Differential Role of von Willebrand Factor and P-Selectin on Microvascular Thrombosis in Endotoxemia

Kavita N. Patel, Said H. Soubra, Ricardo V. Bellera, Jing-Fei Dong, Colleen A. McMullen, Alan R. Burns, Rolando E. Rumbaut

Objective—Endotoxin (lipopolysaccharide [LPS]) enhances microvascular thrombosis in mouse cremaster venules. Because von Willebrand factor (vWF) and P-selectin are suggested to mediate LPS-induced platelet–microvessel interactions, we determined whether vWF and P-selectin contribute to microvascular thrombosis in endotoxemia.

Methods and Results—A light/dye-induced thrombosis model was used in cremaster microvessels of saline or LPS-injected mice (wild-type, P-selectin–deficient, vWF-deficient, or littermate controls). In each strain except vWF-deficient mice, LPS enhanced thrombosis in venules, resulting in ≈30% to 55% reduction in times to thrombotic occlusion. LPS had no effect on thrombosis in vWF-deficient mice, although these mice had similar systemic responses to LPS (tachycardia, thrombocytopenia, and plasma coagulation markers). vWF-deficient mice demonstrated prolonged times to thrombotic occlusion relative to littermates. LPS increased plasma vWF in each strain studied. While immunofluorescence in wild-type mice failed to detect LPS-induced differences in microvascular vWF expression, it revealed markedly higher vWF expression in venules relative to arterioles.

Conclusions—vWF mediates light/dye-induced microvascular thrombosis and endotoxin-induced enhancement of thrombosis in mouse cremaster venules; P-selectin is not required for enhanced thrombosis in response to endotoxin. Enhanced vWF expression in venules relative to arterioles has potential implications for the differences in thrombotic responses among these microvessels. (Arterioscler Thromb Vasc Biol. 2008;28:2225-2230.)

Key Words: endotoxin • platelets • venules • intravital microscopy • sepsis

Sepsis, a systemic response to infection, is a major contributor to mortality in intensive care units, associated with endothelial dysfunction and a prothrombotic state. Endotoxin (lipopolysaccharide [LPS]) is a cell wall component of Gram-negative bacteria, which reproduces many manifestations of Gram-negative sepsis and is used frequently in experimental sepsis models. Endotoxemia promotes platelet adhesion to microvessels in vivo microvascular thrombosis, and enhances light/dye-induced microvascular thrombosis. Although the mechanisms responsible for prothrombotic manifestations of endotoxemia remain to be fully understood, suggested mediators include P-selectin and von Willebrand Factor (vWF). P-selectin is expressed on the surface of activated endothelial cells and platelets and may contribute to a prothrombotic state by several mechanisms. These include: (1) mediating leukocyte-dependent platelet adhesion to microvessels via its ligand P-selectin glycoprotein-1 (PSGL-1), (2) mediating adhesion of ultralarge vWF multimers to endothelium, (3) recruiting microparticles bearing tissue factor and PSGL-1 to developing thrombi, (4) promoting shear-dependent platelet aggregation, and (5) promoting a systemic prothrombotic state via its soluble form. P-selectin expression is increased in various tissues in endotoxemia and has been implicated as a mediator of platelet-microvessel interactions in this and various other inflammatory conditions.

Another potential mediator of these responses is vWF, a multimeric protein produced by endothelial cells and megakaryocytes. vWF is stored in Weibel-Palade bodies of endothelial cells and alpha granules of platelets. In the circulation, or when released from storage granules, vWF may bind to various receptors or counterligands relevant to hemostasis and thrombosis, including Factor VIII, glycoprotein Ibα, integrin αIIbβ3, collagen, and P-selectin. Endothelial vWF release, especially in the hyperactive ultralarge (UL) forms, is enhanced by various inflammatory stimuli, including endotoxin. Further, administration of endotoxin enhances plasma vWF levels in mice and humans. A reactive multimeric form of this molecule, ULvWF, mediates microvascular thrombosis in thrombotic thrombocytopenic
purpura.\textsuperscript{28} ULVWF multimers have also been described in clinical cases of severe sepsis,\textsuperscript{29,30} suggesting a potential role for vWF in microvascular thrombosis in this entity. Given these findings, we tested the hypotheses that vWF and P-selectin mediate the enhanced microvascular thrombosis induced by endotoxin.

