Human Thrombomodulin Knock-In Mice Reveal Differential Effects of Human Thrombomodulin on Thrombosis and Atherosclerosis

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Objective—We sought to develop a murine model to examine the antithrombotic and antiinflammatory functions of human thrombomodulin in vivo.

Methods and Results—Knock-in mice that express human thrombomodulin from the murine thrombomodulin gene locus were generated. Compared with wild-type mice, human thrombomodulin knock-in mice exhibited decreased protein C activation in the aorta (P<0.01) and lung (P<0.001). Activation of endogenous protein C following infusion of thrombin was decreased by 90% in knock-in mice compared with wild-type mice (P<0.05). Carotid artery thrombosis induced by photochemical injury occurred more rapidly in knock-in mice (12±3 minutes) than in wild-type mice (31±6 minutes; P<0.05). No differences in serum cytokine levels were detected between knock-in and wild-type mice after injection of endotoxin. When crossed with apolipoprotein E–deficient mice and fed a Western diet, knock-in mice had a further decrease in protein C activation but did not exhibit increased atherosclerosis.

Conclusion—Expression of human thrombomodulin in place of murine thrombomodulin produces viable mice with a prothrombotic phenotype but unaltered responses to systemic inflammatory or atherogenic stimuli. This humanized animal model will be useful for investigating the function of human thrombomodulin under pathophysiological conditions in vivo. (Arterioscler Thromb Vasc Biol. 2011;31:00-00.)

Key Words: atherosclerosis ■ thrombosis ■ inflammation ■ protein C ■ thrombomodulin

Thrombomodulin is an endothelial cell-surface thrombin receptor that modulates hemostasis and inflammation.1–3 When bound to thrombomodulin, thrombin efficiently catalyzes production of activated protein C (APC), a clinically important endogenous anticoagulant. APC downregulates coagulation by proteolytically inactivating factors Va and VIIIa. In addition to its anticoagulant effects, APC inhibits endothelial inflammatory and cell death pathways through a mechanism that involves protease activated receptor-1 and the endothelial protein C receptor.5–7 Thrombomodulin also modulates inflammation independently of APC, possibly by activating antiinflammatory pathways through its N-terminal lectin domain,8–10 negatively regulating complement,11 or by facilitating the activation of thrombin-activatable fibrinolytic inhibitor, a procarboxypeptidase that may modulate both fibrinolysis and complement-mediated inflammation.2

Several murine models of altered thrombomodulin gene structure have been developed. Homozygous deletion of the murine thrombomodulin gene results in intrauterine lethality before embryonic day (E) 10.5.12 The mechanism of embryonic death of thrombomodulin-null embryos is related to thrombin-induced growth arrest of trophoblast cells.13 Embryonic development is not impaired in mice expressing thrombomodulin mutants with deletions of the C-terminal cytoplasmic or N-terminal lectin domains.8,14 Embryonic development also occurs normally in mice with a thrombomodulin mutation (TM(ab)) that markedly reduces protein C activation and predisposes mice to thrombosis during hypoxia.15 This observation suggests that low levels of APC are sufficient for normal embryogenesis and placental development.

Because of interspecies differences in thrombomodulin structure and function, current murine models provide only limited insights into the pathophysiological role of human thrombomodulin in vivo. We therefore sought to develop knock-in mice that express human thrombomodulin in the absence of murine thrombomodulin and to determine whether such mice can be used to investigate the function of human thrombomodulin under pathophysiological conditions in vivo. Our results indicate that mice expressing human throm-
thrombomodulin from the murine thrombomodulin gene locus are partially protected from embryonic lethality and have normal postnatal development and survival. Compared with wild-type mice, human thrombomodulin knock-in mice have a prothrombotic phenotype characterized by decreased protein C activation and enhanced susceptibility to experimental thrombosis, but they do not exhibit impaired systemic anti-inflammatory responses or enhanced susceptibility to atherosclerosis. These findings reveal differential species-specific effects of murine and human thrombomodulin in regulating thrombosis versus inflammation, and they demonstrate that the human thrombomodulin knock-in mouse is an instructive model for investigating the pathophysiological role of human thrombomodulin in vivo.

Methods

**Generation of Human Thrombomodulin Knock-In Mice**

Two separate lines of human thrombomodulin knock-in mice, designated Hn and Hhn, were generated by targeted replacement of the murine thrombomodulin (Thbd) gene with the homologous gene segment containing the human thrombomodulin (THBD) coding sequence (for details, see Supplemental Methods and Supplemental Figure I, available online at http://atvb.ahajournals.org). Animal protocols were approved by the University of North Carolina or University of Iowa animal care and use committee.

**Generation of Apoe<−/−; Hthm/Hthm Mice**

Apolipoprotein E (ApoE)−/−null mice on the C57BL/6 background were obtained from the Jackson Laboratory (Bar Harbor, ME) and crossbred with Hthm+ mice for 2 generations to generate littermate Apoe<−/− mice that were either Hthm/Hthm or +/+ at the thrombomodulin locus. Starting at 3 weeks of age, the mice were fed either a control diet (LM485, Harlan Teklad, Madison, WI) or high-fat Western diet (TD003159, Harlan Teklad). Starting at 20% fat. Experimental procedures were performed on male mice at 24 weeks of age. Blood from anesthetized mice was collected by cardiac puncture into EDTA (final concentration, 5 mmol/L), and plasma cholesterol was measured using the Infinity Cholesterol Reagent kit (Thermo Electron Corp, Louisville, CO).

**Morphometric Analysis of Atherosclerotic Lesions**

Morphometric analysis of aortic sinus atherosclerotic lesion area was performed as described previously. Briefly, hearts embedded in paraffin were sectioned at 8-μm serial intervals through the entire aortic sinus. The sections were stained by the Verhoeff–van Gieson method. Cross-sectional lesion area was calculated using the mean value of 5 sections, each 80 μm apart, beginning at the aortic valve leaflets and spanning 320 μm.

