Thrombin mediates the extraintestinal thrombosis associated with experimental colitis

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Yoshida H, Russell J, Granger DN. Thrombin mediates the extraintestinal thrombosis associated with experimental colitis. Am J Physiol Gastrointest Liver Physiol 295: G904–G908, 2008. First published September 4, 2008; doi:10.1152/ajpgi.90400.2008.—Recent evidence implicating tissue factor and the protein C pathway in the hypercoagulable state associated with intestinal inflammation suggests that thrombin is likely to contribute to this response. The objective of this study was to assess the role of thrombin in the extraintestinal thrombosis associated with experimental colitis. Thrombus formation was quantified in microvessels of the cremaster muscle in mice with dextran sodium sulfate (DSS)-induced colonic inflammation. The light/dye endothelial injury model was used to elicit thrombus formation in DSS colitic mice treated with either hirudin, heparin, or antithrombin III. The initiation and propagation/stabilization phases of thrombus formation were quantified using the time of onset of the thrombus and time to blood flow cessation, respectively. Thrombus formation was accelerated in arterioles of DSS colitic mice, as exhibited by significant reductions in the time of thrombus initiation and propagation/stabilization. Colitic mice treated with hirudin, heparin, or antithrombin III did not exhibit a significant change in the time of onset of the thrombus compared with untreated colitic mice. However, all three antithrombin agents largely prevented the DSS-induced reduction in the time to flow cessation following light/dye injury, with hirudin offering complete protection. These findings indicate that thrombin plays a major role in the extraintestinal thrombus formation associated with experimental colitis. Thrombin appears to contribute to the propagation/stabilization, rather than initiation, phase of the colitis-associated thrombogenesis at the distant vascular site. The results support the therapeutic use of antithrombin agents for reducing the risk of thromboembolism in patients with inflammatory bowel disease.

coaulation; heparin; hirudin; antithrombin III

ULCERATIVE COLITIS and Crohn’s disease are inflammatory bowel diseases (IBD) that result in significant morbidity and mortality. It is well known that cancer, malnutrition, and infection account for some of the mortality associated with IBD. Thromboembolic (TE) events are also a significant cause of death in patients with IBD (13). There is evidence for thrombus formation both within the inflamed bowel (23) and in extraintestinal tissues (10). It is reported that the overall incidence of systemic TE events in patients with IBD is 6.2%, with a threefold increase in the risk of systemic TE events compared with the general population (22). Deep-vein thrombosis and pulmonary embolus are the most common types of TE events associated with IBD, with fewer reports describing emboli in the brain, retina, and liver (2, 13). Although the mechanisms that underlie the hypercoagulable state in IBD remain poorly defined, there is a growing body of evidence suggesting that coagulation and inflammation are two interrelated physiological processes, with an inflammatory response promoting coagulation and vice versa (6, 7).

The hypercoagulable state and enhanced thrombus formation that is observed in human IBD has been reproduced in animal models of colitis. For example, it has been shown that mice with dextran sodium sulfate (DSS)-induced colitis have elevated level of thrombin-antithrombin complexes in blood (1), and these colitic mice exhibit enhanced thrombus formation in arterioles of extraintestinal tissue (cremaster muscle), compared with control mice (1, 27). These studies of thrombus formation in the microvasculature of colitic mice have also revealed major contributions of tissue factor (1) and activated protein C (27) to the enhanced thrombus formation associated with DSS colitis. Further support for the involvement of the protein C pathway in the pathogenesis of human IBD and experimental colitis is provided by a report by Scaldaferri and coworkers (18), who demonstrated that the expression of endothelial protein C receptor (EPCR) and thrombomodulin is lost in the microvasculature of patients with Crohn’s disease or ulcerative colitis and that DSS colitic mice treated with activated protein C exhibit blunted disease activity, histologic injury scores, and inflammation.

The procoagulation reactions that are elicited by activation of tissue factor and downregulation (or impaired activation) of the protein C pathway during human IBD and experimental colitis should ultimately lead to enhanced generation of thrombin. A role for accelerated thrombin production in IBD is also supported by reports describing significantly reduced antithrombin III (ATIII) levels in plasma of patients with IBD (9, 22). Clinical studies have yielded conflicting results on the therapeutic efficacy of heparin in patients with IBD (4, 22), although the antithrombin agent has shown some benefit in blunting the inflammation and tissue injury in experimental colitis (3, 25). Although the beneficial effects of heparin in human and experimental IBD have been attributed to its anti-inflammatory properties (22), the efficacy of heparin and other antithrombin agents in preventing the thrombus formation associated with IBD has not been previously addressed.

