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Title: Enhanced effect of inhibition of thrombin on endothelium in murine endotoxaemia: specific inhibition of thrombocytopenia.

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Abstract: Introduction: In systemic endotoxaemia, bacterial lipopolysaccharide causes the rapid expression of tissue factor (TF) and disseminated intravascular coagulation and in animal models, anticoagulants limit pathology and promote survival. Recent studies have emphasised the importance of TF expressed by mononuclear cells for initiating thrombin generation during endotoxaemia and suggested that endothelial cell TF is of little relevance. However, the precise importance of endothelium for intravascular thrombin generation has not been established. In this study, we compared the effect of equivalent levels of hirudin tethered to either endothelium or platelets and monocytes.

Materials and Methods: CD31-Hir-Tg mice express a vesicle-targeted, membrane-tethered hirudin fusion protein on endothelium, platelets and monocytes. Bone marrow chimeras between these mice and C57BL/6 were generated The level of intravascular hirudin expressed during endotoxaemia was quantified by inhibition studies using an anti-hirudin antibody and reference to the circulating thrombin anti-thrombin complexes generated in control mice given soluble hirudin.

Results and Conclusions: Antibody inhibition studies indicated that individual chimeras expressed similar levels of hirudin fusion protein on endothelium alone as on platelets and leukocytes combined and accordingly, the levels of thrombin anti-thrombin complexes and fibrinogen in each chimera were similar, indicating equivalent inhibition of thrombin generation. However, mice with hirudin on endothelium alone developed significantly less thrombocytopenia. These results suggest a hitherto unrecognized role of endothelium in thrombin-dependent platelet sequestration during endotoxaemia. The data have implications for the development of therapeutic strategies based on targeted anticoagulation to limit disseminated intravascular coagulation.
Original Article

Enhanced effect of inhibition of thrombin on endothelium in murine endotoxaemia: specific inhibition of thrombocytopenia.

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Abstract

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Keywords: Endothelium; Thrombin; Endotoxin; Thrombocytopenia;
Abbreviations:

C57BL/6 (BL/6)
Endothelial cell (EC)
Endothelial protein C receptor (ePCR)
Factor IX (FIX)
Factor X (FX)
Hirudin Equivalent” (HEQ)
Lipopolysaccharide (LPS)
P-selectin glycoprotein ligand-1 (PSGL-1)
Tissue factor (TF)
Thrombin anti-thrombin (TAT)
Wild-type (WT)
Introduction

In systemic endotoxaemia induced by lipopolysaccharide (LPS), widespread intravascular activation of coagulation by tissue factor (TF) is a critical early pathophysiological event. For that reason, inhibiting either the initiation or propagation phases of clotting [1-8] has a significant impact on morbidity and mortality in experimental models. The mechanisms that trigger intravascular coagulation in endotoxaemia have been studied in depth. TF is important for the initiation phase of coagulation [9], but the source of TF has been controversial. One hypothesis places the pathological activation of the vascular endothelium, by the direct effects of LPS, at the heart of this process [10]. In vitro, endothelial cell (EC) activation by LPS or inflammatory cytokines such as TNFα, results in the expression of TF [11]. However, EC expression of TF has never been convincingly demonstrated in vivo, as discussed by Aird [12].

A second hypothesis with widespread support emphasises the importance of LPS-activated leukocytes and platelets. Leukocyte-derived TF includes the classical membrane-tethered moiety on activated cells (reviewed in [13]) which has been shown to promote thrombosis in vivo in one model of intravascular thrombosis [14]. TF is also found in the alpha granules of platelets [15] and can also be found in blood in the form of leukocyte- or platelet-derived microparticles [15, 16]. These can initiate clotting after P-selectin-mediated transfer of the TF to activated platelet [17] or leukocyte membranes [18, 19] and levels in the blood correlate strongly with the amount of thrombin generated in endotoxaemic mice [20]. A third form of TF, alternatively spliced and lacking a transmembrane sequence has also been described. This soluble protein, produced by CD14+ monocytes, has procoagulant activity in the presence of activated platelets [21]. Recent studies using mice expressing either low levels of TF, human TF or strains in which TF has been selectively deleted from specific cell types, have emphasised that TF expressed by non- haematopoietic elements appears as important as that expressed by
bone-marrow-derived elements, in particular by monocytes [22]. However, selective knockout of TF on endothelium or smooth muscle cells appears to have no impact on thrombin generation during LPS endotoxaemia [23]. Therefore, the non-haematopoietic source of TF remains uncertain, although these studies suggest that it is not expressed by cells in the vessel wall.