**Measurement of Thrombomodulin mRNA by Quantitative Polymerase Chain Reaction**

Total RNA was isolated from lung, heart, or kidney of male mice using Trizol reagent. RNA was treated with DNase I to remove contaminating genomic DNA and then reverse transcribed using TaqMan reverse transcriptase reagents and random hexamer primers. Real-time polymerase chain reaction was performed using a 7700HT sequence detection system (Applied Biosystems) as described previously. Primers and probes were designed using Primer Express software (Applied Biosystems) to be specific for either murine thbd (forward, ATTTCCATGGCAGCCCTG; reverse, TGACTCTCAGCCTGCAG) or human THBD (forward, ATCTCCATCGGAGCCCTTG; reverse, TGACTCTCATTGTGCGG). A common probe (Fam-CGCCCTGCTTCTTGCGCAGGT-Tamra) was used for both murine and human products. Total amounts of thrombomodulin transcripts in Hthm/Hthm and +/+ mice were determined using a probe/primer set that detects regions common to the mouse and human transcripts (forward, GACAGCCAGTTTTCTTTTCA; reverse, TCTGGGATCTCCGGTATT; probe, Fam-CACACTCCGCCCAGGTGTCAG-Tamra). Data were analyzed using the comparative threshold cycle (ΔΔCt) method, with β-actin as the comparator, using Sequence Detection software, version 1.6.3 (Applied Biosystems).

**Immunohistochemistry**

Sections of formalin-fixed, paraffin-embedded lung or tail from adult mice were deparaffinized, and endogenous peroxidase activity was quenched with hydrogen peroxide. Sections were blocked with Power Block (Biogenex, San Ramon, CA) and stained with a 1:50 dilution of mouse monoclonal anti-human thrombomodulin antibody (DakoCytomation, Carpentaria, CA) in PBS. LSAB II linker–horseradish peroxidase (DakoCytomation) was applied, and staining was visualized with diaminobenzidine (DakoCytomation). Sections were counterstained with Harris hematoxylin without acid.

**Thrombomodulin Antigen**

Lung lysates were prepared by homogenization in 0.02 mol/L Tris-HCl, 0.1 mol/L NaCl, 2% Triton X-100 (pH 8.0) as described. Human thrombomodulin antigen was measured by ELISA (Asserachrom Thrombomodulin, Diagnostica Stago, Franchonville, France). To measure mouse thrombomodulin antigen, immunoassay plates (Nunc-Immuno plate, Polysorb surface, flat bottom, Nalge Nunc International, Rochester, NY) were coated overnight at 4°C with 1 μg per well of polyclonal goat anti-mouse thrombomodulin (R&D Systems, Inc, Minneapolis, MN) in 20 mmol/L carbonate (pH 9.2), 0.02% sodium azide. After being washed 3 times, wells were blocked with 1% bovine serum albumin (Research Products International Corp, Prospect, IL) in 50 mmol/L Tris-HCl (pH 7.4), 150 mmol/L NaCl. Lung lysate samples (40 μg of protein per well) or recombinant human thrombomodulin (R&D Systems) standard samples were added and incubated for 1 hour at room temperature. Following washing, monoclonal rat anti-mouse thrombomodulin (0.2 μg/well, R&D Systems) was added and incubated for 1 hour at room temperature. After 3 additional washes, anti-rat IgG-alkaline phosphatase conjugate antibody (0.064 μg/well, Sigma-Aldrich, St Louis, MO) was added and incubated for 1 hour at room temperature. After 3 additional washes, para-nitrophenol phosphate substrate (Sigma-Aldrich) was added, and color development was monitored at 405 nm in a 96-well plate reader (Spectra Max 190, Molecular Devices, Woburn, MA). The standard curve was linear over a range of 0.1 to 2.0 nmol/L recombinant mouse thrombomodulin. No cross-reactivity with recombinant human thrombomodulin was detected at concentrations up to 4 nmol/L. The total protein concentration of lung lysates was measured using a modified Bradford assay (Bio-Rad Laboratories, Richmond, CA).

**Protein C Activation**

Activation of exogenous protein C by thrombin was measured in freshly isolated rings of proximal aorta (1.0 mm in length) or in lung lysates using a 2-stage assay described previously. Activation assays were performed using either 2.6 nmol/L human thrombin and 150 nmol/L human protein C20 or 2.6 nmol/L murine thrombin (Hematologic Technologies, Essex Junction, VT) and 150 nmol/L murine protein C22. Reference curves were generated using rabbit lung thrombomodulin (American Diagnostica, Stamford, CT). One unit of activity was defined as the amount of APC generated in the presence of 1.0 nmol/L rabbit thrombomodulin.

Activation of endogenous protein C was measured in response to infusion of human thrombin in adult mice. Mice anesthetized with sodium pentobarbital (70–90 mg/kg) were secured in a supine position under a dissecting microscope and ventilated mechanically with room air and supplemental oxygen as described previously. A heating pad was used to maintain body temperature at 36°C to 37°C. Human thrombin (Hematologic Technologies) (40 U/kg) or saline was rapidly infused into the right cardiac ventricle in a total volume of 100 μL. After 10 minutes, blood was collected by cardiac...
puncture into a 1/10th volume of 0.1 mol/L sodium citrate containing 0.5 mol/L benzamidine. The concentration of murine APC in citrate/benzamidine-treated plasma was measured as described previously\(^\text{22}\) and reported as percentage of APC in a pool of normal mouse plasma. For experiments performed with Apoe\(^{-/-}\) Hthm/ Hthm mice, murine APC was measured using an enzyme capture assay. Citrate-anticoagulated plasma samples containing 30 mmol/L benzamidine were added to Nunc MaxiSorp plates (Fisher Scientific, Pittsburgh, PA) coated with 5 μg/mL anti-mouse APC (AMGDP61587), a generous gift from Charles Esmon (Oklahoma Medical Research Foundation, Oklahoma City, OK). The activity of bound APC was determined by incubation for 24 hours at 37°C with Spectrozyme PCa (American Diagnostica), followed by acid quenching and determination of absorbance using a microplate reader (Spectra Max 190, Molecular Devices). Mouse APC (Hematologic Technologies) was used to standardize the assay.

