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A murine model of myocardial microvascular thrombosis

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Disorders of hemostasis lead to vascular pathology. Endothelium-derived gene products play a critical role in the formation and degradation of fibrin. We sought to characterize the importance of these locally produced factors in the formation of fibrin in the cardiac microvasculature and microvasculature. This study used mice with modifications of the thrombomodulin (TM) gene, the tissue-type plasminogen activator (tPA) gene, and the urokinase-type plasminogen activator (uPA) gene. The results revealed that tPA played the most important role in local regulation of fibrin deposition in the heart, with lesser contributions by TM and uPA (least significant). Moreover, a synergistic relationship in fibrin formation existed in mice with concomitant modifications of tPA and TM, resulting in myocardial necrosis and depressed cardiac function. The data were fit to a statistical model that may offer a foundation for examination of hemostasis-regulating gene interactions.


Introduction

Disorders of hemostasis lead to vascular pathology. Absence of coagulation factors may lead to an increased propensity for bleeding in various vascular beds. Conversely, altered regulation of hemostasis can be associated with a pronounced tendency toward thrombosis in different vascular beds, which can lead to tissue ischemia and infarction. The regulation of this balance is maintained by systematically circulating plasma proteins, membrane-bound proteins, and the endothelium. The propensity of different vascular beds to develop thrombosis may be due to specific disorders of the endothelium. Local control of hemostasis is under the direction of the endothelium, which itself has some diversity in its expression of various gene products (1, 2). In mice, differential expression of plasminogen activator inhibitor-1 has been observed in different tissues, with high levels in aorta, heart, and adipose tissue, and low levels in the liver, adrenals, and kidneys (3). In the mouse, there are higher levels of expression of thrombomodulin (TM) in the abdominal aorta than in the thoracic aorta (4). Endothelial cells derived from microvascular beds express 100-fold more tissue-type plasminogen activator (tPA) than endothelial cells derived from macrovascular cells (5).

In the Western world, coronary artery thrombosis is usually the result of atherosclerosis. Atherosclerotic plaque rupture is thought to trigger arterial thrombosis mediated by platelets and fibrin, which results in regional ischemia or infarction (6, 7). Atherosclerosis is a disease of the macrovasculature (8). Small vessel thrombosis as a result of endothelial cell dysfunction may also produce regional ischemia (9–11). Multiple genes regulate coagulation (12, 13). We hypothesized that differential gene expression is important for organ-bed specificity with respect to hemostatic balance. Such a biological system would likely be controlled at the endothelium level (14). We demonstrated previously that the cardiac myocardium regulates the specific activity of its endothelial cells, suggesting a potential role in regulating hemostasis (2). Moreover, fibrin deposition in the cardiac vasculature has a profound effect on heart function; thus, we chose to study this organ bed as a model of thrombus regulation. The use of genetically modified animals provides a powerful tool for unraveling the complex interactions of the various proteins involved in coagulation homeostasis in the cardiac circulation. In this study, we investigated the interaction of 3 endothelium-derived genes — TM, tPA, and urokinase-type plasminogen activator (uPA) — and their effects on myocardial thrombosis. TM, a natural anticoagulant, is a membrane-bound protein that facilitates the activation of protein C to activated protein C (APC) when thrombin is also...
bound to it (15). A primary component of the fibrinolytic system is an inactive zymogen called plasminogen, which, after activation by either tPA or uPA, is able to remove fibrin clots (16). Using genetically modified animals, we studied the relative contributions of the above gene products to the maintenance of the hemostatic balance. We showed that the simultaneous reduction of the natural TM-anticoagulant pathway activity and diminution of tPA-mediated fibrinolytic capacity combined to produce severe tissue damage and functional abnormalities in the hearts of mutant mice. A statistical approach was developed that quantitatively describes the effects of the above hemostatic gene defects, and should aid in detecting genetic interactions that are relevant to the physiology and pathophysiology of the cardiovascular system.

Methods

Generation of mice. The mice with single-gene modifications (TM+/–, TMPro/Pro, TMPro/Pro, tPA+/–, and uPA+/–) have all been described previously (4, 17, 18). Mice deficient in uPA or tPA were obtained from P. Mulligan (Children’s Hospital, Boston, Massachusetts, USA). Unless otherwise stated, the background of all mice was mixed C57BL/6 and 129. The wild-type (TM+/+) and TM+/– mice were littermates, and the F1 generation was backcrossed to C57BL/6. The uPA+/– and tPA+/– mice were backcrossed 4 times to C57BL/6. The TM+/– lines were generated from a TMPro/Pro F1 mouse, and a TM+/– mouse was backcrossed 4 times to C57BL/6 (F4). The other lines were generated from the uPA+/–, tPA+/–, TM+/–, and TM+/– lines as described above. The tPA+/–/TMPro/Pro and tPA+/-TMPro/Pro animals were littermates, as were the uPA+/–/TMPro/Pro and uPA+/–/TM+/– animals. The maximum contribution of 129 to the genetic background of the different mouse genotypes ranged from 1.5% to 12.5%. The genotypes of the animals were identified by lacZ reporter gene analysis of tails and by Southern blot analysis. All these studies were performed on young adult mice (3–6 months of age). We have not noticed any shortening of life-span in animals up to 8 months of age.