Only a fraction of the circulating factor IX (FIX) and factor X (FX) are converted to their active forms, FIXa and FXa by TF during initiation of coagulation [24]. In the absence of the activated cofactor factor Va, FXa generates barely detectable levels of thrombin. Although insufficient to initiate significant fibrin polymerization alone, trace amounts of thrombin formed are able to back-activate intrinsic pathway cofactors factor V and VIII, ultimately generating much larger amounts of thrombin which are sufficient to generate a clot. This ‘propagation’ phase of coagulation occurs on plasma membranes and is dependent on the exposure of procoagulant phospholipids [25]. A substantial body of data supports the idea that the activated platelet membrane is an important site of thrombin generation (see for instance, [26]). However, platelets appear not to be required for fibrin generation in a vessel injury model in mice [27].

Activated EC may also contribute to the generation of thrombin, but much less is known about the involvement of EC membranes in this process in vivo. However, activated EC lose many of their anticoagulant functions (reviewed in [28]), consequent upon the downregulation of thrombomodulin expression [29, 30], and shedding of endothelial cell protein C receptor (ePCR) [31] along with surface glycosaminoglycans with resultant loss of non-covalently bound molecules such as antithrombin and tissue factor pathway inhibitor [32]. Some of these changes have been observed in vivo during sepsis [33], indicating their potential relevance.

The series of experiments reported here addresses the relative importance of activated EC, platelets and monocytes for thrombin inhibition during LPS endotoxaemia. We have
used transgenic mice expressing the leech anticoagulant hirudin as a stable, covalently linked membrane fusion protein to inhibit thrombin on these surfaces. In previous work, we showed that EC expression was sufficient to inhibit the development of consumptive coagulopathy after LPS [34]. In this study, we show that expression of the hirudin fusion protein on platelets and leukocytes appears as efficient at inhibiting thrombin as when similar levels are expressed on EC. However, platelet expression was accompanied by profound thrombocytopenia that was not seen when the hirudin fusion protein was expressed on EC. These data suggest that preventing procoagulant changes on activated EC has a more significant influence on pathology than on platelet or leukocyte membranes, despite the fact that both appear effective at inhibiting thrombin.
Materials and methods

**CD31-Hir-Tg mice.** Generation of these mice, their characterisation and phenotype after LPS injection have been described [34]. All mice in this study were heterozygous and were backcrossed for at least 5 generations onto a C57BL/6 (BL/6) background.

**Immunohistochemistry** Organs were embedded in OCT (BDH, Lutterworth, UK) by freezing with dry ice, sectioned and fixed in methanol at –20°C. Frozen sections were immersed in 1% BSA-PBS and 10% goat serum (Sigma, Poole Dorset UK) for 30 min, then incubated overnight at 4°C with one of the following antibodies; sheep anti-hirudin, (Enzyme Research Lab., Swansea, UK), rabbit anti-mouse TF [35], FITC-conjugated rabbit anti-human fibrinogen, (DAKO, Glostrup, Denmark). Second layer staining was with goat anti-rabbit IgG-FITC, donkey anti-sheep IgG-FITC or sheep anti-mouse IgG-FITC (all from Sigma).

**Cell lines** Immortalised porcine EC transfectants expressing hirudin fusion protein have been described previously [36]. EC were maintained in DMEM (Invitrogen, Paisley, UK) supplemented with 10% FCS (Globepharm, Surrey, UK), penicillin, streptomycin, L-glutamine and mycophenolic acid.

**Leukocyte isolation** Mouse blood, collected by tail-tip resection, was diluted 1:20 in ACK buffer (155 mM NH₄Cl / 10 mM KHCO₃ / 0.1 mM EDTA). After 20 min at room temperature, cells were washed and resuspended in 3%FCS/DMEM medium. Monocyte phenotype was confirmed with an anti-mouse CD14 mAb (Pharmingen, San Diego, USA). Splenocytes were isolated from homogenised mouse spleen, suspended in 10 ml of ACK buffer, incubated for 5 min at room temperature and re-suspended in RPMI containing 10% FCS. Cells were activated with Conconavalin A at the final concentration of 5μg/ml for 48 hours at 37°C.

