal dose of Intimatan was used throughout the study.

The dose of dalteparin used in this study was adjusted from the recommended human clinical dose used for treatment of venous thrombosis (70 IU/kg). The pharmacokinetics of dalteparin in humans and dogs are similar (Grebe et al., 2000). The bioavailability of dalteparin after subcutaneous injection is 100% in beagles and 90% in humans (Grebe et al., 2000). Preliminary studies in the dog did not produce inhibition of either arterial or venous thrombosis with 70 IU/kg bolus; additional pilot studies were done in an effort to achieve an antithrombotic effect with dalteparin. At 400 IU/kg, a dose well above that used clinically, we observed an increase in time to thrombosis; however, complete inhibition of thrombosis was not observed. All remaining experiments were performed with 400 IU/kg dalteparin. The subcutaneous route of administration was selected since this is how the drug is administered clinically for prevention of venous thrombosis. In preliminary studies with dalteparin, electrolytic injury was initiated 30 min after subcutaneous injection, and no drug effect was observed. When the time from injection was extended to 2 h, time to thrombosis was extended, indicating an effect with dalteparin.

If thrombotic occlusion developed in less than 3 h, the current and infusion (Intimatan only) were discontinued after 30 min of zero blood flow. If occlusive thrombosis did not occur and blood flow persisted after 3 h of electrolytic stimulation, the current and infusion (Intimatan only) were discontinued and vessel patency was monitored for an additional 2 h.

Hematologic Determinations

Ex Vivo Platelet Aggregation Studies. Blood was taken for platelet aggregation studies at baseline and at specific time points, as indicated in Fig. 1. Venous blood (10 ml) was withdrawn from the right femoral vein into a plastic syringe containing 3.7% sodium citrate as the anticoagulant [1:10 citrate to blood (v/v)]. Platelet-rich plasma (PRP) was obtained by collecting the supernatant from whole blood centrifuged at 140 g for 5 min. Platelet-poor plasma was prepared from the same blood sample by further centrifugation at 2000g for 10 min. Ex vivo platelet aggregation was assessed at 37°C with a four-channel platelet aggregometer (Bio-Data-PAP-4; Bio-Data, Hatboro, PA) by recording the increase in light transmission through a stirred suspension of PRP adjusted to 200,000 platelets/μL. Aggregation was induced with arachidonic acid (AA, 0.65 mM), ADP (20 μM), and γ-thrombin (25 nM). A subaggregatory concentration of epinephrine (550 nM) was used to prime the platelets before the agonists were added. Values are expressed as percentage of aggregation, representing the percentage of light transmission standardized to PRP and platelet-poor plasma samples yielding 0% and 100% light transmission, respectively.

Tongue Bleeding Times. Bleeding times were determined with the use of a SurgiCut device, which makes a uniform incision 5 mm long and 1 mm deep on the upper surface of the tongue. The tongue lesion was blotched with a filter paper every 20 s until the transfer of blood to the filter paper was no longer apparent. The interval, from the time of the tongue incision until the time that blood is no longer transferred to the filter paper, was recorded as the “tongue bleeding time”.

Activated Partial Thromboplastin Time (aPTT). aPTT is a measure of the intrinsic coagulation pathway that involves all the coagulation factors, except factors VIII and VII. The aPTT determinations were performed using 2 ml of citrated whole blood [1:10 citrate/blood (v/v)] injected into OneStep aPTT tubes placed in a Hemochron whole blood coagulation instrument (International Technidyne Corp., Edison, NJ), and time to coagulation was determined automatically.

Statistical Analysis

Data are expressed as mean ± S.E. for all experiments. Comparisons between heart rate and mean arterial blood pressure were performed using a one-way ANOVA followed by Student-Newman-Keuls multiple comparison test. Comparisons between the incidence of occlusion in saline and drug-treated vessels were performed using a χ² test. Changes in time to thrombosis between saline and drug-treated vessels were carried out using paired t tests. Differences in time to thrombosis between Intimatan- and dalteparin-treated animals were made using Student’s t test. Platelet aggregation values, bleeding times, and aPTT values were compared with respective baseline using a one-way ANOVA followed by Dunnett’s post hoc test. All results were considered significant when p < 0.05.

Results

Hemodynamic Data. Table 1 summarizes the systemic hemodynamic data for animals treated with Intimatan or dalteparin. Heart rate (HR) and mean arterial blood pressure (MABP) were unchanged from their respective baseline in each group studied.