Detection of tissue fibrin. Fibrin was measured in mouse tissues by a method that has been described previously (17), with several modifications. The primary antibody was either the monoclonal 59D8 (kindly provided by Marshall Runge, University of Texas Medical Branch at Galveston, Galveston, Texas, USA; ref. 19) or the commercially available mAb NYB T2G1 (Accurate Chemical & Scientific Corp., Westbury, New York, USA) (20). Protein was detected using a chemiluminescence system (either ECL from Amer sham Life Sciences Inc., Arlington, Illinois, USA, or CDP-Star from NEN Life Science Products Inc., Boston, Massachusetts, USA) and was quantitated using a Kodak Image Station (model 440CF; Eastman Kodak Co. Scientific Imaging Systems, New Haven, Connecticut, USA) supplied with 1D Image Analysis Software. Each sample group contained 10 mice, except the following genotypes: uPA+/–/TMPro/Pro (3 mice), uPA+/–/TM+/– (6 mice), tPA+/–/TMPro/Pro (7 mice), and uPA+/–/tPA+/– (8 mice).

Statistical analysis. The statistical relationship between fibrin levels and defects in the TM gene and fibrinolytic genes was analyzed using standard linear modeling techniques (21). Plots of fibrin levels within genotypic groups revealed a slight skewness in the data. Therefore, fibrin levels were natural log-transformed (ln) to normalize their distribution; all analyses were performed on these transformed data. A 3-way cell-means ANOVA model with all pairwise interactions between the TM gene, uPA gene, and tPA gene was first fit to the data, and then F tests were performed to determine whether these factors contributed significantly to the overall fit of the model. In this process, TM was treated as a 4-level factor taking on the values TM+/+, TM+/–, TMPro/Pro, and TM+/–. uPA and tPA were treated individually as 2-level factors, indicating the presence of the wild-type gene or deletion mutation. A simple linear regression model was fit to the data. If the potential interactions between the TM gene and the uPA gene, or between the TM gene and the tPA gene, were significant based on the ANOVA results, then all components of these interactions were considered separately in the regression. All main effects were also included. A backward elimination procedure based on Wald tests was performed (22). The final model consisted of all factors with coefficients significantly different from 0 (P < 0.05). Estimated coefficients from the final regression model were reported to indicate the direction and magnitude of the effect of each term on ln fibrin. Unadjusted prediction intervals for multiple comparisons of fibrin levels were also reported for all observed genotype combinations.

Histology. Murine heart sections from 5 wild-type control mice and 3 tPA+/–/TM+/– animals were prepared for histological analysis as described previously (17, 23). Masson’s trichrome stain (Sigma HT15; Sigma Diagnostics, St. Louis, Missouri, USA) was performed on alternate sections to those that were stained for fibrin (24).

Cell culture. These studies used both wild-type C57BL/6 and FVB mice. Cardiac microvascular endothelial cells and myocytes were isolated and cultured from murine hearts as described previously (25, 26). The endothelial cells were cultured alone or in the presence of cardiac myocytes, in 12-mm (0.4-μm pore) Transwell dishes (Corning-Costar Corp., Cambridge, Massachusetts, USA), as described previously (2). The cells were cultured for 72 hours, with a medium change at 48 hours. The cultures were then washed twice with PBS, and the medium was changed to DMEM with 1% BSA and 20 mM glucose, and incubated for an additional 12 hours. The medium was then collected for secreted-protein analysis.

