Characterization of a Mouse Model for Thrombomodulin Deficiency


Abstract—Mutations in the gene encoding thrombomodulin (TM), a thrombin regulator, are suspected risk factors for venous and arterial thrombotic disease. We have previously described the generation of TM<sup>Pro/Pro</sup> mice carrying a TM gene mutation that disrupts the TM-dependent activation of protein C. Here, it is shown that inbred C57BL/6J TM<sup>Pro/Pro</sup> mice exhibit a hypercoagulable state and an increased susceptibility to thrombosis and sepsis. Platelet thrombosis growth after FeCl<sub>3</sub>-induced acute endothelial injury was accelerated in mutant mice. Vascular stasis after permanent ligation of the carotid artery precipitated thrombosis in mutant but not in normal mice. Mutant mice showed increased mortality after exposure to high doses of endotoxin and demonstrated altered cytokine production in response to low-dose endotoxin. The severity of the hypercoagulable state and chronic microvascular thrombosis caused by the TM<sup>Pro</sup> mutation is profoundly influenced by mouse strain–specific genetic differences between C57BL/6 and 129SvPas mice. These data demonstrate that in mice, TM is a physiologically relevant regulator of platelet- and coagulation-driven large-vessel thrombosis and modifies the response to endotoxin-induced inflammation. The phenotypic penetrance of the TM<sup>Pro</sup> mutation is determined by as-yet-uncharacterized genetic modifiers of thrombosis other than TM.

Key Words: transgenic mice | thrombomodulin | thrombosis | inflammation | thrombosis modifier genes

Inherited defects in the thrombomodulin (TM)-protein C system, such as heterozygous deficiency of protein C and S or the factor V Leiden mutation, are among the most commonly recognized causes of venous thrombosis. TM gene polymorphisms appear to be rare in the general population, and the thrombosis risk associated with these mutations may largely depend on the presence of compounding variables. Prospective studies suggest that low levels of TM expression may be associated with increased incidence of coronary heart disease. The first gene mutation causing reduced TM function (insT1689) was discovered in a kindred with familial myocardial infarction. These observations suggest that reduced TM function contributes significantly to the pathogenesis of arterial thrombosis, whereas protein C deficiency or resistance to activated protein C (APC) is predominantly associated with venous thrombosis. These differences could reflect protein C–independent functions of TM, such as its interaction with the thrombin-activatable inhibitor of fibrinolysis or protein C–dependent processes unrelated to the inactivation of factor Va. Protein C/APC possesses anti-inflammatory activities that depend on the engagement of specific receptors for APC and/or protein C on monocytes and endothelial cells.

We previously introduced a mutation into the mouse TM gene (TM<sup>Pro</sup>, Glu404→Pro) that reduces the expression of TM and disrupts its ability to augment the activation of protein C. TM<sup>Pro/Pro</sup> mice show augmented intravascular fibrin deposition but remain free from overt thrombosis. A superimposed defect in tissue plasminogen activator–dependent fibrinolysis exacerbated fibrin deposition in specific organs of TM<sup>Pro/Pro</sup> mice and caused focal myocardial necrosis.

The goal of the present study was to exploit this mouse model of TM deficiency. First, we compared the consequences of reduced TM expression/function on platelet-dependent thrombus formation after endothelial injury and coagulation-driven thrombus formation after vascular stasis (the 2 dominant pathogenic mechanisms underlying arterial and venous thrombosis, respectively). Second, we sought to determine whether the genetic suppression of TM function in mice was indeed associated with increased sensitivity to
endotoxin-induced septicemia, as predicted from the above observations on the role of the protein C pathway in inflammation. Third, we examined to what extent the phenotypic penetrance of the TM<sup>pro</sup> mutation was determined by the interaction with other mouse strain–specific thrombosis modifier genes.

**Methods**

**Transgenic Animals**

Mouse strains with altered TM genes, referred to as TM<sup>pro</sup>, and TM<sup>wt</sup>, have been described earlier. The mutation in TM<sup>pro</sup> mice causes 2 -3 fold reduced TM expression and disrupts protein C activation. In TM<sup>wt</sup> mice, insertion of the β-galactosidase reporter gene leads to a complete elimination of TM expression. TM<sup>pro</sup> and TM<sup>pro/</sup> mice were derived by inbreeding nonlittermate N9 C57BL/6J TM<sup>pro</sup> mice or TM<sup>pro</sup> mice. The C57BL/6J-derived Y chromosome was introduced by breeding N8 C57BL/6J TM<sup>pro</sup> females with wild-type C57BL/6J males. Normal C57BL/6J and 129SvPas mice were obtained from Charles River Laboratories, Wilmington, Mass. All animal experiments were approved by the institutional animal care and use committee at the Medical College of Wisconsin.