Carotid Artery and Jugular Vein Thrombosis. Electrolytic injury to the carotid artery and jugular vein resulted in specific time points, that progressed to form an occlusive thrombus and cessation of blood flow in all control vessels. As shown in Table 2, left carotid artery and right jugular vein time to thrombosis averaged 87.1 ± 7.9 min and 60.6 ± 7.4 min, respectively, in the Intimatan control group (animals treated with saline, i.v.). In the dalteparin control group (animals treated s.c. with saline), time to thrombosis averaged 64.3 ± 8.2 min in the left carotid artery and 70.3 ± 9.8 min in the right jugular vein. Intravenous administration of Intimatan (9 mg/kg bolus + 300 μg/kg/min infusion) prevented the development of occlusive thrombi in seven of eight right carotid arteries and eight of eight left jugular veins. After 3 h of electrolytic injury, Intimatan infusion and anodal current delivery to the right carotid artery and left jugular vein were terminated. Vessel patency was monitored for an additional hour. Vessels that were patent at the time of drug and current cessation (180 min) continued to maintain blood flow at 240 min, at which time the protocol was concluded. Despite continued arterial or venous blood flow in the patent injured (seven of eight arterial and eight of eight venous) vessels of the Intimatan-treated group, time to thrombosis was recorded as 240 min at the termination of the experiment for the purpose of statistical analysis (see Table 2).

In dalteparin-treated animals (400 IU/kg, s.c.), occlusive thrombosis was prevented in all vessels. At 90 min after the termination of dalteparin infusion, time to thrombosis was the same as saline control (156.8 ± 25.4 min; Table 2). Mean arterial blood pressure was not affected by Intimatan or dalteparin treatment (Table 1). There were no differences in hemodynamic data for animals treated with Intimatan or dalteparin. Heart rate (HR) and mean arterial blood pressure (MABP) were unchanged from their respective baseline in each group studied.

<table>
<thead>
<tr>
<th></th>
<th>HR</th>
<th>MABP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baseline</td>
<td>156.8 ± 8.5</td>
<td>134.4 ± 14.2</td>
</tr>
<tr>
<td>70 min saline</td>
<td>152.8 ± 10.0</td>
<td>138.8 ± 12.4</td>
</tr>
<tr>
<td>70 min Intimatan</td>
<td>142.5 ± 12.5</td>
<td>129.8 ± 14.9</td>
</tr>
<tr>
<td>130 min Intimatan</td>
<td>140.5 ± 11.7</td>
<td>126.9 ± 14.6</td>
</tr>
<tr>
<td>Dalteparin</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>135.3 ± 5.7</td>
<td>132.1 ± 9.5</td>
</tr>
<tr>
<td>70 min saline</td>
<td>145.3 ± 5.8</td>
<td>134.3 ± 9.7</td>
</tr>
<tr>
<td>70 min dalteparin</td>
<td>144.9 ± 6.8</td>
<td>142.9 ± 11.5</td>
</tr>
<tr>
<td>130 min dalteparin</td>
<td>136.0 ± 8.1</td>
<td>140.7 ± 11.8</td>
</tr>
</tbody>
</table>

TABLE 1

Effect of Intimatan (9 mg/kg bolus + 300 μg/kg/min infusion, i.v.) and dalteparin (400 IU/kg, s.c.) on HR and MABP in anesthetized dogs

Data represent mean ± SE for n = 8 with Intimatan and n = 7 with dalteparin.
thrombus formation was prevented in only one right carotid artery. All remaining right carotid arteries (six of seven) and left jugular veins (seven of seven) progressed to form an occlusive thrombus in the presence of dalteparin. Although dalteparin did not inhibit the development of an occlusive thrombus, it did prolong the time to thrombosis significantly in the right carotid artery (122.1 ± 17.5 min), compared with saline-treated controls (64.3 ± 8.2 min). No significant change in time to thrombosis was observed in the left jugular vein after treatment with dalteparin.

The effects of Intimatan and dalteparin on blood flow are summarized in Table 2 and shown graphically in Figs. 2 and 3. Although Intimatan prevented the development of occlusive thrombosis, there was a decrease in right carotid artery and left jugular vein blood flow during the 3 h of electrolytic injury (see Fig. 2). Dalteparin did not prevent occlusive thrombosis; thus, the decrease in blood flow during electrolytic injury approached that of the vehicle-treated controls (see Fig. 3).

Hematologic Measurements. Ex vivo platelet aggregation in response to AA (0.65 mM), ADP (20 μM), and γ-thrombin (25 nM) was determined before and after administration of Intimatan or dalteparin in all animals studied. As shown in Fig. 4, Intimatan decreased significantly platelet responses to γ-thrombin at 70 and 130 min after infusion and to ADP 70 min after initiating the loading dose and infusion. Ex vivo aggregation responses to ADP were also reduced significantly at 130 min after drug administration. Both Intimatan and dalteparin significantly increased aPTT above the respective baseline values at 1 and 2 h after drug treatment (see Fig. 6).

The effects of Intimatan and dalteparin on tongue bleeding time are summarized in Fig. 7. Both drugs produced only limited increases in bleeding time that were not statistically significant from baseline.

Table 2

Incidence of carotid artery and jugular vein occlusion and time to thrombosis after treatment with Intimatan (9 mg/kg bolus + 300 μg/kg/min infusion, i.v.) and dalteparin (400 IU/kg, s.c.)