Protein assay. Cellular and secreted protein samples were isolated from cardiac microvascular endothelial cells cultured as described above. Relative antigenic levels of TM were determined by Western dot blotting with
Table 1
Fibrin deposition in mutant mice

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Fibrin deposition</th>
<th>Fibrin coefficient</th>
<th>Predicted fibrin level</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ng fibrin/4 mg tissue</td>
<td>ln(ng fibrin/4 mg tissue)</td>
<td>ln(ng fibrin/4 mg tissue) (95% prediction interval)</td>
</tr>
<tr>
<td>Wild-type</td>
<td>5.66 ± 0.57</td>
<td>1.56</td>
<td>0.31–2.81</td>
</tr>
<tr>
<td>TM−/−</td>
<td>13.5 ± 2.2</td>
<td>1.08</td>
<td>1.39–3.90</td>
</tr>
<tr>
<td>TMPro/Pro</td>
<td>24.1 ± 5.4</td>
<td>1.28</td>
<td>1.58–4.10</td>
</tr>
<tr>
<td>TM−/−</td>
<td>51.1 ± 4.9</td>
<td>2.82</td>
<td>3.10–5.65</td>
</tr>
<tr>
<td>tPA−/−</td>
<td>90.4 ± 11.7</td>
<td>2.95</td>
<td>3.25–5.76</td>
</tr>
<tr>
<td>uPA−/−</td>
<td>45.3 ± 5.1</td>
<td>2.23</td>
<td>2.53–5.04</td>
</tr>
<tr>
<td>tPA−/− TM−/Pro</td>
<td>331 ± 37</td>
<td>NS</td>
<td>4.34–6.84</td>
</tr>
<tr>
<td>tPA−/− TMPro/Pro</td>
<td>324 ± 58</td>
<td>NS</td>
<td>4.52–7.05</td>
</tr>
<tr>
<td>uPA−/− TM−/−</td>
<td>716 ± 114</td>
<td>-0.90</td>
<td>5.15–7.70</td>
</tr>
<tr>
<td>uPA−/− TMPro/Pro</td>
<td>148 ± 22</td>
<td>NS</td>
<td>3.61–6.12</td>
</tr>
<tr>
<td>uPA−/− TM−/Pro</td>
<td>42.9 ± 5.5</td>
<td>-1.32</td>
<td>2.34–5.15</td>
</tr>
<tr>
<td>uPA−/− TMPro/Pro</td>
<td>163 ± 73</td>
<td>-1.87</td>
<td>3.42–6.05</td>
</tr>
<tr>
<td>tPA−/− uPA−/−</td>
<td>353 ± 51</td>
<td>-1.02</td>
<td>4.44–6.99</td>
</tr>
</tbody>
</table>

Genotypic crosses
- tPA−/− TM−/−
- tPA−/− TMPro/Pro
- tPA−/− TM−/Pro
- uPA−/− TM−/−
- uPA−/− TMPro/Pro
- uPA−/− TM−/Pro
- uPA−/− TMPro/Pro
- tPA−/− uPA−/−

Column 2 is the average calculated for the fibrin deposition in each genotypic group. The values are average ± SD. Column 3 contains the coefficients from the linear regression analysis of the fibrin deposition values for all of the terms. The unadjusted 95% prediction intervals for fibrin levels are reported in column 4. NS, not significant.

Results
Fibrin deposition in mutant mice. The fibrin Western blot assay quantitated the amount of fibrin present in each heart (Table 1, column 2). When the number of fully functional TM alleles was gradually reduced from the wild-type (in TM−/−, TMPro/Pro, and TM−/Pro; ref. 17), the concentration of fibrin increased (from 5.66 in the wild-type to 13.5, 24.1, and 51.1 ng fibrin/4 mg tissue, respectively). Among the single-knockout animals, tPA−/− had the highest concentration of fibrin (90.4 ng fibrin/4 mg tissue). The levels of fibrin in double-knockout animal models also increased, with the highest deposition observed when tPA−/− was combined with the TM-modified animals. The tPA−/− TMPro/Pro mice had the most fibrin (716 ng fibrin/4 mg tissue) of all genotypes.

Figure 1
Cardiac microvascular endothelial cell and cardiac myocyte culture. Protein analysis of the levels of TM, uPA, and tPA from cardiac microvascular endothelial cells cultured alone (open bars) and in the presence of cardiac myocytes (filled bars).
Cardiac myocyte induction of tPA. Cardiac microvascular endothelial cells were cultured alone and in the presence of cardiac myocytes. Cardiac myocytes induced increases in endothelial levels of tPA to 191 ± 13% of baseline values (Figure 1). The communication between the cardiac myocytes and the endothelial cells did not alter TM or uPA levels (92 ± 15% and 103 ± 12%, respectively, compared with baseline values). Levels of TM, tPA, and uPA in cardiac myocytes in monoculture were not detectable.

