Basic Science Reports

Thrombomodulin Overexpression to Limit Neointima Formation

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Background—These studies were initiated to confirm that high-level thrombomodulin overexpression is sufficient to limit neointima formation after mechanical over-dilation injury.

Methods and Results—An adenoviral construct expressing thrombomodulin (Adv/RSV-THM) was created and functionally characterized in vitro and in vivo. The impact of local overexpression of thrombomodulin on neointima formation 28 days after mechanical over-dilation injury was evaluated. New Zealand White rabbit common femoral arteries were treated with buffer, viral control, or Adv/RSV-THM and subjected to mechanical over-dilation injury. The treated vessels (n=4 per treatment) were harvested after 28 days and evaluated to determine intima-to-media (I/M) ratios. Additional experiments were performed to determine early (7-day) changes in extracellular elastin and collagen content; local macrophage, T-cell, and neutrophil infiltration; and local thrombus formation as potential contributors to the observed impact on 28-day neointima formation. The construct significantly decreased neointima formation after mechanical dilation injury in this model. By histological analysis, buffer controls exhibited mean I/M ratios of 0.76±0.06%, whereas viral controls reached 0.77±0.08%; in contrast, Adv/RSV-THM reduced I/M ratios to 0.47±0.06%. Local inflammatory infiltrate decreased in the Adv/RSV-THM group relative to controls, whereas matrix remained relatively preserved. Rates of early thrombus formation also decreased in Adv/RSV-THM animals.

Conclusions—This construct thus offers a viable technique for promoting a locally neointima-resistant small-caliber artery via decreased thrombus bulk, normal matrix preservation, and decreased local inflammation without the inflammatory damage that has limited many other adenoviral applications. (Circulation. 2000;102:332-337.)

Key Words: gene therapy ■ extracellular matrix ■ viruses ■ inflammation ■ thrombosis

Clinically significant restenosis occurs in 40% of balloon angioplasty patients within 6 months and exacts high tolls in survival and medical costs.1 Although processes leading to restenosis are complex, several factors prove critical in underlying neointima formation. Thrombosis represents both an initiator of neointima formation and a significant complication of angioplasty itself. Patients with significant thrombus formation at the time of balloon dilation consistently demonstrate more extensive late lesions,2–4 and therapy to decrease platelet aggregation by use of antibodies to the platelet GP IIb/IIIa receptor decreases rates of restenosis.5 Thrombin activates thrombin receptors on platelets, smooth muscle cells (SMCs), and endothelial cells and results in phenotypic cellular activation, growth factor release, and proliferation of each cell type.6–8 The thrombotic cascade affects phenotypes of endothelial cells and SMCs,2,5 predominantly altering responses of each cell type to mechanical damage and subsequent exposure to growth factor.10,11 Preliminary studies suggest that therapies that use antithrombotic moieties, such as tissue factor pathway inhibitor,12 hirudin,12 NO,13 or prostacyclin,2,14 or inhibiting prothrombotic elements, such as factor VIIa or Xa12 or thrombin,12 limit neointima formation.

Thrombin mediates thrombosis and activates endothelial cells, SMCs, platelets, and macrophages directly to yield enhanced local inflammation and enhanced matrix proteolysis.6–8 The primary physiological buffer for thrombin activity in normal vessels is thrombomodulin (THM).

The surface glycoprotein THM, by binding thrombin on a 1:1 or a 2:1 basis15 and increasing inactivation via antithrombin III,16 removes a catalytically active procoagulant. Bound thrombin undergoes a conformational change altering its
specificity and allowing conversion of protein C to its active form, a powerful anticoagulant. In addition to the anti-thrombotic and anti-inflammatory effects of THM in blocking thrombin, protein C itself adds potent antithrombotic and anti-inflammatory activity. Thrombodastheries have been found to result from relative deficiency of THM or protein C/S, and homozygous deficiency of THM has been shown be lethal in mice. THM is normally present at very high levels on the surface of unperturbed endothelial cells. However, injured endothelial cells express dramatically decreased levels of THM expression, predisposing to thrombotic complications and enhancements of local inflammation and matrix proteolysis. We have previously demonstrated that adenovirus-mediated local overexpression of THM is sufficient to maintain functional THM expression despite certain types of local perturbation. In this work, we examine the impact of local overexpression of THM on the processes leading to neointima formation after mechanical dilation balloon injury in the rabbit common femoral artery. 