The objective of this study was to assess the role of thrombin in the extraintestinal thrombosis associated with experimental IBD and to compare the efficacy of three mechanistically distinct antithrombin agents (heparin, hirudin, and antithrombin III) in this model of inflammation-enhanced thrombosis. To achieve these objectives, the light/dye endothelial injury method was used to induce thrombus formation in arterioles and venules

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of the cremaster muscle in mice with DSS-induced colitis. Our findings are consistent with a major role for thrombin in the extraintestinal thrombosis associated with experimental IBD.

MATERIALS AND METHODS

Animals. A total of 58 male C57BL/6 [wild-type (WT) control strain, 21–28 g body wt] mice (Jackson Laboratories, ME) were used in this study. All mice were housed under specific pathogen-free conditions in standard cages and fed standard laboratory chow and water until the desired age (6–8 wk). All animal procedures were reviewed and approved by the Institutional Animal Care and Use Committee of Louisiana State University Health Sciences Center and were performed according to the criteria outlined by the National Institutes of Health.

DSS-induced colitis. Colitis was induced, as previously described (24), by feeding mice 3% (wt/vol) DSS (mol wt, 40,000; MP Biomedicals, Solon, OH) dissolved in filter-purified drinking water. The first day of DSS feeding was defined as day 0, and the mice were maintained on the DSS until day 6. The 3% DSS regimen, unlike higher DSS doses, is not associated with mortality. Control mice received filtered water alone (without DSS).

Assessment of colitis progression. Body weights, fecal status, presence of occult blood in the stools, and perianal bleeding were observed and recorded every day while the mice received DSS. Occult blood was detected using guiac paper (ColoScreen; Helena Laboratories, Beaumont, TX) (24). Disease activity index (DAI), a measure of disease severity ranging between 0 and 4, was calculated from data collected on stool consistency, presence or absence of fecal blood, and weight loss, as previously described (24). The DAI was monitored to ensure that DSS treatment resulted in clinical responses that are consistent with colitic disease activity.

Cremaster muscle preparation. On day 6 of DSS (colitis) or water (control) treatment, mice were anesthetized using 50 mg/kg pentobarbital sodium intraperitoneally, with supplemental doses (12.5 mg/kg) given as needed. The right internal jugular vein was cannulated for intravenous administration of FITC-dextran. In most experiments, the right carotid artery was cannulated for blood pressure measurement. Body temperature was maintained at 35.5–36.5°C during the entire experiment with a homeothermic blanket and monitored with a rectal temperature probe. The cremaster muscle was prepared for intravital fluorescence microscopic observation, as previously described (21). The surface of the exposed cremaster muscle was suffused continuously with bicarbonate-buffered saline, with a pH of 7.35–7.45. The cremaster muscle preparation was moved onto the stage of an upright fluorescent microscope and was allowed to stabilize for 20–30 min before initiation of the experiment.

Microscopic observations were carried out using an upright microscope (BX51WI; Olympus, Tokyo, Japan) with a 40× water immersion objective lens (LUMPlanFl/IR 40×/0.80 w). The microscopic image was projected onto a monitor (Sony TRINITRON PVM-2030) through a color video camera (Hitachi VK-C150) and recorded using a DVD recorder (JVC SR-MV50). A video timer (Panasonic Time-Date Generator WJ-810) was connected to the monitor to record time and date. The diameters of the cremaster vessels were measured by video analysis software (ImageJ 1.37v, NIH, Public Domain software) on a personal computer (G4 Macintosh, Apple). After the preparation was stabilized, 10 ml/kg of 5% FITC-dextran (150,000 MW; Sigma, St. Louis, MO) was slowly injected intravenously. It was allowed to circulate for 10 min, and then second- or third-order unbranched venules and arterioles with diameters (dV) ranging between 30–50 μm were selected for study. Red blood cell velocity (vRBC) in the microvessels was measured using an optical Doppler velocimeter (Microcirculation Research Institute, Texas A & M University, College Station, TX). Blood flow was calculated from the product of mean red blood cell velocity (Vmean = vRBC/1.6) and cross-sectional area, assuming cylindrical geometry. Wall shear rate (WSR) was calculated on the basis of the Newtonian definition: WSR = 8 (Vmean/dV) (17). The fluorescent microscopic images were received by a charge-coupled device video camera (Hamamatsu XC-77).