Echocardiography. A subset of experimental mice was subjected to echocardiography (Table 3). This subset consisted of wild-type (TM+/+), TM+/–, TM−/Pro, tPA−/–, uPA−/–, and tPA−/– TM−/Pro animals. Two representative echocardiograms of the mice are shown in Figure 2. No effect on cardiac function was seen when TM was mutated to drastically reduce its anticoagulant function (TM−/– and TM−/Pro). A global decrease in cardiac function was observed in tPA−/– mice. When tPA−/– TM−/Pro mice were examined, they were found to have regional myocardial dysfunction suggestive of myocardial infarcts. Examination of the echocardiograms demonstrates that the infarction was localized to the LV free wall in these animals.

Heart histology of tPA−/– TM−/Pro mice. The fibrin Western blot provides a quantitative description of how much fibrin is present in the heart tissue; immunohistochemistry shows where the fibrin is (Figure 3). Comparison of hearts of tPA−/– TM−/Pro mice and wild-type controls showed that fibrin is present in both macrovasculature (Figure 3a) and microvasculature (not shown).

### Table 2

ANOVA table of the genotype interactions

<table>
<thead>
<tr>
<th>Source</th>
<th>DF</th>
<th>Sum of squares</th>
<th>Mean square</th>
<th>F value</th>
<th>Pr &gt; F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Model</td>
<td>12</td>
<td>230.408</td>
<td>19.201</td>
<td>50.64</td>
<td>0.0001</td>
</tr>
<tr>
<td>Error</td>
<td>103</td>
<td>39.050</td>
<td>0.379</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Corrected total</td>
<td>115</td>
<td>269.458</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Source</th>
<th>DF</th>
<th>Sum of squares</th>
<th>Mean square</th>
<th>F value</th>
<th>Pr &gt; F</th>
</tr>
</thead>
<tbody>
<tr>
<td>TM</td>
<td>3</td>
<td>23.87</td>
<td>7.957</td>
<td>20.99</td>
<td>0.0001</td>
</tr>
<tr>
<td>uPA</td>
<td>1</td>
<td>8.942</td>
<td>8.942</td>
<td>23.59</td>
<td>0.0001</td>
</tr>
<tr>
<td>tPA</td>
<td>1</td>
<td>52.246</td>
<td>52.246</td>
<td>137.8</td>
<td>0.0001</td>
</tr>
<tr>
<td>TM × tPA</td>
<td>3</td>
<td>3.91</td>
<td>1.303</td>
<td>3.44</td>
<td>0.0196</td>
</tr>
<tr>
<td>TM × uPA</td>
<td>3</td>
<td>11.845</td>
<td>3.948</td>
<td>10.41</td>
<td>0.0001</td>
</tr>
<tr>
<td>uPA × tPA</td>
<td>1</td>
<td>1.591</td>
<td>1.591</td>
<td>4.2</td>
<td>0.043</td>
</tr>
</tbody>
</table>

DF, degrees of freedom; Pr, probability.
When the heart was examined distal to the area of intravascular fibrin deposition, abnormalities were observed in the trichrome stain of the tPA–/– TM–/Pro mice but not the wild-type mice. Trichrome stains use 3 different dyes to identify and distinguish nuclei (blue to black), collagen (blue), and cytoplasm and muscle (red). When muscle necrosis occurs, fibers invade the necrotic tissue; these fibers are stained with aniline blue. The trichrome stain shows clearly that myocardial infarction is present in the LV myocardium of the tPA–/– TM–/Pro animals. On average, 1 area of muscle necrosis was present in each randomly chosen section of the heart.

Discussion
The heart is an organ in which there is heterogeneity in gene expression in the endothelium (2). Recent data have suggested that tPA is associated only with a distinct subpopulation of the endothelial cells (28), and that the level of tPA can be increased when these cells are exposed to pertinent stimuli (29). The tissues with the highest levels of tPA are the brain and heart (E.G. Levin and K.G. Osborn, unpublished observations; also reported in ref. 29). The tissue with the highest expression level of uPA is the kidney (30). TM has high levels of expression in the vascularized tissues, including the heart (4, 31).