Methods

Recombinant adenovirus containing the Escherichia coli β-galactosidase gene driven by the Rous sarcoma virus (RSV) promoter (Adv/RSV-βgal) was kindly provided by Dr Michel Perricaudet (Institut Gustave Roussy, Ville-Juif, France). Recombinant adenovirus expressing human THM under the control of the RSV promoter (Adv/RSV-THM) was constructed as previously described. All animal experiments were performed in accordance with NIH and institutional guidelines. 

Mechanical Overdilation and In Vivo Adenovirus Delivery
The distal left common femoral artery of each New Zealand White rabbit was cannulated. A 2-mm×2-cm Cordis SAVVY angioplasty balloon was introduced and inflated to 6 atm for 1 minute in 2 cycles. The segment was isolated, and the contents were aspirated before incubation of 400 μL of saline alone, 400 μL of 5×10^10 pfu/mL Adv/RSV-βgal, or 400 μL of 5×10^4 pfu/mL Adv/RSV-THM (number as detailed for each experiment below). 

Local THM Expression Levels
Animals underwent balloon injury and virus delivery as above (n=3 animals for each group) for PBS, Adv/RSV-βgal, or Adv/RSV-THM 3 days before vessel harvesting for immunohistochemical evaluation of sections (1 per segment) with a goat anti-rabbit THM primary antibody (kind gift of K. Wright, M.D. Anderson Cancer Center, Houston, Tex) at 1:1000 dilution overnight. Paired sections were used as before for CD31 (endothelial staining, Santa Cruz Biotech, at a dilution of 1:400) or α-actin (SMC staining, 1A4, DAKO, at a dilution of 1:75) to identify cell-specific THM distribution after treatment. Automated immunohistochemistry was performed on a TechMate 500 (Ventana Medical Systems). A Diagnostic Instruments SPOT true-color digital camera recorded noninterpolated microscopic images of each slide at high resolution. The resulting images were analyzed with the ImagePro Plus analysis system to determine percent THM expression. Images underwent red channel extraction, thresholding at 4 to 200 (=0), addition of paired CD31 and THM or α-actin and THM images, and counting of areas of interest (double-positive areas represented by zero), followed by summation. These results were normalized to total area as determined by area on floating point conversions of the same image and compared with THM total results (single stain) to determine nonendothelial/non-SMC distribution by subtraction. For all experiments, mean and SEM were determined for each group, with statistical comparisons made with 1-factor repeated-measures ANOVA with significance evaluated at 95% and with Bonferroni, Tukey-A, and Student-Newman-Keuls post hoc testing performed in Statview and SPSS 6.1 for the Macintosh (Prentice Hall), and individual probability values were determined as reported in the text. 

Tissue Harvesting and Preparation and Determination of Intima-to-Media Ratios
Cross sections at the 7-day time point evaluated above were obtained (1 per segment) from each vessel and incubated with a primary monoclonal antibody to rabbit macrophage (RAM-11, 1/800 dilution, DAKO), incubated with a primary monoclonal antibody to rabbit CD4 (KEN-4, Spring Valley Laboratories), or subjected to chloroacetate esterase staining to visualize neutrophilic granulocytes as previously reported. Images of each section were acquired to determine the number of positive cells per cross section. RAM-11 and esterase images each underwent red channel extraction, thresholding at 0 to 127, and summation. The results were divided by the total image area and reported as cross-sectional percent matrix. Estain content was determined by floating point conversion, thresholding at 0 to 35, and summation and confirmed by single-stained Verhoeff–van Gieson results. 