**Direct Anticoagulant Activity**

To measure the direct anticoagulant activity of thrombomodulin, lung lysates were incubated with 1 mmol/L human α-thrombin (HT3054, Enzyme Research, South Bend, IL) and bovine fibrinogen (1 mg/mL, Sigma-Aldrich), and the time to clot formation was measured using a fibrometer (BBL FibroSystem, Becton Dickinson, Franklin Lakes, NJ).

**Glycosaminoglycan Hydrolysis**

To determine the effect of glycosaminoglycan (GAG) on thrombo- modulin activity, lung lysates were incubated for 16 hours at 37°C with or without chondroitin sulfate ABC lysate (Seikagaku America, Falmouth, MA) at a final concentration of 0.2 U/mg protein in 50 mmol/L Tris-HCl containing 100 mmol/L NaCl, 0.5 mmol/L sodium acetate, leupeptin (20 μg/mL), pepstatin (10 μg/mL), and 2.5 mmol/L N-acetyl-L-lysine, pH 8.0. The reaction was stopped by incubating the solution at 90°C for 1 minute. To control for the efficiency of chondroitin sulfate lyase activity, a chondroitin sulfate-containing proteoglycan (aggrecan) from rat chondrosarcoma\(^{25}\) was added to the chondroitin sulfate-lyase–treated lysates. The mixtures were electrophoresed using 7.5% SDS-PAGE and stained with Coomassie Blue to detect a characteristic 94-kDa aggrecan peptide devoid of GAG\(^{26}\). Protein C activation and direct anticoagulant activity assays were then performed using chondroitin sulfate–treated or control lung lysates.

**Carotid Artery Thrombosis**

Carotid artery thrombosis was induced by photochemical injury as described\(^{21,22}\). Mice anesthetized with sodium pentobarbital (70–90 mg/kg) were ventilated mechanically with room air and supplemental oxygen. Blood flow in the right carotid artery was measured with a 0.5-PSB Doppler flow probe (Transonic Systems, Ithaca, NY). To induce endothelial injury, the right common carotid artery was transfused intermittently with a 1.5-mV, 540-nm green laser (Melles Griot, Carlsbad, CA) from a distance of 6 cm, and Rose Bengal (25–50 mg/kg) was injected via a femoral vein catheter. Blood flow was monitored continuously for 90 minutes or until stable occlusion occurred. Stable occlusion was defined as the time at which blood flow remained absent for ≥10 minutes.

**Response to Endotoxin**

Endotoxin (*Escherichia coli* bacterial lipopolysaccharide [LPS] se- rotype 055:B5) was purchased from Sigma-Aldrich. Mice were injected intraperitoneally with 8 mg/kg LPS or saline in a volume of 100 μL. After 4 hours, serum was isolated for measurement of interleukin-6, interleukin-1β, and tumor necrosis factor-α by Quan- tikine immunoassay kits (R&D Systems). Plasma and serum were frozen at −80°C until they were assayed.

**Statistical Analyses**

Comparisons between mouse genotypes were performed using the unpaired 2-tailed Student *t* test or 1-way ANOVA. Occlusion times were compared with the Mann-Whitney rank sum test. Viability data were analyzed using the chi² test. Statistical significance was defined as a probability value less than 0.05. Values are reported as mean±SE. Except where indicated, all statistical comparisons were performed between littermates, and all experimental groups included both female and male mice.

**Results**

**Generation and Viability of Human Thrombomodulin Knock-In Mice**

Two separate lines of human thrombomodulin knock-in mice, designated *Hn* and *Hthm*, were generated by targeted replace- ment of the murine *Thbd* gene with the homologous gene segment containing the human *THBD* coding sequence (Supple- mental Figure I). The *Hn* line contained a loxP-flanked (floxed) neomycin resistance gene within the thrombomodulin–3′ untranslated region. The *Hthm* line was generated by crossingbreeding *Hn/+* mice with transgenic mice expressing Cre recombinase\(^{26}\) to excise the neomycin resistance gene.

In both the *Hn* and *Hthm* lines, the gross appearance of weaned heterozygous and homozygous human thrombo- modulin knock-in mice was normal, and no abnormalities in organ morphology or histology were observed. However, the number of viable homozygous *Hn/Hn* or *Hthm/Hthm* mice produced from heterozygous matings was decreased from expected mendelian frequencies (Supplemental Tables I and II). Homozygous *Hn/Hn* mice represented only 5% of weaned offspring (*P*<0.05 versus the expected Mendelian frequency of 25%). The frequency of homozygous *Hthm/Hthm* offspring at weaning (14%) was greater than for *Hn/Hn* homozy- gotes (*P*<0.001) but was still less than the expected mende- lian frequency (*P*<0.05). There were no significant differences in the frequencies of homozygous *Hthm/Hthm* or *Hn/Hn* offspring at weaning between mice on a 129/SvEv inbred background versus mice on a mixed background of 129/SvEv and C57BL/6. *Hn/Hn* and *Hthm/Hthm* mice that survived weaning exhibited normal growth and survival. The mean weights of *Hn/Hn* and *Hthm/Hthm* mice between 12 and 18 months of age did not differ from +/+ mice (data not shown).

Because mice with complete deficiency of thrombomodu- lin uniformly die before day 10 of embryogenesis,\(^{12}\) we sought to determine whether the decreased viability of human thrombomodulin knock-in mice was also due to early embry- onic lethality. The frequencies of *Hn/Hn* and *Hthm/Hthm* fetuses isolated from heterozygous matings on E10.5 were 16% and 23%, respectively (Supplemental Tables I and II), which suggests that early embryonic viability is relatively normal in human thrombomodulin knock-in mice. On E17.5, the frequencies of *Hn/Hn* and *Hthm/Hthm* fetuses had decreased to 6% and 13%, respectively, quite similar to those observed at weaning. These findings suggest that homozy- gous replacement of the murine thrombomodulin allele with the *Hn* or *Hthm* alleles resulted in the partial loss of viability between E10.5 and E17.5. This effect on viability was more severe for *Hn/Hn* mice than for *Hthm/Hthm* mice.

