Microvascular thrombosis is a major cause of organ damage in Shiga toxin-mediated hemolytic uremic syndrome (Stx-HUS). In vitro and clinical studies implicate thrombin-mediated mechanisms in the pathogenesis of Stx microvascular thrombosis. In a greyhound model, administration of 0.03 μg/kg to 0.05 μg/kg Stx1 or Stx2 causes severe bloody diarrhea and HUS with microvascular thrombosis requiring humane euthanasia within 65 hours. Using a greyhound model of Stx-HUS we analyzed early hemostatic changes, and tested the hypothesis that thrombin blockade with lepirudin would prevent lethal Stx effects. Two Stx1-exposed greyhounds were analyzed for hemostatic changes prior to onset of clinical manifestations. Serial hemostasis studies after Stx1 challenge revealed trends of increased aPTT, fibrinogen levels, and prothrombin fragment 1+2, and appearance of abnormally large von Willebrand factor multimers. Three greyhounds were anticoagulated with lepirudin to maintain activated partial thromboplastin times (aPTT) >2.5-fold normal, followed by administration of Stx2 and observation of clinical responses. Among the 3 lepirudin-treated, Stx2-challenged greyhounds, one developed severe illness requiring euthanasia. Remarkably, 2 of the 3 greyhounds developed only hypersalivation and restlessness that resolved (P <.03 compared to 14 historical controls). These two greyhounds were clinically, hematologically and biochemically normal 74 hours after Stx administration, well beyond the time of euthanasia of any previous greyhound. This study suggests that greyhounds exposed to Stx develop procoagulant changes similar to humans, and that thrombin may be a critical factor in the pathogenesis and treatment of Stx-HUS.

Keywords
Shiga toxin, lepirudin, thrombotic microangiopathy, procoagulant.

Microvascular thrombosis is a critical pathological event in Shiga toxin-associated hemolytic uremic syndrome (Stx-HUS). Microvascular thrombosis may result from endothelial injury mediated by Stx and inflammatory factors. Procoagulant effects of these pathogenic factors include downregulation of anticoagulant and profibrinolytic factors and induction of thrombin-generating tissue factor (1-4).

Microvascular thrombi in Stx-HUS contain a conspicuous component of fibrin (5-7). Fibrin polymerization requires thrombin activity, which directly implicates thrombin-mediated pathways in Stx-HUS pathogenesis. Studies of Stx-HUS patient plasma and tissue samples demonstrate consistent evidence of thrombin-mediated coagulation (7-11). These observations suggest that control of thrombin activity could be a key strategy in preventing and treating Stx syndromes.
Hirudins are proteins derived from the medicinal leech that have potent anticoagulant activity (12). Hirudins bind thrombin in 1:1 complexes and block both binding to substrate and the enzymatic active site. Hirudins appear to have singular affinity for, and inhibit all known hemostatic effects of thrombin, including fibrin polymerization, platelet activation, and hemostatic factor activation (12). Lepirudin (Refludan\textsuperscript{®}), is a recombinant form of hirudin approved by the Food and Drug Administration for use in humans as an anticoagulant.

This study utilized a canine model of Stx-HUS that derives from a naturally occurring epidemic disease of kenneled greyhound dogs, associated with exposure to Stx-producing Escherichia coli (E. coli) through the practice of including non-food grade ground beef in the diet (13). In the natural disease, greyhounds present with acute subcutaneous edema and multifocal well demarcated cutaneous ulcers in association with venous thrombosis. Severely affected dogs develop precipitous thrombocytopenia (<20,000 platelets/µl) and microangiopathic hemolytic anemia (Fig. 1) followed by acute renal failure and death from uremia. Glomerular ultrastructural changes resemble those in human HUS, including sequential membrane derangement, necrosis, and detachment of glomerular capillary endothelium, erythrocyte congestion and adhesion of platelets to exposed basement membranes (Fig. 2). As in humans, coagulopathy indicative of disseminated intravascular coagulation is not present in canine E. coli-associated HUS (14).