The fibrin Western blot illustrates the effect of selective gene modification on hemostasis in different vascular beds. Statistical analysis provides a tool for examining the interactions and effects of various gene deletions/modifications in an in vivo mouse model. The combination of modified TM and a gene deletion in the fibrinolytic pathway greatly increases the levels of fibrin. In the animals with a single knockout/modification, removal of uPA causes the same magnitude of effect on fibrin deposition as is seen in TM–/Pro mice. Both uPA and TM have a more general role in the fibrinolytic/coagulation cascade than tPA; only tPA has specificity for the removal of fibrin from the vascular system (16). The removal of tPA appears to have the greatest effect among the animals with a single knockout/modification. This is also reflected in tPA–/– mice having a larger fibrin coefficient than either TM–/Pro or uPA–/– mice. It has been reported (32) that tPA and uPA are able to compensate for one another. This could contribute to the lower levels of fibrin present in the single knockouts. When the tPA–/– characteristic is combined with the TM–/Pro modification, the resulting tPA–/– TM–/Pro mutant mouse has fibrin levels that can be calculated by adding the fibrin coefficients (predicted ln fibrin levels) of the 2 original mutants to the wild-type coefficient (no additional interaction is observed when the 2 genes are modified or deleted). The predicted ln fibrin level of a tPA–/– TM–/Pro/Pro mouse is 5.79, which is simply the sum of the individual ln fibrin levels of a wild-type (1.56), a tPA–/– (2.95), and a TM–/Pro (1.28) mouse. When uPA is removed in the presence of the modified TM, it appears that tPA is able to compensate, producing a negative fibrin coefficient for the interaction of uPA and TM. The predicted ln fibrin level for a uPA–/– TM–/Pro mouse is 4.74, which is less than the sum of the individual ln fibrin coefficients of a wild-type (1.56), a TM–/Pro (2.82), and a uPA–/– (2.23) mouse, which is 6.61. The fibrin levels of the mice with 2 genetic modifications were higher than either of the single knockouts, but not as high as the tPA–/– TM–/Pro mice. Because of the number of mouse lines used in this study, it was not always possible to genetically match the backgrounds of all the

Figure 3
Histology of heart. (a and c) Fibrin immunohistochemistry. Frozen tissue sections of tPA–/– TM–/Pro (a) and wild-type (c) mice were fixed in acidic formalin to wash out soluble fibrinogen/fibrin; reacted with the fibrinogen/fibrin-specific antibody; and counterstained with hematoxylin. The dark-brown horseradish peroxidase reaction product shows fibrin present in the macrovasculature. (b and d) Trichrome stain. Frozen tissue sections of tPA–/– TM–/Pro (b) and wild-type (d) mice were processed according to standard protocols (24). The tPA–/– TM–/Pro section shows myocardial infarct caused by the fibrin present in distal arteries. This tissue death is not present in the wild-type hearts. All images are sections from the LV wall of the heart. ×150.
The above data indicate that tPA is important for normal heart function. We demonstrated previously that other genes are critical to cardiac development and physiology (including VEGF and Flk-1), and are regulated by communication between cardiac myocytes and the microvascular endothelium (2). The present studies suggest that levels of tPA may be regulated by a similar mechanism. It was shown that in the coculture of endothelial cells and myocytes, levels of tPA increased, whereas the uPA and TM protein levels were not affected. It has been suggested (32) that tPA is important for dissolving embolized clots, whereas uPA is more necessary for the healing of wound fields on the skin. Any disruption in communication between the cardiac myocytes and the endothelium could potentially lead to altered levels in tPA, possibly resulting in profound effects on fibrin levels. As judged by echocardiography, the hearts of the animals with the TM defect function normally, while the tPA-deficient animals have global defects in their cardiac function. Because tPA is expressed throughout the heart, it is not surprising that its removal leads to a global cardiac effect (28). The tPA-/- TM-Pro/+ mice exhibited a regional effect, reflected in LV dysfunction. In addition, these mice displayed indications of focal myocardial necrosis that correlate with dramatically increased amounts of fibrin deposited in the myocardial vasculature. We are currently examining larger numbers of animals to develop a model in which gene interactions are related to cardiac function.

Echocardiography is an excellent technique for looking at cardiac function of genetically modified mice. It has been shown in this study that global and regional cardiac defects can be determined noninvasively. This technique could potentially be used as an evaluation tool in genetic manipulation studies of cardiac abnormalities. Because of the noninvasive nature of the technique, it would be of great benefit in studying gene-modified animals as a disease progresses. For instance, we have previously reported an age-related dependence of fibrin deposition in TM-null chimeric mice (41). Echocardiography would provide a means for observing the physiological consequences of the progression of this disease state.

In conclusion, we have produced an in vivo mouse model with lesions associated with microvascular thrombosis. The tPA-/- TM-Pro/+ animals have LV region-
al wall motion abnormalities, as demonstrated using echocardiography. This dysfunction is likely the result of the overwhelming extent of fibrinous vascular occlusion in the hearts of these animals. Thus, it appears that the addition of modified TM is sufficient to convert the biochemically defined hyperfibrinolytic state and global cardiac dysfunction observed in tPA-deficient mice into a mutant mouse with focal myocardial tissue necrosis associated with LV dysfunction. The tPA+/−/TM−/Pro μ mouse should prove to be a good in vivo model of myocardial infarction caused by thrombosis.

Acknowledgments
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