**Expression of Thrombomodulin mRNA**

Levels of total (murine plus human) thrombomodulin mRNA were measured in lung and heart isolated from adult +/+.
Table 1. Quantitative Polymerase Chain Reaction Analysis of Total (Murine+Human) Thrombomodulin mRNA

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<th>Murine TM mRNA (%)</th>
<th>Human TM mRNA (%)</th>
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<tr>
<td>Lung</td>
<td>100±19</td>
<td>72±19</td>
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<tr>
<td>Heart</td>
<td>26±4</td>
<td>20±2</td>
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The total amount of thrombomodulin mRNA was determined using a probe/primer set that detects regions common to the murine and human transcripts. All mice were on an inbred 129/SvEv background and were studied at 4 to 6 mo of age. Data are reported as a percentage of the total amount of thrombomodulin mRNA in the lung of +/+ mice. Values represent mean±SE (n=4–5 in each group). *P<0.05 vs +/+ mice.

Table 2. Species-Specific Quantitative Polymerase Chain Reaction Analysis of Thrombomodulin mRNA

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<thead>
<tr>
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<th>Murine TM mRNA (%)</th>
<th>Human TM mRNA (%)</th>
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<tbody>
<tr>
<td>Lung</td>
<td>100±20</td>
<td>43±13*</td>
</tr>
<tr>
<td>Heart</td>
<td>14±2</td>
<td>6±1</td>
</tr>
<tr>
<td>Kidney</td>
<td>8±1</td>
<td>4±1*</td>
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Levels of individual murine thrombomodulin and human thrombomodulin transcripts were measured using species-specific real-time polymerase chain reaction primers. All mice were on an inbred 129/SvEv background and were studied at 4 to 6 mo of age. Data are reported as a percentage of murine thrombomodulin mRNA in the lung of +/+ mice. Values are mean±SE (n=4–7 in each group). TM indicates thrombomodulin mutation. *P<0.05 vs +/+ mice.

Expression of Human Thrombomodulin Protein

Expression of human thrombomodulin protein in Hthm/Hthm and Hn/Hn mice was demonstrated by immunohistochemistry and ELISA (Figure 1). Immunohistochemical staining of tail cross-sections using a monoclonal anti-human thrombomodulin antibody was strongly positive in endothelium and keratinocytes in Hthm/Hthm mice, moderately positive in Hthm/Hn mice, and negative in +/+ mice (Figure 1A–C). This expression pattern is consistent with that observed previously for thrombomodulin in mice and humans.27,28 Immunofluorescence staining of lung sections also revealed endothelium-specific staining for human thrombomodulin in Hthm/Hthm mice, whereas staining for murine thrombomodulin was detected in +/+ and Hthm/Hn mice (Supplemental Figure III). Colocalization of murine and human thrombomodulin was observed in lung endothelium (Supplemental Figure IV).

Human thrombomodulin antigen levels measured by ELISA in lung lysates were concordant with immunohistochemical observations (Figure 1D). Human thrombomodulin antigen was approximately twice as high in Hthm/Hthm mice compared with Hthm/Hn mice (P=0.01). Murine thrombomodulin antigen levels measured by ELISA in lung lysates were decreased by approximately 70% in Hthm/Hn mice compared with +/+ mice (P<0.01) (Figure 1E).

Protein C Activation

Activation of protein C in aortic rings and lung lysates was measured in vitro using human protein C and human thrombin. Generation of APC was similar in aortic rings from +/+ and Hthm/Hthm mice (Figure 2A). APC generation was decreased by 28% in aortic rings from Hn/Hn mice (P<0.05 versus +/+ mice), and by approximately 60% in aortic rings from both Hthm/Hthm and Hn/Hn mice (P<0.01 versus +/+ mice). With lung lysates (Figure 2B), generation of APC was decreased by approximately 50% for Hthm/Hthm and Hn/Hn mice (P<0.05 versus +/+ mice), 75% for Hthm/Hthm mice (P<0.001 versus +/+ mice), and 90% for Hn/Hn mice (P<0.05 versus +/+ mice). To determine whether differences in protein C activation also occurred with murine reagents, activation of murine protein C was measured with lung lysates from +/+ , Hthm/Hthm, and Hthm/Hn mice using murine thrombin. Compared with lysates from +/+ mice, lysates from Hthm/Hthm mice generated 52% less APC (P<0.05), and lysates from Hthm/Hn mice generated 86% less APC (P<0.001) (Figure 2C).

Activation of endogenous murine protein C in vivo was assessed by measuring plasma levels of APC in +/+ , Hthm/Hthm, and Hthm/Hn mice following infusion of saline vehicle or 40 U/kg human thrombin (Figure 2D). Compared with infusion of saline, infusion of thrombin produced a 2.7-fold increase in plasma APC concentration in +/+ mice (P<0.05) but no significant increase in Hthm/Hn or Hthm/Hthm mice.
Effect of GAG Hydrolysis on Thrombomodulin Activity

To determine whether the decreased thrombomodulin activity of human thrombomodulin knock-in mice was caused by differential effects of GAG attachment to thrombomodulin, lung lysates from \(+/+\), \(Hthm+/\), or \(Hthm/Hthm\) mice were incubated with chondroitin sulfate ABC lyase before protein C activation or direct anticoagulant activity assays were performed. The protein C activation activity of thrombomodulin in lung lysates from \(+/+\), \(Hthm+/\), or \(Hthm/Hthm\) mice was not significantly affected by treatment with chondroitin sulfate lyase (Supplemental Figure VA). In contrast, the direct anticoagulant activity of thrombomodulin (ie, the ability of thrombin to directly inhibit the fibrinogen cleaving activity of thrombin), which is highly sensitive to its GAG content, was decreased by approximately 40% after GAG hydrolysis in both the \(+/+\), \(Hthm+/\), and \(Hthm/Hthm\) samples (Supplemental Figure VB). These results suggested that GAG attachment contributes equivalently to the direct anticoagulant activity of murine and human thrombomodulin expressed in mouse lung.