**Materials and Methods**

Male mice, \( \sim 30 \) g of weight, were studied; all protocols were approved by the Animal Care and Use Committee of Baylor College of Medicine. We studied C57BL/6J (wild-type), P-selectin–deficient,\textsuperscript{31} vWF-deficient\textsuperscript{32} mice, and their littermate controls. P-selectin–deficient mice have been backcrossed onto a C57BL/6 J background for at least 10 generations,\textsuperscript{33} prior to homozygous breeding. vWF-deficient mice and their littermate controls (backcrossed on a C57BL/6 J background for \( \geq 8 \) generations) were generated by heterozygous breeding. Strains originated from the Jackson Laboratories (Bar Harbor, Maine); P-selectin- and vWF-deficient mice were genotyped by polymerase chain reaction (PCR) analysis of tail clippings.

**Animal Preparation**

Mice were anesthetized with an intraperitoneal (IP) injection of pentobarbital (30 mg/kg), with additional doses (12.5 mg/kg) as needed. The mice were then placed on a custom Plexiglas tray and maintained at 37°C with a homeothermic blanket, monitored with a rectal temperature probe (F.H.C.). A tracheotomy was performed to facilitate breathing, an internal jugular vein was cannulated for intravenous drug administration, and a carotid artery was cannulated for blood pressure and heart rate measurement. The cremaster muscle was exteriorized and prepared for intravital microscopy as described previously.\textsuperscript{6,34}

**Intravital Microscopy and Microvascular Thrombosis Model**

The preparation was placed under an upright microscope (BX-50, Olympus, NY) and observed with a 40X water immersion objective (N.A. 0.8) and allowed to equilibrate for 30 minutes. After the equilibration period, fluorescein isothiocyanate (FITC)-dextran (150 kDa, 10 mL/kg of a 5% solution) was injected via the venular catheter and allowed to circulate for \( \sim 10 \) minutes. Thereafter, venular diameter was measured (image 1.6, NIH, public domain software) as well as mean blood cell velocity (\( V_d \) doppler, using an optical Doppler velocimeter, Cardiovascular Research Institute, Texas A\&M University). Venular wall shear rate (\( \gamma \)) was calculated as \( 8(V_d/3.4)/\text{diameter} \).\textsuperscript{35}

After those measurements, light/dye-induced injury was begun by exposing \( \sim 100 \mu m \) of a venule length to epi-illumination, with a 175W xenon lamp (Lambda LS) and a fluorescein filter cube (HQ-FITC, Chroma). Excitation light was monitored daily (IL 1700 175W xenon lamp (Lambda LS) and a fluorescein filter cube (HQ-FITC, Chroma)). Epi-illumination was applied continuously and the following times were recorded: (1) Time of onset of platelet aggregates and (2) Time of flow cessation, for at least 60 seconds. In some cases, following venular flow cessation, an arteriole was selected and the measurements repeated as outlined above.

**Platelet Counts**

In several experiments as indicated in the text, \( \sim 400 \mu L \) of blood were collected via the arterial catheter and placed in an EDTA-coated blood collection tube (Baxter). Blood was diluted with a Unopette collection system (Becton Dickinson) per the manufacturer’s instructions, and platelets were counted with a hemocytometer.

**vWF and P-Selectin Expression**

Expression of vWF protein in cremaster venules and arterioles (identified by smooth muscle \( \alpha \)-actin staining) was assessed by immunofluorescence. Mouse cremasters were excised and fixed in cold acetone (\( -20°C \)) for 20 minutes, washed in PBS, permeabilized in PBS containing 0.1% Triton-X 100, and blocked in PBS containing 1% bovine serum albumin for 15 minutes. The tissue was then coincubated with FITC-labeled antibody against smooth muscle \( \alpha \)-actin (Sigma) and unlabeled rabbit polyclonal antibody against vWF (Dako). Subsequent incubation in Texas Red-labeled goat-antirabbit IgG was used for secondary detection of bound anti-vWF antibody. Nonimmune IgG species-matched equivalents were used in place of primary antibody for determination of nonspecific background fluorescence. Labeled cremasters were mounted in Airvol (Air Products and Chemicals) and digital images were captured on a DeltaVision microscope (Applied Precision) using a 40X oil immersion lens. Fluorescence intensity values for vWF labeling were evaluated using SoftWorx software (Applied Precision). For each vessel we obtained 5 measures of vascular fluorescence intensity, placing a 10 \( \times \)10 \( \mu m \) window on the center of the vessel, and 5 measures of interstitial fluorescence (placing the window 10 \( \mu m \) away from the vessel wall). Final measures were obtained by subtracting background and interstitial from vascular fluorescence values.