Light/dye-induced thrombosis. Photoactivation of FITC-dextran within the microvessels was performed after the fluorochrome was in the circulation for 10 min. Less than 100 μm of vessel length was exposed to epi-illumination using a 175-W xenon lamp (Lambda LS; Sutter, Novado, CA) and a fluorescein filter cube (HQ-FITC; Chroma, Rockingham, VT). The excitation power density was measured daily (ILT 1700 Radiometer, SED033 detector; International Light, Peabody, MA) and maintained within 1% of 0.77 W/cm², as previously described (1, 15, 16, 17). Epi-illumination was continuously applied to the vessels, and thrombus formation was quantified by determining I) the time of onset of platelet deposition/aggregation within the microvessel (onset time, thrombus initiation) and 2) the time required for complete flow cessation for ≥60 s (cessation time, time to thrombus stabilization). Epi-illumination was discontinued once blood flow ceased in the vessel under study. Typically, two to four thrombi were induced in each mouse, and the results of each vessel type (venules, arterioles) were averaged. In mice not receiving FITC-dextran, epi-illumination of the cremaster microvasculature for a period of 30 min did not result in platelet aggregation or thrombus formation in either venules or arterioles. Similarly, vessel diameters and red blood cell velocity were not altered.

Experimental protocols. The light/dye endothelial injury model was used to monitor thrombus formation in the following experimental groups: 1) control mice (no DSS in drinking water), 2) DSS-treated (colitic) mice, 3) control and 4) colitic mice receiving 1 mg/kg of hirudin (Calbiochem, Darmstadt, Germany) 5 min before vessel epi-illumination (19), 5) control and 6) colitic mice receiving 100 IU/kg of heparin (Abbaxis, Chicago, IL) 5 min before vessel epi-illumination (19), and 7) control and 8) colitic mice receiving 50 IU/kg of antithrombin III (Calbiochem) 5 min before vessel epi-illumination (19).

Statistics. Data were analyzed using standard statistical analyses, i.e., one-way ANOVA and Fisher’s post hoc test. Values are reported as means ± SE, and statistical significance was set at P < 0.05.

RESULTS

All mice receiving DSS in drinking water developed clinical signs of colitis (weight loss, perianal gross bleeding, loose stools, or diarrhea), and all mice survived the full course of DSS treatment. Table 1 summarizes the estimated values of WSR (obtained immediately before the induction of light/dye injury) in venules and arterioles in all experimental groups. No significant differences in WSR were noted between groups for either vessel population.

Table 1. Wall shear rates in venules and arterioles before thrombus formation.

<table>
<thead>
<tr>
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<th>Wall Shear Rate, s⁻¹</th>
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<tr>
<td></td>
<td>Venules</td>
</tr>
<tr>
<td>Control</td>
<td>589.6 ± 24.6</td>
</tr>
<tr>
<td>Control + Hirudin</td>
<td>677.0 ± 49.9</td>
</tr>
<tr>
<td>Control + Heparin</td>
<td>899.6 ± 178.2</td>
</tr>
<tr>
<td>Colitic + Hirudin</td>
<td>671.2 ± 36.1</td>
</tr>
<tr>
<td>Control + Heparin</td>
<td>801.3 ± 169.9</td>
</tr>
<tr>
<td>Colitic + Heparin</td>
<td>821.5 ± 106.0</td>
</tr>
<tr>
<td>Control + Antithrombin III</td>
<td>705.95 ± 79.75</td>
</tr>
<tr>
<td>Colitic + Antithrombin III</td>
<td>684.7 ± 65.2</td>
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Values are means ± SE; n = 7 mice per group. *Colitic mice received 3% dextran sodium sulfate (DSS), whereas controls received water only.
Figure 1 summarizes the changes in the time of onset of thrombosis and time to flow cessation in cremaster arterioles of untreated and hirudin-treated control and colitic mice. As previously reported (1, 27), DSS colitis is associated with significant reductions in both the onset of thrombus formation and time to flow cessation. Hirudin treatment did not alter the onset and flow cessation times in control mice, compared with untreated control mice. Colitic mice receiving the same dose of hirudin exhibited an unchanged thrombus onset time compared with untreated colitic mice. However, hirudin treatment did result in a significantly prolonged time to flow cessation after light/dye injury. A similar pattern of responses was noted in mice treated with either heparin (Fig. 2) or ATIII (Fig. 3), i.e., neither heparin nor ATIII altered the onset of thrombus formation in cremaster arterioles of colitic mice; however, both thrombin inhibitors significantly prolonged the time to flow cessation in light/dye-injured arterioles of colitic mice. Although all three thrombin inhibitors were effective in blunting thrombus formation, only hirudin completely inhibited the reduction in time to flow cessation noted in arterioles of colitic mice after light/dye injury, which is consistent with the view that hirudin is the most potent of all thrombin inhibitors.