Carotid Artery Thrombosis

The time to thrombotic occlusion of the carotid artery after photochemical injury was measured in \(+/+\), \(Hthm+/\), and \(Hthm/Hthm\) mice (Figure 3). Occlusion times did not differ between \(+/+\) and \(Hthm+/\) mice, but the mean time to stable occlusion was decreased by more than 60% in \(Hthm/Hthm\) mice (12±3 minutes) compared with \(+/+\) mice (31±6 minutes) \((P<0.05)\).

Response to Endotoxin

The inflammatory response to intraperitoneal injection of a sublethal dose of 8 mg/kg \(E. coli\) LPS was determined by measurement of serum cytokine levels in \(+/+\) and \(Hthm/Hthm\) mice 4 hours after LPS administration. Serum levels of interleukin-6 (Figure 4A), interleukin-1 \(\beta\) (Figure 4B), and tumor necrosis factor-\(\alpha\) (Figure 4C) all increased significantly and equivalently after LPS administration in \(+/+\) and \(Hthm/Hthm\) mice \((P<0.05\) versus saline).

Phenotype of \(Apoe^{-/-}\) \(Hthm/Hthm\) Mice

To determine the effects of hypercholesterolemia in human thrombomodulin knock-in mice, \(Apoe^{-/-}\) mice were cross-bred with \(Hthm+/\) mice for 2 generations to generate littermate \(Apoe^{-/-}\) mice that were either \(Hthm/Hthm\) or \(+/+\) at the thrombomodulin locus. Starting at 3 weeks of age, male mice were fed either a control diet or high-fat Western diet until they were studied at 24 weeks of age. Compared with the control diet, the high-fat diet produced a similar 2-fold increase in plasma total cholesterol levels in \(Apoe^{-/-}\) and \(Hthm/Hthm\) mice \((P<0.01)\) (Figure 5A). The high-fat diet also produced similar increases in cross-
sectional atherosclerotic lesion area, measured in the aortic sinus, compared with the control diet in Apoe<sup>−/−</sup> and Apoe<sup>−/− Hthm/Hthm</sup> mice (P<0.01) (Figure 5B). Following infusion of thrombin, circulating plasma levels of APC were significantly lower in Apoe<sup>−/− Hthm/Hthm</sup> mice compared with littermate Apoe<sup>−/−</sup> mice fed either the control or high-fat diets (P<0.01) (Figure 5C). Notably, the amount of circulating APC generated in Apoe<sup>−/− Hthm/Hthm</sup> mice was significantly lower with the high-fat diet than the control diet (P<0.05). Finally, the time to stable thrombotic occlusion of the carotid artery after photochemical injury was decreased markedly in Apoe<sup>−/−</sup> or Apoe<sup>−/− Hthm/Hthm</sup> mice fed the high-fat diet (P<0.05) (Figure 5D), confirming that hypercholesterolemia induces a prothrombotic phenotype in mice expressing either murine or human thrombomodulin.

Figure 2. Protein C activation. Thrombomodulin-dependent activation of protein C was measured in aortic rings (A) or lung lysates (B) of +/+, Hthm/+ , Hthm/Hthm , Hn/+ , and Hn/Hn mice using 2.6 nmol/L human thrombin and 150 nmol/L human protein C. Mice were studied at 3 months of age. Values represent mean±SE (n=4 for Hn/Hn mice; n=19–22 for all other groups). *P<0.05 vs +/+ mice. C, Thrombomodulin-dependent activation of protein C was measured in lung lysates of +/+, Hthm/+ , or Hthm/Hthm mice using 2.6 nmol/L murine thrombin and 150 nmol/L murine protein C (n=3–8 in each group). *P<0.05 vs +/+ mice. D, Plasma levels of murine APC were measured following infusion of either saline vehicle or 40 U/kg human thrombin. Results are reported as percent of APC in pooled normal mouse plasma (n=5–8 in each group). *P<0.05 vs saline. APC indicates activated protein C.

Figure 3. Carotid artery thrombosis following photochemical injury. Susceptibility to thrombosis in +/+, Hthm/+ , or Hthm/Hthm mice was assessed by measuring the time to stable occlusion after photochemical injury of the carotid artery in anesthetized, ventilated mice. Mice were studied at 6 months of age. Thrombosis was induced by injection of 35 to 50 mg/kg Rose Bengal. Values represent mean±SE (n=9–10 in each group). *P<0.05 vs +/+ mice.

Figure 4. Response to intraperitoneal injection of endotoxin. Blood samples were obtained 4 hours after intraperitoneal injection of 8 mg/kg lipopolysaccharide (LPS) or saline vehicle in +/+, Hthm/Hthm, and Hthm/Hthm mice, and were assayed for serum interleukin (IL)-6 (A), serum IL-1β (B), and serum tumor necrosis factor-α (TNF-α) (C). Mice were studied at 12 to 18 months of age. Values represent mean±SE (n=5–13 in each group). #P<0.05 vs saline.
Expression of human thrombomodulin in place of murine thrombomodulin partially bypassed the uniform embryonic lethality that occurs with complete deficiency of murine thrombomodulin. Approximately 50% of mice homozygous for the Hthm allele and 80% of mice homozygous for the Hn allele died between E10.5 and E17.5 (Supplemental Table I). Thrombomodulin is expressed in both trophoblasts and developing endothelium early in embryogenesis. The mechanism of early embryonic death of thrombomodulin-null embryos appears to be caused by growth arrest of trophoblast cells. Reconstitution of murine thrombomodulin expression in placental tissue rescues thrombomodulin-null embryos from early embryonic lethality, and reveals a secondary developmental block between days 12.5 and 16.5 that is caused by lack of thrombomodulin expression in endothelium. Our findings suggest that human thrombomodulin can functionally substitute for murine thrombomodulin to bypass the early trophoblast-dependent developmental block but only partially bypass the later, endothelium-dependent developmental block. The more severe embryonic loss in Hn/Hn mice compared with Hthm/Hthm mice may be due to slightly lower expression of human thrombomodulin in some tissues (Table 1), likely caused by the presence of the neomycin resistance gene within the 3’ untranslated region of the Hn allele.