**Measurement of Plasma vWF and Coagulation Markers**

Plasma vWF was measured with a commercially available ELISA kit (Ramco Laboratory), according to the manufacturer’s instructions. This kit was developed for human vWF, but the goat antihuman antibody used in the kit cross-reacts with mouse vWF. Plasma vWF was measured in saline- and LPS-injected mice of each strain except vWF-deficient mice. For these experiments, mice did not receive FITC-dextran and did not undergo light/dye-induced thrombosis. Plasma coagulation markers were measured in saline- and LPS-injected mice of each strain. Prothrombin time (PT) and activated partial thromboplastin time (aPTT) were measured using a STAGO STA-R analyzer (Diagnostica Stago). Plasma fibrinogen levels were determined using STA Fibrinogen Kit (Diagnostica Stago), thrombin-antithrombin (TAT) complexes were obtained using a commercially available ELISA kit (Enzymost, Siemens), according to the manufacturer’s instructions. The TAT kit uses antihuman antibodies; however, there is cross-reactivity with murine specimens. As in the case of plasma vWF measurements, these mice did not receive FITC-dextran and did not undergo light/dye-induced thrombosis protocols.

**Experimental Groups**

To determine the influence of endotoxemia on microvascular thrombosis, mice were injected intraperitoneally with either endotoxin (LPS) from *Escherichia coli* serotype 0111:B4 (Sigma \#L3024, endotoxin content 1X10\(^6\) EU/mg) at 4 or 5 mg/kg in 0.5 mL of sterile, pyrogen-free isotonic saline, 4 hours before photoactivation. For all experiments, the investigator performing intravital microscopy was blinded with regards to the injected agent (saline versus LPS). In all cases except experiments done in P-selectin–deficient mice, the investigator was also blinded to the mouse genotype, because the microvascular phenotype of P-selectin–deficient mice precluded blinding (ie, virtual absence of rolling leukocytes in venules).

**Statistics**

All data are expressed as mean±SE except for data not distributed normally, which are shown as median±interquartile range. Comparisons by genotype within each test group (ie, LPS versus saline) were done with 1-way analysis of variance with Fisher’s posthoc test, and nonparametric Mann-Whitney \( U \) test, as appropriate, using Statview 5.01 statistical software (SAS Institute, NC). A probability value of <0.05 was considered statistically significant.

**Results**

Intravital microscopy was performed on 72 mice with weight of 29.6±0.5 g, venule diameter of 44.4±0.3 \( \mu m \) and wall...
shear rate of 465±13 s⁻¹. There were no statistical differences in weight, microvessel diameters and wall shear rates within each comparison group (data not shown).

**Endotoxemia Enhanced Microvascular Thrombosis Independent of P-Selectin**

Figure 1 depicts the influence of endotoxin on kinetics of light/dye-induced thrombosis in venules of both wild-type (WT) and P-selectin–deficient mice. In WT mice, LPS enhanced both the time of onset and time to flow cessation as shown previously. In P-selectin–deficient mice, preliminary experiments revealed lower success using the same LPS dose as in WT mice, attributable to frequent cremaster preparations with sluggish microvascular flow, hypotension, or mortality. However, a 20% lower dose of LPS was not associated with the above-mentioned problems in P-selectin–deficient mice, and resulted in enhanced microvascular thrombosis, both in time of onset and flow cessation, as shown in Figure 1. Mean arterial pressure did not differ statistically between the saline- and LPS-treated mice shown in Figure 1, although heart rate was ≈30% to 40% higher in LPS-treated mice in both genotypes ($P<0.01$ in each case).

To determine whether LPS-induced enhancement of thrombosis occurred in arterioles in a manner analogous to venules, thrombotic responses of arterioles in saline- and LPS-treated wild-type mice (with similar wall shear rate of 1411±105 s⁻¹ and 1438±127 s⁻¹, respectively) were compared. Endotoxin resulted in a 24% reduction in time to flow cessation (from 32±2.5 minutes to 24.5±2.8 minutes, $P<0.05$) with no difference in time of onset of thrombosis. Given the predominance of LPS-induced responses in venules as compared to arterioles, also noted previously, subsequent assessments of the influence of LPS on thrombosis was limited to venules.