**Purification of platelets** Platelet-rich plasma was obtained from blood by centrifugation at 80 g for 10 min, followed by dilution 1 in 20 with 1% ammonium oxalate (Sigma) and 2.5
mM of Gly-Pro-Arg-Pro peptide (Sigma). Samples were placed in a counting chamber in a moist petri dish, allowed to settle and the platelets in 1 mm² counted (=N). The number of platelets per litre of blood = 2N x10⁹. Platelet phenotype was confirmed with an anti-CD41 mAb (Pharmingen).

**Flow cytometry analysis** 1x10⁵ cells or platelets were incubated with primary antibodies for 30 min on ice, followed by secondary antibodies for 30 min. Antibodies used were as described above. Stained cells were analysed on a cytofluorometer (Becton Dickinson, Franklin Lakes, USA).

**In vitro clotting assay** Porcine EC transfectants expressing hirudin fusion protein were included in a recalcified mouse plasma clotting assay, similar to that previously described [37]. The hirudin fusion protein variants expressed by these clones lacked a cytoplasmic motif from P-selectin, so were expressed constitutively at the cell membrane, avoiding the need for activation and relocation, as would have been the case had primary mouse EC been used [36]. Plasma was collected from BL/6 mice. EC were seeded at a density of 10⁵ cells/ml in a 6-well plate and grown to near confluence before addition of PMA for 12 hours. After washing three times with serum-free DMEM, cells were detached with EDTA before a further wash. 5X10⁶ EC were added to 200μl of plasma, recalcified and the time to clot measured.

**Endotoxic shock** All mice weighed 25g +/- 1g. Experiments were performed under terminal anaesthesia and conformed to UK national and institutional guidelines. Mice were anaesthetized with 60 ng/g of sagatal (Rhone Merieux Limited, Harlow, UK), and given a single injection of LPS (Escherichia coli serotype 0127:B8 Sigma) 2 mg/kg or saline (control) intraperitoneally (IP). L-NAME (Alexis Corp., Nottingham, UK) (50mg/kg) was administered IP 30 min before LPS and again at time 0, 2 and 4 hours post-LPS. This model was used to maximise the thrombotic manifestations of endotoxin, as previously described [34, 38] during the short time-interval available whilst the mice were under
terminal anaesthesia. In some experiments, soluble hirudin (Enzyme Research Labs) or anti-hirudin mAb were administered at variable doses with LPS. Mice were sacrificed 4.5 hours post-LPS or saline and blood samples were withdrawn by puncture of the heart into 0.1 volume of 3.8% sodium citrate (Sigma).

**Bleeding time assay** Anaesthetised mice were maintained in a restrainer (Becton Dickinson) and a distal 2-mm segment of tail was severed. The tail was immersed in 0.9% saline at 37˚C. Bleeding time was defined as the time required for the bleeding to stop [39].

**Fibrinogen assay.** Fibrinogen levels were measured using the Clauss method with bovine thrombin (Sigma). Assays were terminated at 20 mins.

**Bone marrow (BM) reconstitution** BM was flushed from the excised ends of long bones from euthanized mice and re-suspended at 5X10⁷ cells/ml in fresh RPMI. Recipient mice were irradiated with 1200 Rad, before injection of 1 x 10⁷ BM cells into a tail vein. Mice were isolated for four weeks before further experimentation.

**Determination of thrombin-antithrombin III (TAT) complexes** Blood was collected by tail-tip resection and centrifuged at 13000g for 10mins at 4˚C to obtain platelet-poor plasma. TAT levels were determined using a commercial ELISA-based kit (Enzygnost TAT, Dade Behring AG, Germany) and microtiter plate reader (Titertek Multiskan®, Labsystems, Finland) according to the manufacturer’s instructions. All estimations were in triplicate.