Previous experiments developed a reproducible Stx-HUS model by injecting healthy greyhounds with purified Stx. In the direct Stx-challenge model, dogs injected with 0.03 µg/kg to 0.05 µg/kg purified Stx1 or Stx2 invariably develop severe bloody diarrhea and a HUS-like illness within 48 to 52 hours. Development of HUS is preceded by a precipitous decline in platelet count and microangiopathic anemia. Renal failure and thrombotic microangiopathy occur late in the time course. Ultrastructural studies reveal microvascular lesions in colon and kidney identical to those observed in the natural disease. At these doses of Stx the illness is uniformly fatal by 65 hours, requiring humane euthanasia. The LD100 dose range of Stx1 or Stx2 (0.03 to 0.05 µg/kg) was demonstrated in 14 previous greyhound experiments in which all of 14 greyhounds exposed to Stx1 or Stx2 dosages in the above range required humane euthanasia within 65 hours. The specificity of the pathogenic effect of Stx was demonstrated when administration of heat-denatured Stx had no pathogenic effect, and pre-treatment of Stx-challenged greyhounds with plasma from Stx-immunized dogs prevented HUS. Stx1 and Stx2 have indistinguishable clinical effects in the greyhound model.(15) Several other breeds were demonstrated to be susceptible to similar clinical effects of Stx at varying doses. Greyhounds are ideal for clinical studies because of the ease of venous access and their docile nature, which allows easy handling and clinical observation.

In this study we report preliminary experiments in two greyhounds challenged with Stx in which we observed hemostatic changes consistent with thrombin activation. In addition, in two healthy greyhounds we demonstrated that lepirudin anticoagulated plasma in vitro, and prolonged clotting times in vivo. We therefore hypothesized that thrombin activity may be critical in Stx-HUS, and that blockade of thrombin activity with lepirudin would prevent lethal Stx effects. We tested our hypothesis by anticoagulating three greyhounds with lepirudin followed by Stx challenge.
Materials and methods

Coagulation studies in two Stx-challenged greyhounds

Preliminary experiments determined the effect of Stx1 on hemostasis parameters in two greyhounds. Two adult greyhounds were administered 0.03 µg/kg Stx1 (gift of David Acheson, Tufts New England Medical Center) intravenously, and serial sodium citrate anticoagulated blood samples were obtained at time points up to 48 hours. Samples were obtained from a central venous catheter and immediately centrifuged. Plasma was immediately frozen at −80°C until use. All samples were thawed and assayed concurrently using human clinical assay reagents. Samples were assayed on an MLA 900C instrument for prothrombin time (PT) using thromboplastin C reagent (Dade Behring), activated partial thromboplastin time (aPTT) using plateletin LS reagent (bioMérieux), fibrinogen concentration using Clauss method (Dade Behring), and prothrombin fragment 1+2 (f 1+2) by enzyme linked immunosorbant assay using Enzygnost F 1+2 microkit (Dade Behring). Von Willebrand factor (VWF) multimer analyses were performed by electrophoresis in 0.65% agarose with detection using a mixture of 9 125I-labeled monoclonal anti-VWF antibodies, as described previously (16). Data plots and linear regression analysis and 95% confidence intervals were performed using SigmaPlot version 7.0.

Lepirudin anticoagulation of two healthy greyhounds

To determine the in vitro anticoagulant effects of lepirudin in greyhound plasma, PT and aPTT were determined in plasma samples to which different concentrations of lepirudin (Refludan® - Berlex, Montville, NJ) were added.
Based on preliminary in vitro and in vivo dose response data, Refludan was given to healthy greyhounds at an initial dose of 5.0 mg/kg body weight by intravenous infusion over a period of 15 to 20 seconds, followed by a continuous intravenous infusion of 2.0 mg/kg using an infusion pump. The response to Refludan was monitored using the aPTT ratio. Base-line and sequential aPTT determinations were performed at 4-hour intervals after the start of the Refludan infusion. Refludan infusion was continued up to 60 hours. Four hours after stopping the infusion, aPPT ratios were again determined. Complete blood counts and serum chemistry values were determined prior to, once during, and daily for three days following the experimental period.

**Lepirudin treatment in Stx2-challenged greyhounds**

Three 20 to 30 kg healthy adult greyhounds (> 1 year old) each received intravenous bolus infusions of 5 mg/kg lepirudin followed by 2 mg/kg/hr continuous infusions to maintain aPTT levels >2.5 fold the upper limit of normal throughout the experiment. Emerging data indicated that Stx2 is most frequently associated with HUS in humans (17, 18). Therefore, in lepirudin treatment studies we utilized Stx2 at the upper range of LD100 dosage to maximally challenge the therapeutic intervention. Eight hours after beginning anticoagulation, each dog received an intravenous injection of 0.05 µg/kg purified Stx2 (gift of David Acheson). After Stx2 administration, greyhounds were monitored for clinical signs. Blood samples for laboratory studies were obtained at regular intervals. All greyhounds were humanely euthanized at the end of the experiment as required by the conditions approved by the institutional animal care and use committee. Survival outcomes of the 3 lepirudin treated greyhounds were statistically compared to 14 Stx1 or Stx2-exposed, non-treated, historical control greyhounds, using StatView for Windows (SAS Institute, Inc., Cary NC).