Evaluation of Local Extracellular Matrix Elements
Elastic lamina and collagenous connective tissue were demonstrated with a combination of Verhoeff stain and Masson’s trichrome stain for collagen (sections from 7-day experiment above). Total collagen was evaluated after extraction of red channel, thresholding from 0 to 127, and summation. The results were divided by the total image area and reported as cross-sectional percent matrix. Elastin content was determined by floating point conversion, thresholding at 0 to 35, and summation and confirmed by single-stained Verhoeff–van Gieson results. 

Evaluation of Local Inflammatory Cell Infiltrate
Cross sections at the 7-day time point evaluated above were obtained (1 per segment) from each vessel and incubated with a primary monoclonal antibody to rabbit macrophage (RAM-11, 1/800 dilution, DAKO), incubated with a primary monoclonal antibody to rabbit CD4 (KEN-4, Spring Valley Laboratories), or subjected to chloroacetate esterase staining to visualize neutrophilic granulocytes as previously reported. Images of each section were acquired to determine the number of positive cells per cross section. RAM-11 and esterase images each underwent red channel extraction, thresholding at 0 to 127, and application of a 3×3 circular closing filter once before counting. The resulting processed images were used to count the number of positive cells per cross section. 

Reproducibility
Neointima formation at 28 days was confirmed in duplicate experiments with Cordis SAVVY angioplasty balloons and TEGwire balloons. Across experiments from 2 institutions, 2 different groups of interventionists, and 2 types of angioplasty balloons, local delivery of Adv/RSV-THM exhibited statistically significant decreases in neointima similar to those presented here.

Results

Local THM Expression Levels After Gene Delivery
At virus doses that yielded 20% marker expression in preliminary β-galactosidase experiments, total cross-sectional THM antigen expression rose to 320% of buffer controls (P<0.0012) and 233% of viral controls (P<0.0026), whereas both controls were statistically similar to one another (P>0.05), as presented in Table 1. Significant increases in
TABLE 1.  THM Expression After Local Adenovirus-mediated Gene Delivery of THM

<table>
<thead>
<tr>
<th>CD31</th>
<th>α-Actin</th>
<th>Other</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Buffer</td>
<td>3.50±0.54</td>
<td>7.59±0.92</td>
<td>2.90±0.37</td>
</tr>
<tr>
<td>βgal</td>
<td>4.67±0.64</td>
<td>9.59±1.94</td>
<td>3.29±0.53</td>
</tr>
<tr>
<td>THM</td>
<td>5.33±0.66</td>
<td>30.74±7.67</td>
<td>8.65±1.40</td>
</tr>
</tbody>
</table>

THM levels were determined by cell type (endothelial, SMC, other) as described 3 days after balloon dilution and delivery of saline, Adv/RSV-βgal, or Adv/RSV-THM and normalized to total cross-sectional area for each artery for tabulation. Values represent mean±SEM.

THM expression were observed (P<0.05 for all comparisons except THM versus βgal endothelial staining) in endothelial cells, SMCs, and “other” (endothelial cells or SMCs with weak antigen levels below threshold or other cell types) relative to control groups that were comparable to one another (P>0.05 for all comparisons).

Intima-to-Media Ratios After Overexpression of THM

As presented in Figure 1, right, local overexpression of THM in an in vivo rabbit model decreases degree of neointima formation after mechanical overdistension by 37% relative to balloon-injured controls after 28 days (P=0.003 and P=0.010, respectively, relative to buffer and viral controls, which were comparable to one another, P>0.05). This treatment group also exhibits decreases in neointimal hyperplasia by 43% of buffer control values after only 7 days in these same experiments, as presented in Figure 1, left (P=0.009 and P=0.048, respectively, relative to buffer and viral controls, which were comparable to one another, P>0.05). Figure 2 presents representative photomicrographs of Verhoeff–van Gieson/Masson–stained sections depicting the predominantly cellular neointima observed at 28 days.

Intra-Arterial Thrombus Formation After Local Overexpression of THM

The degree of intra-arterial thrombus formation was quantified 7 days after mechanical balloon dilatation and gene delivery as described above and summarized as Figure 3. Local overexpression of THM leads to statistically significant decreases in intra-arterial thrombus formation relative to both viral and buffer controls (P=0.0324 relative to buffer controls and P=0.0134 relative to viral controls).