**Method to quantitate expression of hirudin fusion protein.** The concentration of anti-hirudin mAb required to completely inhibit the anticoagulant phenotype of 1x10⁵ porcine EC transfectants expressing hirudin-CD4 fusion protein in 1 ml of recalcified mouse plasma [37] was determined by titration. This amount of anti-hirudin mAb was incubated with various concentrations of soluble hirudin in 1ml for 60 min at room temperature. The mix was then added to 1x10⁵ transfectants and incubated for 30 min on ice. Antibody bound to the transfectants was analysed by flow cytometry. The concentration of soluble hirudin that
reduced antibody binding to zero was taken as the equivalent of that expressed by $1 \times 10^5$ transfectants
Results

Analyis of relative hirudin fusion protein expression in Tg mice and BM chimeras.

To enable a quantitative analysis of the amount of hirudin fusion protein expressed by CD31-Hir-Tg mice, we first characterised the effect of soluble recombinant hirudin on parameters of coagulation in wild-type BL/6 mice. In this way, a mathematical relationship between hirudin and plasma thrombin anti-thrombin (TAT) in endotoxaemic wild-type BL/6 mice was defined (figure 1a). Data was analysed using the regression analysis facility of Sigmaplot, and data fit analysed by R² values. In this way, TAT levels in subsequent experiments could be defined in terms of an equivalent amount of hirudin given to wtBL/6 mice; we called this measure of TAT a “Hirudin Equivalent” (HEQ).

BM chimeras between CD31-Hir-Tg and wild-type BL/6 mice were generated (‘BMHir-wtB6’ and BMwtB6-Hir mice) along with BMwtB6-wtB6 and BMHir-Hir controls. TAT concentrations after injection of LPS/L-NAME into these mice are shown in table 1, along with HEQ values derived from figure 1a.

To estimate the amount of functional hirudin in the BMHir-wtB6 and BMwtB6-Hir mice, chimeras were injected with increasing concentrations of an anti-hirudin monoclonal antibody known to inhibit thrombin binding. The TAT levels and their HEQs are shown in table 1. HEQ values from these experiments were plotted against the dose of antibody, as shown in figure 1b. At the point on these curves where 50% of HEQ activity was inhibited (=[HEQ]50%), the amount of antibody administered is directly proportional to the concentration of surface membrane-expressed hirudin fusion protein. As shown, this concentration was almost identical in the two types of chimera, indicating no significant difference in the amount of functionally active surface exposed hirudin fusion protein expressed in BMwtB6-Hir and BMHir-wtB6 mice.

Inhibition of thrombin and parameters of DIC in BM chimeric mice.
Although the TAT and fibrinogen levels in the BMwtB6-Hir and BMHir-wtB6 chimeric mice were not significantly different at baseline (table 1), bleeding times and particularly platelet counts were significantly better in the BMwtB6-Hir compared to BMHir-wtB6 mice (table 2). HEQs derived from these three parameters (fibrinogen, bleeding times and platelet count) and plotted in a similar way to that shown in figure 1b confirmed no significant difference between the amount of anti-hirudin required to achieve [HEQ]_{50\%} in each of the chimeras (5.43±0.74 ng/g in BMwtB6-Hir vs. 4.43±0.54 ng/g in BMHir-wtB6. P=NS), confirming similar levels of hirudin fusion protein expression at cell surfaces in the two types of chimera.

To verify these data, we went back to samples collected from a previous series of BM chimeras [34] and measured TAT levels. This analysis was consistent with the data shown here (data not shown).

**No evidence for enhanced potency of hirudin fusion proteins expressed on EC.**

To address the theoretically possible (but biologically implausible) explanation that a given amount of hirudin fusion protein gained increased potency when expressed on EC membranes, in vitro experiments using porcine EC transfectants were conducted. The null hypothesis tested by these experiments was that EC-expressed hirudin fusion protein would be more potent than an equivalent amount of soluble hirudin added alongside control (untransfected) EC in *in vitro* clotting assays using recalcified human plasma. A ‘cold competition’ approach described above was used to determine the amount of hirudin fusion protein antigen expressed by a fixed number of transfectants (equivalent to 80ng of soluble hirudin). Next, clotting time assays in small volumes of recalcified human plasma were performed. These results are shown in figure 1c.