**Results**

**Coagulation studies in two greyhounds challenged with 0.03 µg/kg Stx1**

In the 48 hour time period after Stx1 challenge, prior to onset of any clinical symptoms, there was no significant change in PT (mean 4% increase) and a 4.4 second increase in mean aPTT (Fig. 3). Mean fibrinogen levels increased 39%, and prothrombin f 1+2 increased a maximum of 64%, then declined, consistent with a pre-symptomatic period of thrombin activation (Fig. 3). Linear regression analysis indicated trends of increased aPTT, prothrombin f 1+2 concentration, and fibrinogen concentration over the experimental time period. Despite variability between lanes, the overall intensity of VWF multimer staining increased from earlier to later time point in both dogs, suggestive of endothelial cell stimulation and VWF release (Fig. 4). Both dogs subsequently developed bloody diarrhea and manifestations HUS requiring humane euthanasia.

<table>
<thead>
<tr>
<th>[Lepirudin]</th>
<th>Human Plasma</th>
<th>Canine Plasma</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 mg/ml</td>
<td>28.8 sec</td>
<td>&lt;14.0 sec</td>
</tr>
<tr>
<td>0.25 mg/ml</td>
<td>&gt;300 sec</td>
<td>&gt;300 sec</td>
</tr>
<tr>
<td>0.025 mg/ml</td>
<td>79.1 sec</td>
<td>16.3 sec</td>
</tr>
</tbody>
</table>
Lepirudin anticoagulation in two healthy greyhounds

In samples of greyhound and pooled normal human plasma, concentrations of lepirudin were added, followed by aPTT measurements. Baseline greyhound aPTT measurements were markedly shorter than human, and less prolonged with lepirudin (Table 1). At high concentrations, lepirudin addition markedly prolonged both greyhound and human plasma. In healthy greyhounds infused with Refludan up to 60 hours, prolonged aPTT ratios of not less than 2.5-fold the upper limit of normal were maintained for 60 hours. No adverse clinical, hematologic, or biochemical alterations were noted. Greyhounds required much larger doses of lepirudin to achieve the same degree of anticoagulation as humans.

Effects of lepirudin in three greyhounds challenged with 0.05 µg/kg Stx2

Among three dogs anticoagulated with lepirudin followed by Stx2 challenge, one developed systemic symptoms approximately 48 hours after Stx2 challenge. Fulminant HUS evolved, necessitating humane euthanasia 53 hours after Stx challenge. In marked contrast to all previous experimental results, 2 of 3 greyhounds developed only minimal symptoms of hypersalivation and restlessness approximately 48 hours after Stx2 challenge. Symptoms resolved over several hours, and the greyhounds were clinically normal 21 hours after euthanasia of the non-protected animal (74 hours post Stx2 challenge). Samples were not obtained for coagulation studies. Fisher’s Exact test was used to compare 1 death and 2 survivors in the lepirudin-treated group to 14 deaths and 0 survivors in historical control non-protected animals. Fisher’s Exact test P-value = .0221. Although the number of lepirudin treated animals is small, the results suggest a statistically significant impact of lepirudin treatment on survival of Stx2 challenge in the greyhound model.

Discussion

In this pilot experiment, the blockade of thrombin activity with the specific thrombin inhibitor lepirudin markedly blunted clinical effects of an LD100 dose of Stx2 in 2 of 3 dogs. The dramatic attenuation of the effects of Stx2 by lepirudin in this study implicates a fundamental role of thrombin activity in the pathogenesis of Stx-HUS. The observed evidence of endothelial cell stimulation and thrombin activation in the presymptomatic period is remarkably consistent with recent studies of human Stx illness. A recent study of a large cohort of children with enteric E. coli O157:H7 infections demonstrated that development of HUS correlated significantly with higher levels of prothrombin f 1+2 in the prodromal period, consistent with a pathogenic role of thrombin activation (9). Other coagulation factors, including plasminogen activator inhibitor type 1, tissue plasminogen activator inhibitor, and plasmin-antiplasmin complexes were also elevated in children that developed HUS compared to those that did not. Similar results in other studies (19) strongly implicate thrombin inhibition in the pathogenesis of HUS microvascular thrombosis. The similarities in prothrombotic alterations between children with HUS and the greyhound model of Stx-HUS support the authenticity of greyhounds in modeling human Stx illness.