**Acute Carotid Injury Model**

FeCl<sub>3</sub>-induced arterial injury was induced according to published procedures. The right and left common carotid arteries were exposed by blunt dissection, and blood flow was recorded with miniature Doppler flow probes (model 0.5VB, Transonic Systems) positioned around the distal end of the arteries. Ten minutes after probe placement, a 1.0 x 0.6-mm<sup>2</sup> strip of filter paper soaked in 20% FeCl<sub>3</sub> was applied to the adventitial surface of the artery for 1 minute. The field was flushed with saline, and flow was continuously monitored. In some experiments, the procedure was then repeated on the right common carotid artery. Occlusion times were expressed as t<sub>50</sub> (the time at which pulse amplitude flow dropped to 50% of the initial flow, defined as the average flow occurring between minute 2 and minute 4 after injury). Mixed-models ANOVA was used to assess the relationship of genotype, side, and genotype-side interaction on outcome variables. To adjust for the potential effect of variations in initial flow on t<sub>50</sub>, t<sub>50</sub> was used as the dependent variable, and genotype, initial flow, and side were used as potential predictors.

**Carotid Artery Ligation**

The left common carotid artery was dissected and ligated near the carotid bifurcation as described. After 4 weeks, all animals were perfused at physiological pressure with 4% paraformaldehyde in 0.1 mol/L sodium phosphate buffer, pH 7.3. The left and right common carotid arteries were dissected and embedded in paraffin, and serial sections (5 μm thick) were cut at 150-μm intervals spanning the entire length of the vessel. Digitized images of these vessels were analyzed by using image analysis software (NIH Image 1.60) as described.

**Lipopoly saccharide Challenge**

Animals were injected intraperitoneally with the indicated amounts of bacterial endotoxin (Escherichia coli serotype 055:B5, Sigma Chemical Co) in 200 μL sterile PBS. At 0.5, 2.0, 6.0, or 24 hours after endotoxin injection, the mice were anesthetized, and blood was collected via cardiac or retro-orbital puncture as described below.

**Determination of Serum Cytokine Levels, TAT Complexes, D-Dimer, Fibrinogen, FPA, PAP Complexes, and Cross-Linked Tissue Fibrin**

For cytokine analysis, blood was collected via cardiac or retro-orbital puncture, and concentrations of tumor necrosis factor (TNF)-α, interleukin (IL)-1β, and IL-6 were measured by using Quantikine M mouse immunoassay kits according to the manufacturer’s instructions (R&D Systems). Ten minutes before the collection of samples for analysis of fibrin deposition in tissues, total plasma fibrinogen, D-dimer, and the thrombin-antithrombin (TAT) complex, mice were injected intraperitoneally with 8000 U heparin (unfractionated porcine heparin, sodium salt, molecular weight 6000 to 30 000, Life Technologies). Blood samples for measurement of D-dimer and TAT complex were then collected into 1/10 volume of 3.2% sodium citrate and analyzed by ELISA systems (Asserachrom D-Di, American Bioproducts Co; Enzygnost TAT micro, Behring Diagnostics Inc). Values given reflect the apparent levels of mouse TAT and D-dimer obtained by comparison to a standard curve with human TAT and D-dimer. With the use of purified D-dimer prepared from mouse fibrin, the anti-human D-dimer assay system was found to detect the murine antigen with ~20-fold reduced sensitivity. Fibrinogen levels were measured by sandwich ELISA with the use of rabbit anti-human fibrinogen antibody (Dako Corp) and peroxidase-conjugated goat anti-mouse fibrinogen antibody (Accurate International). The assay was calibrated with purified mouse fibrinogen. The amount of cross-linked fibrin present in mouse tissues was determined by semiquantitative Western blot analysis essentially as described in a previous publication. Detection of fibrinopeptide A (FPA) and plasmin-antiplasmin (PAP) complex was accomplished as described earlier.