Addition of 80ng of soluble hirudin to control EC prolonged the clotting time to a level that was indistinguishable to that observed when transfected EC were used. In separate wells,
saturating concentrations of anti-hirudin antibody reduced the clotting time back to that seen with control EC. These results were inconsistent with the null hypothesis and suggested that soluble hirudin and membrane-expressed hirudin fusion protein were equipotent when present at equivalent concentrations on the EC surface.

**Pattern of TF expression in endotoxaemic mice.**

Organs harvested 4.5 hours after LPS/L-NAME injection were compared to control animals that received saline. In both wtBL/6 (figure 2a) and CD31-Hir-Tg (not shown) saline-treated mice, TF was found only in the adventitia of blood vessels. However, after LPS/L-NAME, TF was detected intraluminally in both strains, located on the luminal aspect of the vessel wall. By this analysis, the distribution of TF expression appeared the same in wtBL/6 and CD31-Hir-Tg mice. No quantitative analysis was undertaken. Monocytes and platelets from wtBL/6 and CD31-Hir-Tg saline-injected animals were negative for TF whereas those from both types of LPS/L-NAME-treated mice stained positive for TF (figure 2b). Monocytes and platelets from LPS/L-NAME but not saline-treated CD31-Hir-Tg mice expressed the hirudin fusion protein, detectable by flow cytometry (data not shown).
Discussion.

Animal studies have indicated TF-induced thrombin generation as a critical event in the early pathophysiology of endotoxaemia and sepsis and shown a significant reduction in morbidity and mortality in animals administered anticoagulants. However in humans disappointing results from phase III trials of anticoagulants have added to uncertainty about the importance of coagulation and of the clinical role of coagulation inhibitors [40-42]. One of the many difficulties with research in this field up until recently was a lack of understanding of the microanatomy and kinetics of how coagulation is initiated and propagated in vivo. One school of thought has the endothelium at the heart of the process but another emphasises the importance of LPS-activated monocytes and activated platelet membranes. Much has been learned recently from the intravital imaging of real-time clot formation in models of vascular injury, particularly about the pathways of platelet activation, though a key question remaining is where thrombin generation occurs [43]?

A related question concerns the biological significance of anticoagulant modulation and shedding from EC membranes during EC activation. There are two potential alternatives. First, anticoagulant shedding by EC might act as a mechanism to protect against intravascular thrombosis, by raising the plasma levels of circulating anticoagulants (as has been observed for TFPI in sepsis [44] and EPCR in endotoxaemia [31]). On the other hand, anticoagulant down regulation and shedding from the EC surface might occur to promote intravascular clotting to promote unregulated thrombin generation on the endothelium.

In this paper, we have used a strain of mice expressing a hirudin fusion protein on activated EC, platelets and leukocytes under the control of the CD31 promoter. As we have described elsewhere [34], cell membrane surface expression was regulated by inclusion of a P-selectin cytoplasmic sequence, which directed resting fusion protein
expression to secretory granules of CD31-positive cells.

Previously, we showed that these mice were resistant to the coagulopathy that developed after injection of LPS/L-NAME and it was apparent from bone marrow reconstitution studies that expression of the fusion protein on EC alone was sufficient to provide near-maximal protection against LPS. One criticism of this early work was that we did not exclude the possibility that a quantitative difference in the amount of functional hirudin expressed by the different chimeras was the reason for these results.

In this study, we have addressed this criticism. Our new data suggest no quantitative difference between the amounts of hirudin fusion protein expressed by the two chimeras. Importantly, having shown this, we can state that there was equivalent inhibition of thrombin when the hirudin fusion protein was expressed on either endothelium alone or on platelets/leukocytes. However, the data also indicates that the hirudin fusion protein had an enhanced potency in vivo on EC membranes, particularly at inhibiting the profound endotoxaemia-induced thrombocytopenia. Our accompanying in vitro data suggests that soluble hirudin was equipotent to the fusion protein when present at high concentrations in the proximity of the EC membrane.

Immunohistology data suggested that TF-expressing LPS-activated monocytes and platelets, which could be detected in the circulation, were sequestered at the site of clot formation in endotoxaemia, placing them in close proximity to the endothelium. There were no gross differences in the patterns of intravascular TF between the different mice used here. Therefore, our interpretation is that the enhanced effect of the hirudin fusion protein on EC is because there is an important interaction between endothelium and platelets during the propagation phase of coagulation during endotoxaemia. This is consistent with what has been shown during real-time imaging of thrombus formation after vascular injury [45] and with data showing that endothelial secretion of protein disulphide isomerase is more important than platelet secretion for fibrin formation in vivo during vascular injury,
even though activated platelets are recruited to the vessel wall [46].