<table>
<thead>
<tr>
<th>Incidence of CA Oclusion</th>
<th>CA Time to Thrombosis</th>
<th>Incidence of JV Oclusion</th>
<th>JV Time to Thrombosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>CA 8/8 (100%)</td>
<td>87.1 ± 7.9</td>
<td>JV 8/8 (100%)</td>
<td>60.6 ± 7.4</td>
</tr>
<tr>
<td>Intimatan i.v. 1/8 (12.5%)**</td>
<td>226.0 ± 14.0**</td>
<td>JV 0/8 (0%)**</td>
<td>240.0 ± 0.0**</td>
</tr>
<tr>
<td>Saline s.c. 7/7 (100%)</td>
<td>64.3 ± 8.2</td>
<td>JV 7/7 (100%)</td>
<td>70.3 ± 9.8</td>
</tr>
<tr>
<td>Dalteparin s.c. 6/7 (86%)</td>
<td>122.1 ± 17.5*</td>
<td>JV 7/7 (100%)</td>
<td>91.9 ± 12.2</td>
</tr>
</tbody>
</table>

CA, carotid artery; JV, jugular vein.
* Significant difference from respective saline control.
** Significant difference between Intimatan and dalteparin (p < 0.05).

Discussion

This study demonstrates that Intimatan is an effective antithrombotic agent in preventing formation of arterial and venous thrombi in response to deep vessel wall injury in the
dog. Furthermore, the antithrombotic effects of Intimatan were achieved with only a minimal increase in bleeding time. Dalteparin, a clinically approved glycosaminoglycan with inhibitory effects on factor Xa and thrombin, had little or no effect on arterial and venous thrombosis in our model.

Thrombin is produced predominately on the surface of circulating platelets by the proteolytic activation of prothrombin (Fenton, 1986). When tissue damage occurs, factor VII is activated, which activates factor X, which in turn binds activated Factor V on the platelet membrane surface to form the prothrombinase complex (see Rosenburg and Bauer, 1994 for review). Membrane-bound prothrombinase catalytically activates prothrombin. Thrombin modulates thrombus formation by activating platelets, converting fibrinogen to fibrin, and by activating blood coagulant factors V, VII, and VIII, thereby promoting systemic hypercoagulation and increased vessel wall thrombogenicity (see Schafer, 1994 for review). Thrombin activated at distant sites in the systemic circulation also participates in thrombus formation. Two plasma protease inhibitors, antithrombin III and heparin...
cofactor II, regulate thrombin activity. Heparin cofactor II combines with thrombin at exosite I on the enzyme surface to form a complex in which the catalytic site is blocked (Becker et al., 1999). Intimatan targets the vessel wall and fibrin clots, then catalyzes the inhibition of surface-bound thrombin by specific activation of heparin cofactor II (Buchanan and Brister, 1998, 1999, 2000). The prevention of arterial and venous thrombosis by Intimatan supports the hypothesis that inhibition of surface-bound thrombin represents an important pharmacological target for achieving an effective antithrombotic effect. Intimatan increased aPTT, along with an anticipated, albeit modest, increase in tongue bleeding time. The latter observations may be significant in that Intimatan was effective in preventing fibrin-dependent venous thrombosis, while at the same time having a beneficial effect in modulating platelet-dependent arterial thrombus formation.

Preclinical studies suggest that Intimatan is more effective than heparin as an inhibitor of thrombin generation in a pig model of cardiopulmonary bypass, of neointimal hyperplasia in balloon-injured rabbit aortae, and following injury to the carotid artery (Brister et al., 1996; Schwartz et al., 1998; Yang et al., 1999). It was also determined that Intimatan can influence activated protein C activity (Fernandez et al., 1999).

Subcutaneous low molecular weight heparins such as dalteparin are effective alternatives to intravenous unfractionated heparin for treatment of venous thrombosis (Koopman et al., 1996; Levine et al., 1996). The low molecular weight heparins have the added advantage of use outside of the hospital setting without the need for laboratory monitoring. Results of clinical studies, however, demonstrate that dalteparin is only as effective as heparin (Lopaciuk et al., 1999; Koopman et al., 1996; Levine et al., 1996). In this study the dose of dalteparin (400 IU/kg, s.c.) was approximately 5 times greater than the recommended human clinical dose (70 IU/kg). Dalteparin altered ex vivo platelet reactivity and produced a modest increase in tongue bleeding time, but failed to prevent occlusive thrombosis; despite having been administered in what may be viewed as an excessive dose. The apparent lack of efficacy may be related to the experimental model in which the extent of electrocytic injury results in deep vessel wall injury. In the same experimental model, Intimatan, derived from chemical modification of dermatan sulfate, was observed to prevent both venous and arterial thrombus formation. Dermatan sulfate has been suggested to promote fibrinolytic activity by inducing the release of tissue plasminogen activator from endothelial cells (Abbadini et al., 1987). Whether Intimatan possesses a similar action is not known. Thus, further investigation into the profibrinolytic effects and antithrombotic effects of Intimatan is warranted.

References