**Results**

**Characterization of Hemostatic System Activity in Inbred C57BL/6J TM<sup>pro</sup> Mice**

Inbred C57BL/6J TM<sup>pro</sup> mice with a ≥88% contribution of the C57BL/6J genetic background were generated by repeated crosses with wild-type C57BL/6J mice (see Methods). Plasma levels of TAT, D-dimer, FPA, and PAP complex levels were, on average, higher in TM<sup>pro</sup> mice than in wild-type mice (respective values are as follows: TAT, 21.0±6.6 versus 18.4±4.1 μg<sub>apparent</sub>/L, P<0.05; FPA, 3.4±0.9 versus 2.2±1.4 ng/L, P<0.03; D-dimer, 16.6±2.6 versus 8.1±2.2 μg<sub>apparent</sub>/L, P<0.002; and PAP, 5.2±6.1 versus 2.5±1.1 μg<sub>apparent</sub>/L, P<0.22). The amount of soluble TM antigen, measured by ELISA, was reduced in the plasma of mutant mice (6.5±2.6 versus 13.9±4.6 A<sub>405</sub> · min<sup>-1</sup> · mL<sup>-1</sup>, P<0.05). Plasma fibrinogen concentration and range of concentration were similar in both groups of animals. Bleeding after standardized tail transection was determined as a crude measurement of the overall hemostatic response. Initial (t=5 minutes) blood loss was somewhat greater in TM<sup>pro</sup> mice (P<0.08), but over time (30 minutes), mutant and normal mice controlled bleeding from the wound to an identical extent.

**TM Deficiency Accelerates Arterial Thrombus Growth After Acute Endothelial Injury**

Platelet-rich occlusive thrombi formed in TM<sup>pro</sup> mice after FeCl<sub>3</sub>-induced injury were histologically grossly indistinguishable from those in wild-type mice. TM<sup>pro</sup> mice exhibited a statistically significant reduction in t<sub>50</sub> (wild-type mice, n=31, 9.7±2.5 [mean±SD] minutes; TM<sup>pro</sup> mice, n=13, 7.6±1.3 minutes; P<0.05). In a subgroup of 5 TM<sup>pro</sup> animals, the right and the left artery were injured consecu-
Thrombus Formation

The left common carotid arteries of 12 wild-type and 19 hypercoagulable TM Pro/Pro mice were permanently occluded by ligation near the carotid bifurcation. All animals recovered from the surgical intervention without complications. In all wild-type mice, thrombotic occlusion was restricted to within 1 mm from the site of ligation. In contrast, 11 TM Pro/Pro mice developed far more extensive thrombotic lesions extending over the entire length of the artery (n=5). The majority of occlusive thrombi exhibited extensive cellular organization, canalization, and/or vascular remodeling at the time of analysis (Figure 1b and 1c). In the remaining 8 TM Pro/Pro mice, the average distance from the ligature at which the vessel was still occluded was significantly longer than that in wild-type mice. (1.81±0.5 versus 0.25±0.08 [mean±SEM] mm, respectively; P<0.005). In 6 of these mice, clot remnants or a highly irregular intimal architecture suggestive of the previous presence of mural thrombi was observed (Figure 1e and 1f). The quantitative assessment of vessel wall architecture in thrombus-free vessel segments in the latter group of TM Pro/Pro mice, compared with wild-type mice, showed a tendency toward increased neointimal mass in the ligated artery (6500±2712 [mean±SD] versus 3333±1214 μm², respectively; P=0.29). Medial area and lumen diameter of the left (control) carotid artery were similar in both groups of animals. Immunohistochemical staining of the arteries 2 or 4 weeks after ligation failed to demonstrate significant injury-induced synthesis of TM antigen in vascular smooth muscle cells, whereas endothelial cells retained robust TM expression (not shown).

TM Deficiency Causes Increased Mortality and Altered Cytokine Production in Lipopolysaccharide-Induced Septicemia

Under ambient husbandry conditions, the TM Pro mutation was not associated with systemically increased plasma levels of IL-1β, TNF-α, or IL-6 (data not shown). No significant differences in steady-state mRNA levels of various mouse cytokines, endothelial cell–leukocyte adhesion molecules, or fibrinolytic regulators were detected in tissues from wild-type and mutant mice (data not shown). Intravital fluorescence microscopy was used to compare endothelial cell–leukocyte rolling interactions in mutant mice with those in control mice. The fraction of rolling leukocytes and the frequency distribution of rolling velocities in small venules of the cremaster muscle and ear skin were indistinguishable in normal and mutant mice (percent rolling cells in total leukocyte flux in ear venules, 61.2±17.1% [mean±SD] versus 60.8±18.8% in wild-type mice; percent rolling cells in total leukocyte flux in cremaster venules, 39±16.5% versus 36±19% in wild-type mice). Antibody-mediated blocking of E-selectin led to an increase in average rolling velocities, the magnitude of which was identical in both animal groups and tissues. The fraction of rolling leukocytes was again identical in normal and mutant mice (data not shown). These data indicate that the constitutive or surgical trauma–induced expression of endothelial selectins and of their carbohydrate ligands on circulating leukocytes is not affected by the TM Pro mutation.