The interaction between P-selectin and its ligand PSGL-1 is known to be an important determinant of the thrombocytopenia that develops in this model, which has been shown to be due to platelet sequestration within pulmonary alveolar capillaries, involving platelet-expressed P-selectin [47]. P-selectin is expressed on the surface of activated platelets and endothelial cells during endotoxemia and thrombin is known to enhance expression by both [48, 49]. Our data, by indicating that direct inhibition of thrombin on platelets is less efficient than inhibition on endothelial cells at preventing thrombin-dependent platelet sequestration, highlights the complexity of the platelet interaction with endothelium in endotoxaemia. We have not addressed the kinetics of TF expression and fusion protein-relocation to the membrane in each of the three compartments studied, but think it unlikely that our results are due to temporal differences, even if they do exist.

In interpreting this data, it is interesting to speculate why organisms have evolved such a response to endotoxin, as widespread intravascular coagulation and thrombosis is inherently disadvantageous if it results in multiple organ dysfunction and ultimately death. One possibility is that it may bestow a survival advantage in animals that are in the early stages of developing sepsis and exposed to low concentrations of endotoxin. Alternatively, it may represent a ‘design flaw’ in the inflammatory response to pathogens, tolerated because the physiological clotting response is efficient at preventing haemorrhage after vascular injury. Evidence that heterozygous carriers of factor V Leiden mutation have a distinct advantage in sepsis and endotoxaemia appears to support the former of these two possibilities [50].

If generating thrombin during early sepsis endows a survival advantage, the gain must be sufficiently significant to justify risking widespread intravascular thrombosis, infarction and organ failure during the later stages of infection. The conventional explanation for why intravascular thrombosis develops during sepsis is that fibrin clots may serve to limit the
spread of certain types of infection by isolating infected tissue [51, 52]. We have previously proposed another potential advantage, based on activated clotting factor-mediated signalling through protease-activated receptors on endothelial cells being required to initiate local chemokine gradients for leukocyte recruitment [53]. We speculate that any potential advantage will be lost in the CD31-Hir-Tg mice, so understanding how these mice respond to infectious challenge will be one focus of our future work.
Acknowledgements

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Thanks to Afzal Chaudhry, Cliburn Chan and Andrew George for help with statistics and interpretation of antibody inhibition studies.

Conflict of Interest statement

The authors declare no conflicts of interest
References


Table 1: Plasma TAT concentrations in bone marrow chimeras 4.5 hours after LPS/L-NAME injection.

<table>
<thead>
<tr>
<th>Anti-Hir mAb (ng/g)</th>
<th>BMwtB6-wtB6 [TAT]*</th>
<th>BMHir-Hir [TAT]*</th>
<th>BMwtB6-Hir [TAT]*</th>
<th>BMHir-wtB6 [TAT]*</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>174 (±5)</td>
<td>4</td>
<td>84 (±3)</td>
<td>239 (±5)</td>
</tr>
<tr>
<td>0.1</td>
<td>175 (±0.3)</td>
<td>4</td>
<td>84 (±0.3)</td>
<td>239 (±0.3)</td>
</tr>
<tr>
<td>0.3</td>
<td>177 (±0.3)</td>
<td>3</td>
<td>84 (±0.3)</td>
<td>212 (±0.3)</td>
</tr>
<tr>
<td>1</td>
<td>175 (±0.3)</td>
<td>4</td>
<td>87 (±0.3)</td>
<td>200 (±0.3)</td>
</tr>
<tr>
<td>3</td>
<td>176 (±0.3)</td>
<td>3</td>
<td>89 (±0.3)</td>
<td>180 (±0.3)</td>
</tr>
<tr>
<td>10</td>
<td>176 (±0.3)</td>
<td>3</td>
<td>103 (±0.3)</td>
<td>97 (±0.3)</td>
</tr>
</tbody>
</table>

* [TAT] in ng/ml
† HEQ in ng/g
‡ p=NS compared to equivalent values in BMwtB6-Hir
§ P<0.01 compared to equivalent values in BMwtB6-Hir
Table 2: Plasma fibrinogen, bleeding times and platelet counts in bone marrow chimeras 4.5 hours after LPS/L-NAME injection.