Compared with wild-type mice, human thrombomodulin knock-in mice were hypomorphic for thrombomodulin activity and exhibited a prothrombotic phenotype, with a 50% shortening in the time to carotid artery occlusion following photochemical injury. The prothrombotic phenotype of human thrombomodulin knock-in mice contrasts with the antithrombotic phenotype of human thrombomodulin transgenic mice reported recently by Crikis et al. An important distinction between these models is that the transgenic mouse model described by Crikis et al is a thrombomodulin overexpression model in which the human thrombomodulin transgene is expressed in addition to endogenous murine thrombomodulin. Overexpression of (human + murine) thrombomodulin in these mice results in increased APC generation and protection from thrombosis. In contrast, the human thrombomodulin knock-in mouse model reported here is a hypomorphic model in which human thrombomodulin is expressed instead of murine thrombomodulin. Compared with +/+ mice, Hthm/Hthm mice had similar levels of thrombomodulin mRNA expression (Table 1) and higher levels of thrombomodulin antigen in the lung (Figure 1). These findings suggest that the prothrombotic phenotype (decreased protein C activation and enhanced susceptibility to arterial thrombosis) in Hthm/Hthm mice is unlikely to be caused by an alteration in thrombomodulin expression. Instead, the findings suggest that human thrombomodulin may be a less active anticoagulant than murine thrombomodulin in vivo.

We explored the possibility that differences in protein C activation between wild-type and human thrombomodulin knock-in mice may be related to differences in modification by O-linked chondroitin sulfate GAG chains. Thrombomodulin’s GAG chains provide secondary thrombin binding sites by O-linked chondroitin sulfate GAG chains. Thrombomodulin knock-in mice may be a less active anticoagulant than murine thrombomodulin.
on protein C activation were observed (Supplemental Figure V). The direct anticoagulant function of both murine and human thrombomodulin was decreased by GAG removal, but the effects were similar for +/+ and Htih/Htih mice. These results indicate that GAG attachment plays an equivalent role in the anticoagulant activity of murine and human thrombomodulin and suggest that the prothrombotic phenotype of human thrombomodulin knock-in mice is not due to differential effects of GAG. It is more likely, therefore, that structural changes due to amino acid differences are responsible for the more potent antithrombotic activity of murine compared with human thrombomodulin.

It is possible but unlikely that the decreased protein C activation in human thrombomodulin knock-in mice is related to the presence of the Ala455Val polymorphism within the sixth epidermal growth factor domain. This common human thrombomodulin polymorphism has been found to be associated with arterial thrombosis in some studies, but several large population studies have failed to confirm this association. Several other candidate prothrombotic thrombomodulin mutations have been identified in human subjects with venous or arterial thrombosis, but the clinical significance of these gene variants remains uncertain. Mutations that impair the function of thrombomodulin were recently described in some patients with atypical hemolytic uremic syndrome. The thrombomodulin knock-in approach described in this study could potentially be used to directly examine the pathophysiological consequences of these and other human thrombomodulin gene variants.

Despite considerable progress in defining the role of thrombomodulin and the protein C activation pathway in susceptibility to thrombosis and modulation of inflammatory responses, the impact of this anticoagulant pathway on atherosclerosis is less well understood. Eitzman et al observed accelerated atherogenesis in Apoe−/− mice expressing the murine equivalent of the human factor V Leiden mutation, which confers resistance to APC. Seehaus et al found that expression of a hypomorphic murine thrombomodulin mutation (TM4Pro) in Apoe−/− mice had paradoxical effects on atherosclerosis: the atherosclerotic lesions were larger but also had histological features suggestive of enhanced plaque stability. These observations raise the question of whether human thrombomodulin confers protection from or susceptibility to atherosclerosis. Our findings indicate that mice expressing human or murine thrombomodulin have similar susceptibilities to atherosclerosis when crossed to the Apoe−/− background despite their differing anticoagulant activities. The contrasting effects on atherosclerosis of the murine TM4Pro mutant studied by Seehaus et al and human thrombomodulin in our study could be due to a relatively lower anticoagulant activity of the TM4Pro mutant. Alternatively, these findings might suggest that human thrombomodulin has protective effects on atherosclerosis that are independent of its anticoagulant activity. In either case, our results suggest that the human thrombomodulin knock-in mouse may be an informative in vivo model for future studies investigating the role of human thrombomodulin in atherosclerosis and other pathophysiological conditions.

A limitation of many mouse models is that there often are species-specific functional differences between human and murine proteins that may alter responses to pathophysiological conditions or pharmacological interventions. The generation of human thrombomodulin knock-in mice is a step toward humanizing the entire protein C system in mice. Such humanization might facilitate the discovery of small molecules that modify the human anticoagulant system and the study of in vivo consequences of such modifications. The current model is limited because only the thrombomodulin gene locus was humanized, and it is not possible to directly compare the functional activities of human versus murine thrombomodulin because of differences in their expression levels. Nevertheless, the availability of this model, as well as other similar models, such as the humanized tissue factor mouse, may enhance the clinical relevance of mouse models of human thrombotic disease.

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Disclosures
Dr Lentz has served as a consultant for Novo Nordisk A/S and has an equity ownership interest in Celgene Corporation.

References


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Supplemental Methods

*Generation of human thrombomodulin knockin mice*

The intronless human thrombomodulin (*THBD*) gene maps to chromosome 20p11.2 (OMIM 188040, UniGene NM_000361, Locus Link 7056). A vector containing the coding sequence of the *THBD* gene was used to achieve targeted replacement of the murine thrombomodulin (*Thbd*) locus (Supplemental Figure I). To generate the targeting vector, mouse genomic DNA spanning the *Thbd* locus was isolated from a 129/SvEv genomic DNA λ phage library. The targeting vector contained a 1.2 kb *XbaI/SphI* 5'-homology fragment followed by the coding sequence of the human *THBD* gene and a 6 kb 3' homology fragment that included the murine 3' UTR. The human *THBD* coding sequence was derived from pUC19TM15, which contains a full-length human *THBD* cDNA.\(^1\) This cDNA contains a common single nucleotide polymorphism, 1418C→T, which results in an amino acid change from alanine to valine at position 455 within the 6\(^{th}\) EGF domain.\(^2\) The *THBD* cDNA was placed between the start and stop codons of the murine sequence using PCR-based mutagenesis. A neomycin resistance gene flanked by loxP sites was inserted into a BamH1 site within the murine 3' UTR.