Intraperitoneal injection of 40 mg lipopolysaccharide (LPS)/kg caused death after 72 hours in 50% of wild-type mice and was subsequently used to compare the survival of TM Pro/Pro and wild-type mice after challenge with an LD₅₀ of LPS. The survival of TM Pro/Pro mice was significantly impaired compared with survival of control mice (P=0.012, by Mantel-Cox log-rank test): 5 of 12 wild-type mice recovered completely from LPS-induced septicemia, whereas the same dose of LPS caused earlier death in TM Pro/Pro mice, and none of the mutant mice survived beyond 5 days. Peak TNF-α serum levels induced by an LD₅₀ LPS dose were comparable in both groups of mice (≈20 ng/mL).
A sublethal dose of 5 mg LPS/kg body mass caused a transient increase in IL-1β, TNF-α, and IL-6 levels in all experimental animals and resulted in significant genotype-dependent differences in the magnitude and time course of the cytokine response (Figure 2). TMPro/Pro mice exhibited significantly higher serum concentrations of IL-6 and IL-1β and earlier maximal IL-1β release. The mean TNF-α level was 2-fold lower in TMPro/Pro mice than in wild-type mice. In both animal groups, plasma fibrinogen concentration remained unaltered at least until 6 hours after LPS challenge but was elevated 1.7-fold after 24 hours. D-dimer levels increased in all mice as early as 2 hours after LPS administration and continued to rise until the next day. The relative increase in D-dimer levels over baseline values was similar in wild-type and mutant mice. Mean PAP levels were at all time points higher in TMPro/Pro mice than in control mice (at 6 hours, 27.5 ± 1.1 [mean ± SD] versus 7.9 ± 3.5 ng/mL, respectively; P < 0.001). Because of a relatively large (compared with baseline levels) variability, the difference in FPA levels did not cause significant intravascular fibrin deposition in some, but not all, organs of mutant mice.18 In contrast, fibrin deposition in the lung, spleen, and heart of inbred C57BL/6J TMPro/Pro mice was not different from that observed in wild-type C57BL/6J mice (Figure 4). Mating of C57BL/6J TMPro/Pro with wild-type 129SvPas mice and subsequent intercrossing of the resulting F1 hybrids yielded TMPro/Pro mice with a mixed 129SvPas/C57BL/6J composition identical to that present in the animal population subjected to our previous analysis. Reintroduction of the 129SvPas genetic component restored the markedly augmented fibrin deposition in the heart, lung, and spleen observed earlier (Figure 4).

The amount of TMPro antigen was 1.3-fold higher in lung tissue extracts of C57BL/6J TMPro/Pro mice than of mixed background TMPro/Pro mice (739 ± 42 versus 591 ± 51 [mean ± SD] A405 · min−1 · g−1). To determine whether fibrin deposition was caused by these strain-specific differences in TM expression levels, TM antigen levels and fibrin deposition were measured in C57BL/6J TMPro/Pro with wild-type 129SvPas/129 genetic background, the TMPro mutation was associated with significant intravascular fibrin deposition in some, but not all, organs of mutant mice.18 In contrast, fibrin deposition in the lung, heart, spleen, and lung was more pronounced in mutant mice (Figure 3). LPS did not elicit detectable fibrin deposition in the brain at either dose (ie, LD50 and 5 mg/kg).

Mouse Strain–Specific Genetic Modifiers Determine the Severity of the Hypercoagulable State and Microvascular Thrombosis in TMPro/Pro Mice

In our previous analysis of TMPro/Pro mice with a mixed 129/C57 genetic background, the TMPro mutation was associated with significant intravascular fibrin deposition in some, but not all, organs of mutant mice.18 In contrast, fibrin deposition in the lung, spleen, and heart of inbred C57BL/6J TMPro/Pro mice was not different from that observed in wild-type C57BL/6J mice (Figure 4). Mating of C57BL/6J TMPro/Pro with wild-type 129SvPas mice and subsequent intercrossing of the resulting F1 hybrids yielded TMPro/Pro mice with a mixed 129SvPas/C57BL/6J composition identical to that present in the animal population subjected to our previous analysis. Reintroduction of the 129SvPas genetic component restored the markedly augmented fibrin deposition in the heart, lung, and spleen observed earlier (Figure 4).