**Role of vWF in Microvascular Thrombosis in Endotoxemia**

Figure 2 illustrates thrombotic responses in venules of vWF-deficient (vWF⁻⁻) mice and littermate controls (vWF⁺⁺). Saline-injected vWF⁻⁻ mice demonstrated significant delay in times to thrombotic occlusion as compared to littermates. Further, endotoxin had no effect on microvascular thrombosis in vWF⁻⁻ mice, whereas it enhanced thrombosis in vWF⁺⁺ mice. Despite a lack of a prothrombotic response in LPS-injected vWF⁻⁻ mice, these mice had comparable systemic responses to LPS as their littermate controls with regards to reductions in circulating platelet counts and enhanced heart rate (Figure 3). Mean arterial pressure did not differ between saline- and LPS-injected mice (data not shown), consistent with prior data in this model of endotoxemia. To exclude the possibility that the reduction in platelet counts was influenced by the light/dye-induced thrombosis model, we measured platelet counts twice in a separate group of 8 mice, first during the equilibration phase and again at the conclusion of the experiments. Platelet counts were unaffected by the thrombosis protocol, platelet counts expressed as a pre-to-post thrombosis ratio were 1.02±0.05 (N.S.). Further, we measured platelet counts in a separate group of 12 saline- and LPS-injected wild-type mice, which did not receive FITC-dextran or light/dye-induced thrombosis; LPS resulted in a 20% reduction in platelets at 4 hours in these mice ($P<0.05$).
Influence of Endotoxin on Plasma vWF and Coagulation Markers

In the presented study, it was determined whether the present model of endotoxemia results in increased plasma vWF, as described by others. As shown in Figure 4, LPS resulted in significant increases in plasma vWF in each genotype (not done in vWF−/− mice) 4 hours after injection, the time at which mice were studied for microvascular thrombosis. To determine whether systemic coagulation responses to LPS differed among the various groups, we measured plasma PT, aPTT, fibrinogen, and thrombin-antithrombin complexes (TAT) in saline- and LPS-treated mice of each genotype. As shown in Table, LPS resulted in prolonged aPTT and markedly enhanced TAT levels in each genotype. Although mean PT was slightly prolonged in LPS-treated mice of each genotype, the difference was only statistically significant in WT mice. LPS had no significant effect on plasma fibrinogen in any of the groups.

Table. Changes in Coagulation Markers in Wild-Type, P-Selectin−/−, VWF+/+, and VWF−/− Mice Exposed to LPS or Saline

<table>
<thead>
<tr>
<th></th>
<th>PT, sec</th>
<th>aPTT, sec</th>
<th>Fibrinogen, mg/dl</th>
<th>TAT, μg/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Saline</td>
<td>13.3±0.3</td>
<td>26.6±0.8</td>
<td>163.9±5.2</td>
<td>5.5±2.0</td>
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<tr>
<td>LPS</td>
<td>14.9±0.4*</td>
<td>35.9±2.0*</td>
<td></td>
<td>20.5±2.3†</td>
</tr>
<tr>
<td>P-Selectin−/−</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Saline</td>
<td>12.2±0.2</td>
<td>24.7±0.6</td>
<td>171.7±4.8</td>
<td>9.4±2.7</td>
</tr>
<tr>
<td>LPS</td>
<td>12.8±0.3</td>
<td>28.6±1.2*</td>
<td></td>
<td>30.3±1.7†</td>
</tr>
<tr>
<td>VWF+/+</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Saline</td>
<td>12.3±0.3</td>
<td>24.9±1.1</td>
<td>191.5±5.9</td>
<td>9.0±2.7</td>
</tr>
<tr>
<td>LPS</td>
<td>13.5±0.3</td>
<td>32.1±2.6*</td>
<td></td>
<td>31.1±10.5*</td>
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<tr>
<td>VWF−/−</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Saline</td>
<td>12.7±0.2</td>
<td>30.9±1.1</td>
<td>174.3±14.3</td>
<td>5.1±1.5</td>
</tr>
<tr>
<td>LPS</td>
<td>13.5±0.4</td>
<td>39.3±3.5*</td>
<td></td>
<td>29.9±10.3*</td>
</tr>
</tbody>
</table>

Data shown as mean±SE of plasma prothrombin time (PT), activated partial thromboplastin time (aPTT), fibrinogen, and thrombin-antithrombin complexes (TAT). *P<0.05, †P<0.005, for comparison with saline group.