The targeting construct was linearized with *NotI* and electroporated into 129/SvEv ES cells, and positive and negative selections for homologous recombination were performed with G418 and gancyclovir as described previously.\(^3\) ES cell clones correctly targeted to the *Thbd* locus were identified by PCR and confirmed by Southern blot analysis (Supplemental Figure II). Three correctly targeted ES cell clones were injected into C57BL/6 blastocysts and the resulting chimeric mice were mated with 129/SvEv mice to generate F1 heterozygotes. Germline transmission of the targeted allele was confirmed by PCR. The *Thbd* allele encoding the human thrombomodulin coding sequence and the neomycin resistance gene in its 3' UTR was
designated as $Hn$, and knockin mice heterozygous or homozygous for this allele as $Hn^+/+$ or $Hn/Hn$, respectively.

To eliminate the neomycin resistance gene from the 3'UTR, $THBD$ allele-containing mice were bred with transgenic mice expressing the $Cre$ recombinase gene under the control of the adenovirus EIIa promoter. The $Cre$ transgenic mice had been backcrossed to 129/SvEv mice for at least 10 generations. The $Thbd$ allele in which the neomycin resistance gene was excised was designated as $Hthm$, and knockin mice heterozygous or homozygous for this allele as $Hthm^+/+$ or $Hthm/Hthm$ respectively. Knockin mice were either maintained on the 129/SvEv genetic background or serially crossed with C57BL/6 mice up to 6 generations. Genotyping was performed by PCR using a common forward primer (TAATCCGAGAACGCAGCTTC) with reverse primers specific for the wild-type (CCAGCACACCCAGAAAGAAA) or targeted (CAGTCCGTCGCAGATCTGA) alleles. Animal protocols were approved by the University of North Carolina or University of Iowa Animal Care and Use Committees.

**Genotyping of embryos**

To determine the genotypes of embryos of different embryonic ages, timed matings between heterozygous breeding pairs were performed. E0.5 was defined as the first day following detection of a vaginal plug. On E10.5 or E17.5, pregnant female mice were anesthetized with sodium pentobarbital (70-90 mg/kg) and placental and embryonic tissues were removed. Embryos were dissected from the yolk sac and washed in PBS. DNA was extracted from whole fetuses using the DNeasy tissue system (Qiagen, Inc., Valencia, CA) and subjected to real-time PCR as described previously. PCR primers and 6-carboxy fluorescein-labeled probes for murine GAPDH (Mm99999915_g1), murine thrombomodulin (Mm00437014_s1), and human thrombomodulin (Hs00264920_s1) were purchased from Applied Biosystems (Foster City, CA). DNA was incubated with TaqMan Universal PCR mix (Applied Biosystems) and PCR primers and probes at 50ºC for 2 minutes and then at 95ºC for
10 minutes followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute using the 
Applied Biosystems 7700 sequence detection system.

**Immunofluorescence**

Lung tissue was fixed in 2% paraformaldehyde/PBS, serially equilibrated in sucrose/PBS 
(10%, 20%, 30%), and then frozen in Tissue Freezing Medium (Triangle Biomedical Sciences, 
Durham, NC). For detection of murine thrombomodulin, frozen sections (10 μm) were blocked 
with BSA and normal goat serum, and then incubated with rat anti-mouse thrombomodulin 
antibody (MAB3894; R&D Systems, Minneapolis, MN) followed by goat-anti-rat antibody 
conjugated to Alexa 568 (Invitrogen, Carlsbad, CA). For detection of human thrombomodulin, 
sections were blocked sequentially with egg substitute, dry milk, and mouse Ig, and then 
incubated with mouse anti-human thrombomodulin antibody (Clone 1009, DakoCytomation). 
Secondary antibody conjugated with biotin (Vector M.O.M., Vector Laboratories, Burlingame, 
CA) was added, followed by streptavidin- Alexa 568 (Invitrogen). For co-localization of human 
TM and murine TM, sections were first blocked with egg substitute and dry milk, incubated with 
anti-human thrombomodulin antibody, biotin-conjugated secondary antibody, and streptavidin-
Alexa 568, and then blocked with normal goat serum (10%), followed by addition of rat anti-
mouse thrombomodulin antibody and goat anti-rat Alexa 647 (Invitrogen). Sections were 
mounted in Vectashield with DAPI (Vector Laboratories, H-1200). Images were captured on a 
Zeiss (model 710) confocal microscope.
References


**Supplemental Table I. Genotypes of viable mice from \textit{Hn}+/+ x \textit{Hn}+/+ matings at embryonic days 10.5 and 17.5 and at weaning**

<table>
<thead>
<tr>
<th>Age</th>
<th>+/+</th>
<th>\textit{Hn}/+</th>
<th>\textit{Hn}/\textit{Hn}</th>
</tr>
</thead>
<tbody>
<tr>
<td>E10.5†</td>
<td>11 (22.4%)</td>
<td>30 (61.2%)</td>
<td>8 (16.3%)</td>
</tr>
<tr>
<td>E17.5†</td>
<td>19 (35.8%)</td>
<td>31 (58.5%)</td>
<td>3 (5.7%)*</td>
</tr>
<tr>
<td>Weaning‡</td>
<td>109 (32.2%)</td>
<td>213 (62.8%)</td>
<td>17 (5.0%)*</td>
</tr>
</tbody>
</table>

†All mice genotyped at E10.5 and E17.5 were on a mixed background of 129/SvEv and C57BL/6.

‡Includes both mice on an inbred 129/SvEv background (n=145) and mice on a mixed background of 129/SvEv and C57BL/6 (n=194).