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Figure 2. Endotoxin-induced alterations of cytokine production and hemostatic parameters. Mice were injected intraperitoneally with 5 mg LPS/kg body wt, and the plasma concentration of cytokines and coagulation/fibrinolysis products was determined at the indicated time points as detailed in Methods. Columns represent the mean ± SD of duplicate analysis of 4 animals each. Compared with wild-type C57BL/6J mice (open bars), C57BL/6J TMPro/Pro mice (solid bars) exhibit increased IL-6 and IL-1β production but reduced TNF-α elaboration. *Statistically significant at the P < 0.05 confidence level.

Figure 3. LPS-induced tissue fibrin deposition. Tissues were harvested 6 hours after administration of LPS/PBS, and fibrin-enriched fractions were resolved by SDS-PAGE, and subjected to Western blot analysis with fibrin-specific antibodies. Varying amounts of purified mouse fibrin were included as reference standards in lanes 5 to 10. Lanes 1 to 4 and 11 to 14 contain samples from 4 individual animals, each treated either with PBS (control, lanes 1 to 4) or LPS (lanes 11 to 14). Compared with wild-type (wt) C57BL/6J mice, C57BL/6J TMPro/Pro mice exhibit augmented fibrin deposition in the lung and kidney. LPS treatment did not cause significant intravascular fibrin deposition in the brain of normal or mutant mice. The signal visible in lane 4 of the top panel is an artifact due to shifting of the film during exposure.
C57BL/6J TM Pro/Pro mice and 1.5-fold lower than in TM Pro/Pro mice with a mixed background. Yet, the amount of cross-linked tissue fibrin present in the lung, spleen, and heart was not altered by this manipulation (Figure 4, bottom panel). This result demonstrates that the augmented fibrin formation/deposition seen in TM Pro/Pro mice with a mixed genetic background must be caused by genetic variance in a gene(s) other than TM.

TAT and D-dimer levels were substantially elevated in mixed background TM Pro/Pro mice compared with inbred C57BL/6 TM Pro/Pro mice (for TAT, 58 ± 16 [mean ± SD] versus 21.1 ± 6 μg apparent/L, respectively; for D-dimer, 220 ± 36 versus 16.6 ± 6 μg apparent/L, respectively; n = 10 animals per group; P < 0.001). A survey of hemostatic and hematologic parameters in wild-type C57BL mice and 129SvPas mice showed marginally shortened prothrombin time (10.8 ± 0.4 versus 10.6 ± 0.4 seconds, respectively; n = 10 per group) and activated partial prothrombin time (16.1 ± 1.4 versus 15.7 ± 1 seconds, respectively) in 129SvPas mice. In 129SvPas mice compared with C57BL/6 mice, hematocrit and mean corpuscular red blood cell volume were larger (hematocrit 45.2 ± 2% versus 49.9 ± 2%, respectively [P < 0.005]; mean cell volume 44.6 ± 0.5 versus 48.5 ± 0.5 fL, respectively [P < 0.001]), and platelet counts were 2-fold lower (0.91 ± 0.1 × 10^6 /L versus 0.52 ± 0.2 × 10^6 /L, respectively). In TM Pro/Pro mice with a mixed genetic background, red blood cell volume and hematocrit approached the values seen in wild-type 129SvPas mice (mean cell volume 48.4 ± 1.4 fL, hematocrit 48.7 ± 1.6%, whereas platelet counts were similar to those in wild-type C57BL/6 animals (0.6 ± 0.1 × 10^6 /L). Despite reduced platelet numbers in wild-type 129SvPas mice, occlusion time after FeCl3-induced injury was not significantly prolonged compared with C57BL/6 mice (for 129SvPas mice, 11 ± 2.7 minutes, and for C57BL/6 mice, 9.7 ± 2.5 minutes; P = 0.18). Occlusion time in mixed background TM Pro/Pro mice was comparable to that in C57BL/6 TM Pro/Pro mice (8.4 ± 1.3 and 7.6 ± 1.3 minutes, respectively; P = 0.3) and remained signifi-cantly different from that measured in C57BL/6 wild-type mice (P < 0.05).