Discussion

In this study, vWF expression in cremaster venules and arterioles was assessed using immunofluorescence. Microvascular thrombosis in wild-type, P-selectin−/−, and vWF−/− mice was compared to saline controls. In vWF-deficient mice, it had no effect on microvascular thrombosis, whereas LPS enhanced thrombosis in WT and P-selectin−/− mice. However, vWF−/− mice had comparable systemic responses to LPS as vWF+/+ mice, with regards to platelet counts and heart rate, consistent with a recent report. Similarly, vWF−/− mice had comparable LPS-induced changes in systemic coagulation markers as the other mouse genotypes. Thus, the role of vWF in microvascular thrombosis appears distinct from the systemic coagulation changes evident in endotoxemia. The enhanced plasma levels of vWF in WT mice at the time of thrombosis induction are also consistent with a role for vWF in enhanced thrombosis in this model. For vWF in enhanced thrombosis in endotoxemia is supported by the observation that LPS administration to healthy humans results in enhanced plasma vWF and ULvWF. Similarly, enhanced levels of ULvWF have been described in both adult and pediatric patients with sepsis and ischemic organ failure. Whether ULvWF newly secreted from endothelium, as opposed to plasma vWF, mediates the enhanced thrombosis to LPS could not be distinguished in the present study.

In addition to a lack of response to endotoxin, vWF−/− mice had delayed microvascular thrombosis under control conditions, consistent with data obtained in a ferric chloride model of thrombosis with this strain and those obtained with mice deficient in the vWF receptor GPIbα. The findings of this study support the hypothesis that vWF mediates light/dye-induced microvascular thrombosis, as suggested previously in a rat mesentery model. In our experiments, vWF−/− mice
demonstrated delayed times to thrombotic occlusion, but comparable times of onset of thrombosis. These findings are consistent with data obtained in a laser injury model, in which vWF was not required for platelet activation, but participated in platelet accumulation in a tissue factor-mediated pathway. Further, the greater vWF expression of mouse cremaster venules relative to arterioles (Figure 5) is consistent with the known predisposition of mouse cremaster venules to light/dye-induced thrombosis described previously. Of interest, despite the low vWF expression in arterioles of WT mice, vWF+ mouse demonstrated prolonged times to thrombotic occlusion in arterioles as compared to littermates. Although these findings are consistent with a role for plasma (or platelet) vWF in this model of microvascular thrombosis, we cannot exclude a contribution of arteriolar endothelial cell vWF in these responses. Differential vWF expression in mouse venules and arterioles has been identified in other vascular beds. It is interesting to speculate that the predominance of vWF expression in cremaster venules may be related to the enhanced venular thrombotic response to LPS described in this study and previously.

The observation that P-selectin is not required for enhanced microvascular thrombosis in endotoxemia is interesting, given findings that P-selectin mediates endotoxin-induced platelet-microvesicle recruitment as well as the procoagulant activity associated with its soluble form. The results appear to contradict previous studies showing that recruitment of platelets to inflamed microvessels in endotoxemia (in the absence of light/dye-injury) depend on adherent leukocytes, primarily neutrophils. However, we showed recently that neutrophils were not required for LPS-induced enhancement of microvascular thrombosis in mouse cremaster venules. Further, the magnitude of leukocyte-dependent platelet adhesion in mouse cremaster venules was estimated to result in 2% of endothelial surface covered by platelets, likely having a minimal contribution to light/dye-induced occlusive thrombi. Of interest, Falati et al described a role for P-selectin in a laser-induced model of microvascular thrombosis, via PSGL-1 and tissue factor-bearing microparticles. In that report, P-selectin–deficient mice that were backcrossed for 10 generations onto a C57BL6/J background demonstrated markedly greater expression in venule. B. Superimposed Green (smooth muscle α-actin) and Red fluorescence of the same image shown in panel A confirms smooth muscle content of arteriole. C, IgG control, red fluorescence. Bar = 30 μm.

In conclusion, these data demonstrate a differential role of vWF and P-selectin in microvascular thrombosis in endotoxemia in a light/dye-induced model in mouse cremaster venules. The findings support an important role for vWF in the endotoxin-induced enhancement of thrombosis, which is independent of P-selectin. The influence of vWF on microvascular thrombosis in clinical sepsis remains to be clarified.

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Disclosures
None.

References

Figure 5. Immunofluorescence staining for vWF in a cremaster venule and arteriole obtained from an LPS-treated mouse. A, Red fluorescence (vWF) demonstrates markedly greater expression in venule. B, Superimposed Green (smooth muscle α-actin) and Red fluorescence of the same image shown in panel A confirms smooth muscle content of arteriole. C, IgG control, red fluorescence. Bar = 30 μm.


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