Evaluation of Vessel Wall Matrix After Overexpression of THM

Quantification of elastin and collagenous extracellular matrix was undertaken at the 7-day time point (Table 2). Immunohistochemical evaluation was avoided to evaluate class-wide effects on collagenous extracellular matrix and elastin content via histochemical analyses. Elastin content in the Ad/RSV-THM group increased by 65% relative to viral controls (P=0.001) and was statistically similar to buffer control levels (P>0.05). Collagenous extracellular matrix was significantly preserved relative to both buffer (66% increase, P=0.005) and viral controls (54% increase, P=0.034). Significant matrix preservation occurred in the Adv/RSV-THM group relative to controls. These effects could be due to decreases in local inflammatory infiltrate in the THM group or secondary to decreased cellular activation of endothelial cells, SMCs, and macrophages via thrombin receptor. To evaluate the relative contribution of inflammation, local infiltrate was examined by cellular subtype.

Local Inflammatory Responses After Overexpression of THM

Infiltration of granulocytes, CD4+ T cells, and macrophages was evaluated at 7 days, with the results presented in Table 3. Adv/RSV-THM vessels did not exhibit statistically significant alterations in granulocyte infiltration relative to buffer controls (P>0.05). Viral controls revealed increased infiltration of potentially elastase-degrading granulocytes relative to both Adv/RSV-THM (P=0.040) and buffer controls (P=0.049). THM prevented the ADV-mediated increase in granulocyte infiltration observed in viral controls. No statistically significant differences in number of CD4+ T cells occurred between groups (P>0.05 for all comparisons). Interestingly, substantial decreases in local macrophage infiltration occurred with Adv/RSV-THM treatment relative to both viral and buffer controls (P=0.001 for each).

Discussion

A great deal of recent evidence suggests that thrombin-mediated proteolytically activated receptor (PAR) activation...
may play roles in thrombosis, atheroma development and progression, and neointima formation. Thrombin activates these receptors on platelets, SMCs, and endothelial cells and results in phenotypic cellular activation, growth factor release, and proliferation/migration of each cell type.\textsuperscript{6–8} In normal arteries, THM binds thrombin and prevents PAR activation while also catalytically activating protein C, which adds additional antithrombotic and anti-inflammatory effects.

In this work, we demonstrate that local overexpression of THM limits neointima formation at 7 and 28 days. Potential contributions to these effects include significant reductions in local thrombus, preserved local extracellular matrix, and decreased local inflammation after THM treatment.

Previous work has validated the use of low-dose adenovirus as a delivery system that can be used with minimal adverse impact on THM expression levels.\textsuperscript{23} Although the virus doses used here yielded 20\% marker expression in preliminary \(\beta\)-galactosidase experiments, total THM antigen expression rose to 320\% of buffer controls and 233\% of viral controls with significant percentage of expression that is non-endothelium-based, as described in previous adenovirus-mediated gene transfer experiments after angioplasty. Thus, high levels of Adv/RSV-\(\beta\)-gal, or Adv/RSV-THM, THM expression were achieved throughout these injured arteries.

Differences between levels of THM expression and the level of marker expression are most likely due to decreases in control THM expression after injury, as well as to high-level expression achieved per transfected cell in THM treatments. However, because the antibody used recognizes both the human and rabbit antigens, the differences between marker expression.