**Discussion**

We have shown previously that the introduction of a point mutation (Glu404→Pro) in the EGF4/5-interdomain loop of the mouse TM gene disrupts the ability of TM to enhance the thrombin-mediated activation of protein C. The defect was associated with increased intravascular fibrin deposition, but mutant mice remained free from overt thrombosis and exhibited a normal life span. In the present study, we chose 2 experimental models of vascular injury to determine the effects of the TM Pro/Pro mutation on the 2 major pathways leading to large-vessel thrombosis, ie, platelet-predominant thrombosis after acute endothelial injury and clot formation after stasis-induced activation of the coagulation system. Ligation of the common carotid artery reduces blood flow to a residual pulsatile mode and induces reproducible alterations of arterial wall function by a combination of neo-intimal hyperplasia and remodeling.23,28 The model is distinguished by the continued presence of a functional endothelium and is, therefore, uniquely suited to investigate the contribution of endothelial surface–associated factors to thrombosis prevention. In animals with normal coagulation systems, thrombus deposition remains localized at the site of the ligation.23,29 In contrast, in TM Pro/Pro mice, the model elicited extensive and persistent thrombotic occlusion with subsequent remodeling and thrombus organization. Thus, in mice, functional TM defects constitute a strong risk factor for thrombosis caused by unfettered coagulation activation under conditions of reduced blood flow. Vascular injury has been reported to cause transient TM expression by smooth muscle cells, which might influence vascular wall remodeling. Indeed, we observed induction of TM gene transcription in nonendothelial vascular wall cells in some of the ligated arteries (data not shown), and the morphometric analysis of nonoccluded vessel segments showed a tendency toward increased intimal hyperplasia in mutant mice. However, TM antigen expression in neo-intimal and medial smooth muscle cells was below the limit of detection with TM-specific antibodies. Therefore, the vascular pathology observed in TM Pro/Pro mice after ligation-induced stasis is likely dominated by the reduced anticoagulant capacity of the vascular endothelium. In contrast to ligation-induced injury, the topical application of a FeCl3-solution results in acute damage of the endothelial cell layer and formation of an occlusive platelet-rich thrombus. Reduced TM activity caused a small acceleration of platelet-thrombus growth, indicating that diminished levels of soluble and/or endothelium-associated TM limit, to some extent, initial thrombus growth at sites of severe endothelial dysfunction or acute injury. Compared with the consequences of the TM Pro/Pro mutation on stasis-induced thrombosis, the effect of the mutation on platelet adhesion and/or aggregation is likely of minor importance in TM Pro/Pro mice.

We have shown further that reduced TM expression/function in mice was associated with an altered response to inflammatory challenges. Under basal conditions, there was no evidence of endothelial dysfunction or activation, because endothelial cell–leukocyte interactions were normal, as was the expression of endothelial cell–derived gene products other than TM and levels of proinflammatory cytokines.