It has previously been shown that the enhanced light/dye-induced thrombus formation in the cremaster microcirculation of mice with DSS colitis largely occurs in arterioles, with no significant differences noted in either the onset or flow cessation times in venules of normal and colitic mice (1, 27). The results on the venular responses obtained in the present study (Table 2) are consistent with these previous reports.

The data summarized in Table 2 indicate that the thrombotic responses of venules to light/dye injury in control and colitic mice were largely unaffected by treatment with either hirudin, heparin, or ATIII. None of the thrombin inhibitors significantly affected the time of onset of the thrombus. Although there was a tendency for prolongation of the time to flow cessation in venules of mice (control and colitic) receiving a thrombin inhibitor, this increase in flow cessation time reached statistical significance only for colitic mice treated with hirudin.

DISCUSSION

Efforts to better understand the processes of hemostasis and coagulation in different pathological conditions have revealed an intimate link between coagulation and inflammation (5, 8). It is now appreciated that certain inflammatory mediators (e.g., cytokines) and signaling pathways (e.g., Toll-like receptor-4 and CD40/CD40L) can shift hemostatic mechanisms in favor of thrombosis by upregulating tissue factor while downregulating the natural anticoagulation pathways, i.e., the heparin-antithrombin system, the tissue factor pathway inhibitor system, and the protein C anticoagulant pathway. An important consequence of these responses of the coagulation system to inflammation is an accelerated production of thrombin, which promotes thrombus formation by converting fibrinogen to fibrin, and through feedback activation of factors V, VIII, and XI. The hypercoagulable state that is manifested in patients with IBD and in animal models of experimental colitis likely reflects the impact of inflammatory mediators on procoagulant reactions, including an enhanced production of thrombin (7, 9, 22). The present study represents the first systematic effort to define the contribution of thrombin to colitis-enhanced extraintestinal thrombogenesis. Our findings indicate that thrombin plays a critical role in colitis-enhanced thrombogenesis, and the data suggest that thrombin inhibitors may reduce the risk of thromboembolism in patients with IBD.

Three direct thrombin inhibitors (hirudin, heparin, and ATIII) with distinct mechanisms of action were used to assess...
the role of thrombin in DSS colitis-enhanced thrombus formation in cremaster arterioles after light/dye-induced endothelial injury. Hirudin, the most potent naturally occurring inhibitor of thrombin derived from medicinal leeches, binds thrombin in a 1:1 fashion at each of two sites on the enzyme, i.e., the fibrinogen recognition domain and the catalytic domain. This binding of hirudin to thrombin inhibits all of the biological functions of thrombin but does not inhibit other enzymes in the coagulation or fibrinolytic pathways (12). Heparin, a highly sulfated glycosaminoglycan secreted by mast cells, binds to and results in a 2,000-4,000-fold activation of ATIII, which in turn functions to inactivate thrombin and factors Xa and IXa (14). All three thrombin inhibitors have been shown to exert an influence on thrombus formation in different animal models (19, 26). For example, a comparison of the efficacy of hirudin, heparin, and ATIII in a light/dye model of microvascular thrombosis in the ear of hairless mice has revealed that heparin, hirudin, and ATIII prevent thrombotic vessel occlusion in 62%, 43%, and 100% of arterioles, respectively (19), suggesting that ATIII is the most effective inhibitor. ATIII was also shown to afford protection against light/dye-induced arteriolar thrombus formation in a mouse model of endotoxemia, with ATIII treatment yielding a fourfold delay in thrombus formation (20). Unlike the findings reported in mice with endotoxemia, the results of the present study indicate that hirudin, heparin, ATIII are all very effective in delaying colitis-enhanced arteriolar thrombogenesis following light/dye injury in the cremaster microcirculation. However, hirudin proved to be the only thrombin inhibitor that completely inhibited the reduction in time to flow cessation noted in arterioles of colitic mice after light/dye injury, which is consistent with the view that hirudin is the most potent naturally occurring inhibitor of thrombin.