![Figure 2. Representative low- (×10) and high- (×100) magnification photographs of sections obtained 28 days after mechanical overdilation and delivery of PBS, Adv/RSV-\(\beta\)-gal, or Adv/RSV-THM. Sections were stained with a combination of Verhoeff elastica stain and Masson’s trichrome.](image)

![Figure 3. Intra-arterial thrombus formation after local overexpression of THM. Cross-sectional percent thrombus formation 7 days after balloon dilation and delivery of PBS, Adv/RSV-\(\beta\)-gal, or Adv/RSV-THM.](image)

**TABLE 2. Extracellular Matrix**

<table>
<thead>
<tr>
<th></th>
<th>Elastin</th>
<th>Collagen</th>
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<tbody>
<tr>
<td>Buffer</td>
<td>3.94±0.48</td>
<td>9.40±0.58</td>
</tr>
<tr>
<td>(\beta)gal</td>
<td>2.36±0.23</td>
<td>10.12±0.77</td>
</tr>
<tr>
<td>THM</td>
<td>3.89±0.40</td>
<td>15.64±1.75</td>
</tr>
</tbody>
</table>

Cross-sectional elastin and collagenous extracellular matrix content 7 days after mechanical overdilation and delivery of PBS, Adv/RSV-\(\beta\)-gal, or Adv/RSV-THM.
expression levels and THM epitope availability could be due in part to differential antibody reactivity.

Because thrombus formation has been shown to enhance both neointima formation and clinical restenosis after angioplasty, degree of intra-arterial thrombus formation was evaluated histologically. In the present model, local overexpression of THM results in decreased local thrombus bulk. Given that thrombosis represents a significant clinical complication in up to 20% of angioplasies, this therapy may eventually offer survival benefits beyond even the observed reduction in neointima formation.21

Significant early collagen and elastin preservation were also observed in the THM group relative to controls. Normal extracellular matrix provides a primary functional and architectural barrier to the smooth muscle and inflammatory cell migration that, in part, characterize neointima formation. Moreover, several elements of normal extracellular matrix have been shown to prevent smooth muscle mitogenesis directly, even blunting responses to proliferative stimuli as strong as platelet-derived growth factor (PDGF)-BB.26–28 Degraded matrix elements, in turn, actually directly stimulate smooth muscle cell migration and proliferation under certain conditions.26–29 Matrix-degrading enzymes, such as matrix metalloproteinases and elastase, have been implicated in neointima formation, and therapies targeting each of these enzymes have significantly decreased neointima formation after mechanical over dilation injury.30,31 Local overexpression of THM thus not only offers antithrombotic effects but also provides significant matrix preservation, which may blunt neointima formation.

Previous work has demonstrated that adenovirus doses substantially higher than those presented here result in vigorous local inflammatory responses,32,33 whereas our relatively low-dose strategies achieve therapeutic effects without these high rates of inflammation.33 Consistent with these conclusions, no increases in local neutrophil or CD4+ T cells per cross section 7 days after balloon dilation and delivery of PBS, Adv/RSV-βgal, or Adv/RSV-THM.

<table>
<thead>
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<th>TABLE 3. Local Inflammatory Infiltrate</th>
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<tbody>
<tr>
<td>Esterase</td>
</tr>
<tr>
<td>Buffer</td>
</tr>
<tr>
<td>βgal</td>
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<tr>
<td>THM</td>
</tr>
</tbody>
</table>

Number of neutrophilic granulocytes, RAM-11–positive rabbit macrophages, or CD4+ T cells per cross section 7 days after balloon dilation and delivery of PBS, Adv/RSV-βgal, or Adv/RSV-THM.

Given that therapies that individually target inflammation, thrombosis, and matrix remodeling have met with success in limiting neointima formation, such multitarget strategies as THM may offer significant improvements. Realistically, all these processes probably play roles in patients undergoing balloon angioplasty. However, one process may prove to be central in some patients, whereas another becomes more important in other patients. Because it will be difficult to identify each group a priori, therapies that target only one may be relatively limited in a broad cross section of patients. In contrast, such therapies as THM may prove effective in limiting neointima formation in each of these environments and may thus be applied more broadly.

Acknowledgments

This work was supported in part through NIH HL-50422 (Dr Woo), Swiss National Science Foundation Fellowship (Dr Hilfiker), and the Holderbank Foundation (Dr Hilfiker).

References


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Circulation. 2000;102:332-337
doi: 10.1161/01.CIR.102.3.332

Circulation is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2000 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7322. Online ISSN: 1524-4539

The online version of this article, along with updated information and services, is located on the World Wide Web at:
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