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**Figure 4.** Fibrin deposition in C57BL/6J- and 129/C57-TM Pro/Pro mice. Western blot analysis of fibrin deposition in the lungs of TM Pro/Pro mice is shown. Top, On a C57BL/6J background, amounts of cross-linked tissue fibrin are similar in TM Pro/Pro and wild-type mice. Varying amounts of purified mouse fibrin were included as reference standards. Bottom, On a mixed background (Pro/Pro Pro/Pro Pro/Pro), the TM Pro/Pro mutation is associated with augmented fibrin deposition. The 2-fold reduction of TM levels in C57BL/6J TM Pro/Pro mice does not cause augmented fibrin deposition. Similar results were obtained with heart and spleen (not shown). Each lane contains a sample derived from 1 individual, equivalent to 4 mg of initial wet tissue weight.
These findings suggest that despite increased activation of coagulation, the critical threshold required for overt endothelial activation is not achieved. The LPS response in TM Pro/Pro mice differed from that in mice with normal TM function in several aspects: (1) An LD$_{50}$ of LPS caused 100% mortality of TM Pro/Pro mice. (2) Mutant mice exhibited a blunted TNF-$\alpha$ response. (3) The production of IL-6 and IL-1$\beta$ was transiently augmented, and peak IL-1$\beta$ elaboration occurred earlier. (4) Coagulation system activity appeared enhanced in TM Pro/Pro mice, as judged from increased tissue fibrin deposition. The latter indicates that the procoagulant effect of reduced protein C activation is more pronounced than the potential profibrinolytic effect of reduced activation of the thrombin-activatable inhibitor of fibrinolysis under these conditions. Interestingly, the brain appeared protected from fibrin deposition even after a challenge with lethal LPS doses. This observation is congruent with our earlier findings demonstrating a remarkable resilience of the brain microvasculature toward prothrombotic challenges, which appears to be largely based on a TM-independent mechanism. Of note, endotoxin caused a similar reduction of total tissue TM antigen to 60% to 70% of baseline levels in the lung and brain of mutant and normal mice, but total tissue antigen in the kidney was unaffected, confirming earlier findings demonstrating a tissue-restricted sensitivity of TM expression to LPS. The increased elaboration of IL-6 and IL-1$\beta$ in TM Pro/Pro mice corroborates results from in vivo studies, in which infusion of soluble TM or APC into endotoxin-treated animals inhibits coagulation, prevents organ injury, and suppresses the elaboration of proinflammatory cytokines, including IL-8, IL-6, and TNF-$\alpha$. In vitro studies have shown that APC can inhibit TNF-$\alpha$ production by LPS-stimulated monocytes/macrophages but may elicit production of IL-6 and IL-8 in endothelial cells. The reduced production of TNF-$\alpha$ in TM Pro/Pro mice, which occurs concomitantly with increased IL-6/IL-1$\beta$, is difficult to reconcile with these findings. Interestingly, infusion of recombinant antithrombin III into baboons challenged with a lethal dose of Esherichia coli has a similar disparate effect on the release of TNF-$\alpha$ and IL-6, augmenting the former but diminishing the latter. It warrants further experimental analysis to determine whether TM and antithrombin III modulate cytokine production through a related mechanism. These observations add to a body of evidence that different anticoagulants considered for therapeutic intervention in sepsis all seem effective in dampening the coagulation reaction, yet they exert astounding variable effects on the production of cytokines and the regulation of fibrinolysis (see review). Our analysis provides compelling evidence that the phenotypic penetrance of the TM$\text{P}^{\text{wo}}$ mutation is strongly influenced by mouse strain-specific genetic differences. In contrast to TM Pro/Pro mice with a mixed genetic background (ie, 129SvPas/C57BL/6J), inbred C57BL/6J TM Pro/Pro mice did not exhibit augmented microvascular fibrin deposition. Reintroduction of the 129SvPas genetic component reconstituted the significantly augmented fibrin deposition observed earlier. The protective effect of the C57BL/6J background cannot be explained by strain-specific differences in the level of TM expression but must, therefore, depend on the presence of modifier loci of thrombosis other than TM. Despite the absence of microvascular thrombosis, C57BL/6J TM Pro/Pro mice show alterations in plasma markers of coagulation (ie, FPA and D-dimer) that indicate a hypercoagulable state with a severity similar to that reported in humans carrying the insT1689 mutation in the TM gene. Reintroduction of the 129SvPas genetic component resulted in drastically increased levels of D-dimer and TAT in TM Pro/Pro mice, suggesting that augmented fibrin deposition in mixed background TM Pro/Pro mice is not caused by diminished fibrinolysis but results from augmented thrombin generation. Wild-type C57BL/6J and 129SvPas mice differ with respect to several parameters affecting hemostatic system function, most notably, platelet numbers and red blood cell volume. Although the latter might contribute to some extent to increased congestion and impediment of blood flow in the microvasculature, the mechanism underlying the mouse strain-specific differences in the phenotypic penetrance of the TM$\text{P}^{\text{wo}}$ mutation remains to be experimentally established. The demonstrated genetic basis of this phenomenon should allow one to pinpoint the thrombosis modifier gene loci interacting with the TM mutation and to define epigenetic variables that determine the severity of thrombosis in this animal model. Our findings validate the TM Pro/Pro mouse strain as a genetically defined animal model for the thrombophilic state secondary to reduced function of the TM–protein C pathway, which elicits a pronounced hypercoagulable state and sensitizes the animal to physiologically relevant thrombotic challenges, such as stasis, endothelial injury, and inflammation. In light of the recently demonstrated benefits of recombinant activated protein C in the treatment of acute sepsis, mice carrying the TM$\text{P}^{\text{wo}}$ mutation could be a valuable resource for the in vivo assessment of such novel therapeutic modalities.

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