<table>
<thead>
<tr>
<th></th>
<th>BMwtB6-wtB6</th>
<th>BMHir-Hir</th>
<th>BMwtB6-Hir</th>
<th>BMHir-wtB6</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fibrinogen*</td>
<td>3 (±0.4)</td>
<td>23 (±3)</td>
<td>18 (±2)</td>
<td>14† (±2)</td>
</tr>
<tr>
<td>Bleeding time (minutes)</td>
<td>19 (±3)</td>
<td>7 (±1)</td>
<td>9 (±1)</td>
<td>13‡ (±1)</td>
</tr>
<tr>
<td>Platelet count (x10⁹/l)</td>
<td>19 (±3)</td>
<td>191 (±10)</td>
<td>173 (±7)</td>
<td>88† (±6)</td>
</tr>
</tbody>
</table>

*% fibrinogen relative to control mouse (no LPS)

†P=NS compared to BMwtB6-Hir

‡P<0.05 compared to BMwtB6-Hir

†P<0.01 compared to BMwtB6-Hir
Table Legends

Table 1:
The hirudin equivalent (HEQ) values are derived from the data represented in figure 1A. Mice were injected with an inhibitory monoclonal antihirudin antibody at time of LPS injection. Each value is the mean of TAT concentrations estimated in triplicate from each of three individual mice.

Table 2
Each value is the mean of triplicate estimations from each of three individual mice.

Figure Legends

Figure 1
A: Relationship between plasma TAT concentration ([TAT]) and administered dose of soluble hirudin in wtBL/6 mice. Each point is data derived from three mice. Error bars = SEM. $R^2$ estimated using Sigmaplot.

B: Plasma TAT concentrations from BMwtB6-Hir and BMHir-wtB6 mice expressed as “hirudin equivalents” or HEQ, using values calculated from the data in figure 1A (see table 1) and plotted against the dose of anti-hirudin antibody. Each data point derived from the TAT concentrations of 3 mice. The concentration of antibody at the point where 50% of the HEQ has been inhibited (HEQ$_{50}$) is shown on abscissa as a boxed figure. At this point, the dose of antibody is directly proportional to the amount of hirudin inhibited. $R^2$ estimated using Sigmaplot.

C: In vitro clotting assay using recalcified human plasma in presence or absence of immortalised porcine control EC or EC transfected with hirudin-CD4 construct.
Assays performed in total volume of 100 µl with 1x10⁶ EC. Saturating dose of antihirudin mAb and dose of soluble hirudin equivalent to that expressed on transfected EC were determined by a flow cytometric competition assay as described in materials and methods. Bars show clotting time in seconds + SEM (which are too small to see). Based on analysis of triplicate wells. * p = NS.

**Figure 2**

A: Immunohistology of heart from wtBL/6 and CD31-Hir-Tg animals stained with an antibody against murine TF (green). All sections have been stained with DAPI nuclear stain (blue). Arrows indicate luminal aspect of a blood vessel.

B: Flow cytometric analysis of platelets and monocytes from BL/6 and CD31-Hir-Tg mice after saline or LPS/L-NAME injection as indicated. Cells stained with isotype-matched control mAb (filled profiles) or anti-TF mAb (open profiles). Platelet and monocyte phenotype was confirmed by staining with anti-CD41 and anti-CD14 respectively (data not shown).
Figure 1

A. [TAT] (ng/ml) vs [Hirudin] (ng/g).

B. HEQ vs Anti-hirudin Ab (ng/g).

C. Clotting time (seconds) for different conditions.
Figure 2

A

Saline-treated

C57BL/6

LPS/L-NAME-treated

C57BL/6

CD31-Hir-Tg

B

Saline-treated

LPS/L-NAME-treated

C57BL/6

platelets

FL1-H

CD31-Hir-Tg

platelets

FL1-H
**Conflict of Interest Form**

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<tr>
<th>Manuscript Name:</th>
<th>Enhanced effect of inhibition of thrombin on endothelium in murine endotoxaemia: specific inhibition of thrombocytopenia.</th>
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<tr>
<td>Authors:</td>
<td>Daxin Chen, John H. McVey and Anthony Dorling</td>
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<td>Details of nature of conflict of interest:</td>
<td>none</td>
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7th July 2013

Dear Sirs,

Re. TR-D-13-00241
Enhanced effect of inhibition of thrombin on endothelium in murine endotoxaemia: specific inhibition of thrombocytopenia. First author Daxin Chen.

