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Regular Article

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.

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

Abbreviations: BL/6, C57BL/6; EC, Endothelial cell; ePCR, Endothelial protein C receptor; FIX, Factor IX; FX, Factor X; HEQ, Hirudin Equivalent"; LPS, Lipopolysaccharide; PSGL-1, P-selectin glycoprotein ligand-1; TF, Tissue factor; TAT, Thrombin anti-thrombin; WT, Wild-type.

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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 NH_4Cl/10 mM $KHCO_3$/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 5 μg/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 mm^2 counted (= N). The number of platelets per litre of blood = $2\,N \times 10^9$. Platelet phenotype was confirmed with an anti-CD41 mAb (Pharmingen).

Flow Cytometry Analysis

1×10^5 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 10^5 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. 5×10^6 EC were added to 200 μl of plasma, recalcified and the time to clot measured.

Endotoxic Shock

All mice weighed 25 g +/- 1 g. 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) (50 mg/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