*P < 0.05 vs. expected Mendelian frequency of 25%.
Supplemental Table II. Genotypes of viable mice from $Hthm^+/+ \times Hthm^+/+$ matings at embryonic days 10.5 and 17.5 and at weaning

<table>
<thead>
<tr>
<th>Age</th>
<th>+/+</th>
<th>$Hthm^+/+$</th>
<th>$Hthm^+/Hthm$</th>
</tr>
</thead>
<tbody>
<tr>
<td>E10.5†</td>
<td>13 (27.7%)</td>
<td>23 (48.9%)</td>
<td>11 (23.4%)</td>
</tr>
<tr>
<td>E17.5†</td>
<td>19 (40.4%)</td>
<td>22 (46.8%)</td>
<td>6 (12.8%)*</td>
</tr>
<tr>
<td>Weaning‡</td>
<td>254 (36.3%)</td>
<td>348 (49.7%)</td>
<td>98 (14.0%)*</td>
</tr>
</tbody>
</table>

†All mice genotyped at E10.5 and E17.5 were on a mixed background of 129/SvEv and C57BL/6.

‡Includes both mice on an inbred 129/SvEv background ($n = 238$) and mice on a mixed background of 129/SvEv and C57BL/6 ($n = 462$).

*P < 0.05 vs. expected Mendelian frequency of 25%.
Supplemental Figure Legends

Supplemental Figure I. Construction of the human thrombomodulin targeting vector. The structure of the intronless murine thrombomodulin (Thbd) gene, with its 5’ flanking intracisternal A particle (IAP) repeat, the murine thrombomodulin (TM) coding sequence, and its 3’UTR, is shown at top. An alternative polyA addition signal site is shown in parenthesis. The targeting vector contained a thymidine kinase (TK) cassette, a 1.2 kb 5’-homology fragment, the full length human thrombomodulin (TM) coding sequence, and a 6 kb 3’-homology fragment that included the 3’UTR and a neomycin resistant gene flanked by loxP sites. The targeted allele encoding the human thrombomodulin coding sequence and the neomycin resistance gene in its 3’ UTR was designated as Hn. The targeted allele with the neo gene deleted is designated Hthm. Restriction enzyme cutting sites are B, BamH1; N, Not1; R, EcoR1; X, Xba1. The position of a 500 bp probe (used in the Southern blot shown in Supplemental Figure II) is indicated by **.

Supplemental Figure II. Targeting modification of the thrombomodulin 3’ UTR sequence in ES cells. A Southern blot of EcoR1 digested genomic DNA isolated from individual neomycin-resistant ES cell colonies (lanes 1 through 5) was hybridized with a 500bp PCR fragment corresponding to the proximal promoter region of the mouse thrombomodulin gene. Primers used are 5’-GAACTGTGCTTTGCTGACA-3’ and 5’-AATCCCAAGCATGCTGTCCA-3’. A 14 kb band represents the endogenous locus and an 8 kb band represents the modified locus as indicated in Supplemental Figure I. Lanes 1-4 are correctly modified colonies, lane 5 is a colony with a random integration of the targeting construct, and lane 6 is the parental ES cells. The blot demonstrates: (i) near equal intensity of the targeted band (8kb) and the endogenous band (14kb) in correctly targeted cells in lanes 1-4, (ii) reduced intensity of the endogenous band (14 kb) in lanes 1-4 compared to cells with the unmodified locus in lanes 5 and 6, (iii) random,
nonhomologous integration of the targeting construct giving an additional hybridizing band of about a half intensity compared to the endogenous band (with no reduction of intensity of the endogenous band) in lane 5, and (iv) no additional bands indicative of random integration in the targeted cells (lanes 1-4).

**Supplemental Figure III.** Immunofluorescence staining of murine and human thrombomodulin in lung. Sections of lung tissue from +/+ (A, D), Hthm/+ (B, E), or Hthm/Hthm (C, F) mice were stained for murine thrombomodulin (A, B, C) or human thrombomodulin (D, E, F). Staining for murine thrombomodulin is detected in endothelium of +/+ and Hthm/+ mice, whereas staining for human thrombomodulin is detected in Hthm/+ and Hthm/Hthm mice.

**Supplemental Figure IV.** Co-localization of murine and human thrombomodulin in lung. Sections of lung tissue were stained for murine thrombomodulin (left panel, red) or human thrombomodulin (middle panel, green). The right panel shows the merged image, which confirms co-localization of murine and human thrombomodulin in lung endothelium (yellow).

**Supplemental Figure V.** Effect of GAG hydrolysis on thrombomodulin activity. (A) Thrombomodulin-dependent activation of protein C was measured in lung lysates of +/+ or Hthm/Hthm mice after incubation with or without chondroitin sulfate (CS) ABC lyase, using 2.6 nM human thrombin and 150 nM human protein C (n = 5 in each group). Mice were studied at 3 months of age. Values represent mean ± SE. (B) The direct anticoagulant activity of thrombomodulin was measured in lung lysates of +/+ or Hthm/Hthm mice after incubation with or without chondroitin sulfate (CS) ABC lyase. Lung lysates of +/+ mice (40 µg protein/ml) or Hthm/Hthm mice (100 µg protein/ml) were treated with or without CS lyase, added to thrombin (1 nM) and fibrinogen (1 mg/ml), and the prolongation of the clotting time was recorded. The
baseline clotting time in the absence of thrombomodulin was 134 seconds. Values represent mean ± SE (n = 7 to 9 in each group). *P < 0.05 vs. +/+ mice; #P < 0.05 vs. without CS lyase.
Supplemental Figure II
Supplemental Figure III

murine TM

human TM

+/+

Hthm/+ 

Hthm/Hthm

A

B

C

D

E

F
Supplemental Figure IV

anti-murine TM  anti-human TM  Merge

20 µm
Supplemental Figure V

A

Human APC (U/µg protein)

+/- Hthm/Hthm

Prolongation of Clotting Time (seconds)

0
100
200
300

without CS lyase
with CS lyase

*
*
#
#

B

without CS lyase
with CS lyase

A

B