Thrombin has been shown to induce apoptosis in several types of cultured cells through activation of the protease activated receptor (PAR) PAR1. Intriguing recent work in vitro and in vivo in a mouse model showed that activation of PAR, by thrombin or specific agonists caused intestinal epithelial cell apoptosis and disruption of tight junctions with increased colonic epithelial permeability (20). These studies suggest that thrombin could be pathogenic against colonic epithelium, and may contribute to barrier disruption and facilitation of Stx access to the bloodstream. Thrombin inhibition may therefore protect against colonic epithelial injury as well as microvascular thrombosis.

Although therapeutic trials with heparin and other antithrombotic agents in human HUS patients and animal models have not been convincingly successful (21), our results with lepirudin provide renewed optimism for the potential efficacy of antithrombotic treatment for HUS. However, because this is a small pilot study in which lepirudin was administered before toxin exposure, considerable further experimentation is necessary to determine any potential treatment efficacy of lepirudin in clinical HUS.

Mechanisms of anticoagulation differ between agents. Heparin anticoagulation occurs by an indirect mechanism involving antithrombin III, whereas hirudins bind directly to thrombin and block both substrate binding and the proteolytic domain (12). Hirudins inhibit polymerization of fibrin, cellular activation through protease-activated receptors, and feedback activation of coagulation factors. Unlike heparin, hirudins inhibit thrombin activity of clot-bound as well as fluid phase thrombin (12). Clinically, hirudins have been shown to decrease mortality and prevent deposition of fibrin in kidney and liver in animal models of lipopolysaccharide-induced DIC (22). In human leukemia-associated DIC, and healthy volunteers exposed to lipopolysaccharide, hirudin caused marked blunting of clinical and laboratory markers of coagulopathy (12).

In this pilot study, we showed that lepirudin effectively anticoagulates canine plasma in vitro and in vivo. The dosage of lepirudin required for therapeutic range anticoagulation is about 10 times that required for human plasma, consistent with other human agents tested in dogs, such as recombinant human activated protein C (23).

The potential risk of adverse events that may be associated with use of potent anticoagulation in thrombocytopenic HUS patients remains a critical issue. Extensive preclinical animal studies will be necessary before consideration of human clinical
trials. The observation that none of the three lepirudin treated, Stx-challenged dogs were observed to have unexpected hemorrhage supports further exploration of the experimental model.

When administered prior to a lethal dose of Stx2, lepirudin markedly blunted Stx effects in 2 of 3 dogs. Whether the failure to protect one dog reflects biological variability, experimental error, or stochastic events, remains to be determined. However, based on previous experience, the survival of 2 dogs was unanticipated, and warrants additional experimentation to determine the reproducibility of the effect, and to determine if lepirudin is protective when administered at times after a Stx challenge.

Animal models are a critical resource for studying Stx illness. Rabbits, mice, pigs, calves, baboons, guinea pigs and chickens have been evaluated as potential experimental models.(15) An outbreak of E. coli infection-associated illness in Dutch belted rabbits exhibiting renal thrombotic microangiopathy suggests the possibility of a future model. Experience in greyhounds, however, has shown that reproducing natural disease from live bacteria challenge is difficult. A recent study found renal thrombotic microangiopathy in gnotobiotic piglets after oral infection with Stx-producing E. coli (24). However, hematological and creatinine changes were not observed, and the possibility of bacteremia causing disseminated intravascular coagulation (as observed in previous studies) was not addressed. The baboon has been suggested as an optimal Stx illness model because the distribution of GB3 in the baboon is similar to that in humans (25, 26). The greyhound reaction to purified Stx is essentially identical to the Stx-HUS in the baboon (15). The potential advantages of the greyhound model over the baboon are that dogs do not have to be anesthetized for the duration of the study, are more available, less expensive, easier to work with, and evoke a lesser degree of ethical objection. Unlike smaller animals, dogs are large enough to allow repeated blood sampling without significant physiological impact.

The availability of the practical greyhound model that recapitulates human Stx-HUS is a major experimental advance. Our results indicate that exploration of thrombin-mediated pathways using the greyhound model of Stx-HUS could lead to major advances in the understanding and treatment of Stx illness.

Acknowledgments

We thank the staff of the Hemostasis Reference Laboratory of The Blood Center of Southeastern Wisconsin for their assistance in performing hemostasis assays.

References