Many thanks for the opportunity to submit a revised version of this manuscript. I herewith present a detailed response to the reviewer’s comments.

Reviewer #1:
The study conducted by Daxin Chen et al. is interesting but data is insufficient to support the main conclusion. The authors show that in mice with hirudin, not surprisingly, activation of the coagulation system during sepsis is less severe. The main claim is that in the mice with hirudin, the endothelial level of TF is reduced, shown by immunostaining, while the level of TF on platelets and monocytes is similar to wild type. This point has to be dealt with thoroughly with more substantiating data. The immunostaining (2A) is without additional staining to endothelial marker (CD31, vWF) inorder to be sure that what is shown is truly the endothelium. In addition, staining of CD31-Hir-tg saline treated control is missing (it is possible that TF endothelial level in this mutated mice is lower than the wild type). We apologise if the main claims of the manuscript were not clear to the reviewer. This manuscript does not address the source of TF and we are not claiming that endothelial TF is reduced. The data presented in Figure 2 was an attempt to show the opposite, that there are no gross differences in the distribution of intravascular TF expression in two strains of mice. We have added a sentence to the discussion (page 15) to make this clearer. Rather, what we demonstrate is the differential effect of targeted delivery of an anti-coagulant to either BM derived or endothelial cells. Our results indicate that whilst equi-potent in inhibiting thrombin, endothelial targeted thrombin inhibition is significantly better at preventing thrombocytopenia.

Reviewer #2:
The authors have evaluated the differential effect of thrombin inhibition on either platelets or leucocytes or endothelial cells upon endotoxemia on coagulation activation and platelet concentration. They conclude that endothelial cell-associated thrombin is more important for platelet sequestration than other surfaces. This is an interesting observation that is original and the experiments appear to be done well. However, a few
concerns should be addressed:

1. In the abstract the authors mention tissue factor expression on "myeloid leucocytes". It may be more precise to use the term mononuclear cells. This has been addressed as suggested by the reviewer.

2. The authors have employed rather artificial in vitro and ex vivo models of cell culture and blood/plasma assays. In the absence of in vivo evidence they should be much more careful with their conclusions as in reality things may be very different from these purified models. The study would be significantly strengthened and the conclusions much more robust if in vivo evidence would be added to this set of experiments. We apologise if our experimental approach was not clear. The data presented is all derived in vivo, from mice treated with LPS/L-name to induce endotoxemia. The only in vitro data presented is that shown in Figure 1C. All other data are the results of in vitro tests/measurements of various haemostatic variables (TAT, fibrinogen, platelet counts) in plasma samples obtained from mice. In addition the bleeding time (Table 2) was performed on mice.

3. The authors have used a thrombin-antithrombin assay for detection of human thrombin-antithrombin complexes employing murine monoclonal antibodies. How can they be sure this assay is accurate in murine models? The Enzygost TAT micro ELISA was first shown to cross-react with murine thrombin-TAT complexes by Ravant et al (Blood Coagul Fibrinolysis 1995 6: 446-455) and has subsequently been used by a number of groups to measure TAT levels in murine plasma. For example:
Xu et al 2009 7: 851-856
Unruh et al Thrombosis Journal 2005 3: 21

4. In the discussion the authors seem to ignore the role of platelet P-selectin in upregulation and expression of tissue factor. The text in the Discussion section (page 16) has now been modified to address the importance of P-selectin in the platelet sequestration that occurs in endotoxaemia. However, the role of platelet derived TF is controversial, whilst human platelets have been shown to express TF mouse platelets do not and may only acquire TF from TF positive microparticles. For this reason and because it is not fundamental to this manuscript we have omitted a discussion of the contribution of platelet TF.

I do hope that you’ll now consider this revised manuscript suitable for publication.

Yours faithfully

A. Dorling