Thrombogenesis in an injured blood vessel occurs in three stages, initiation, propagation, and stabilization (11). The initiation phase involves the rapid binding of platelets to the injured vessel wall and activation of the coagulation cascade by tissue factor. A variable measured in our study that likely reflects the initiation phase of thrombus formation following light/dye injury is onset time. The propagation phase involves the recruitment of more platelets and amplification of the coagulation cascade via the intrinsic pathway. Stabilization of the thrombus occurs as a result of fibrin deposition (11). The time to flow cessation measured in microvessels after light/dye injury reflects the time to thrombus stabilization and likely includes both the propagation and stabilization phases of thrombogenesis. The present analysis of colitis-induced thrombogenesis indicates that the three direct thrombin inhibitors (hirudin, heparin, and ATIII) exert no influence on the time of onset of the colitis-enhanced thrombus following light/dye injury, suggesting that thrombin does not contribute to the initiation phase in this model of thrombogenesis. These findings are consistent with a recent report (27) describing no improvement (extension) of the onset time for thrombus formation in cremaster arterioles after light/dye injury in DSS colitic WT mice treated with activated protein C and in EPCR transgenic mice (EPCR-Tg) with DSS colitis. However, it has been reported (1) that immunoblockade of tissue factor largely prevents the reduction in thrombus onset time that is associated with DSS colitis, which supports the view that tissue factor expression is a key event in the initiation phase (onset) of arteriolar thrombogenesis (11).

All three thrombin inhibitors were effective in prolonging the time to flow cessation following light/dye injury in DSS colitic mice, suggesting that thrombin is an important contributor to the propagation and stabilization phases of thrombogenesis in this model. This finding is consistent with a previous report showing a similar prolongation of the time to flow cessation following light/dye injury in cremaster arterioles of WT DSS colitic mice treated with activated protein C and in colitic EPCR-Tg mice (27). Collectively, the two sets of data raise the possibility that an impaired protein C pathway that has already been implicated in human and experimental colitis (18) contributes significantly to the enhanced thrombin generation that mediates the propagation and stabilization phases of thrombus formation. The previously reported finding that tissue factor antibody treatment does not affect the time to flow cessation in this model (1) suggests that tissue factor is not a major contributor to the propagation/stabilization phases of colitis-enhanced thrombogenesis in cremaster arterioles.

The identity of the chemical and/or cellular signal produced by the inflamed gut that enhances thrombus formation in arterioles of skeletal muscle remains unknown. However, it is possible that cytokines released into the blood stream by the inflamed bowel mediates this distant tissue response. For example, tumor necrosis factor-α and interleukin-1β, which have been implicated in pathogenesis of IBD, are known to downregulate the expression of EPCR on microvascular endothelial cells and reduce the capacity of these cells to activate protein C (18). Alternatively, blood cells such as monocytes or platelets that flow through microvasculature of the inflamed gut may be activated to express tissue factor, which is then delivered via blood to extraintestinal vascular beds, such as skeletal muscle, that are rendered vulnerable to thrombus formation (1). More work is needed to determine how the inflamed gut signals thrombogenesis in distant tissues.

In conclusion, the results of this study indicate that thrombin inhibitors confer significant protection against the extraintestinal thrombosis that is associated with colonic inflammation. These thrombin inhibitors appear to exert their actions on the propagation and stabilization phases of thrombogenesis. In view of the previously reported role of tissue factor in mediating the initiation phase of colitis-induced thrombogenesis, the combined use of tissue factor and thrombin inhibitors may provide a therapeutic advantage in reducing the morbidity and mortality from thromboembolic events in patients with IBD.

### Table 2. Light/dye-induced thrombus formation in venules of control and colitic mice

<table>
<thead>
<tr>
<th>Time, min</th>
<th>Onset</th>
<th>Cessation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.3±0.1</td>
<td>13.8±5.1</td>
</tr>
<tr>
<td>Colitic*</td>
<td>0.4±0.1</td>
<td>11.5±2.9</td>
</tr>
<tr>
<td>Control + Hirudin</td>
<td>0.5±0.1</td>
<td>40.17±19.6</td>
</tr>
<tr>
<td>Colitic + Hirudin</td>
<td>0.5±0.1</td>
<td>56.2±18.3**</td>
</tr>
<tr>
<td>Control + Heparin</td>
<td>0.6±0.1</td>
<td>44.5±18.7</td>
</tr>
<tr>
<td>Colitic + Heparin</td>
<td>0.5±0.1</td>
<td>33.3±13.6</td>
</tr>
<tr>
<td>Control + Antithrombin III</td>
<td>0.5±0.1</td>
<td>12.8±3.5</td>
</tr>
<tr>
<td>Colitic + Antithrombin III</td>
<td>0.3±0.1</td>
<td>20.3±5.1</td>
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</table>

*Values are means ± SE; n = 7 per group. *Colitic mice received 3% DSS, whereas controls received water only. **P < 0.05 vs. colitic mice.
GRANTS

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REFERENCES


