Local Overexpression of Thrombomodulin for In Vivo Prevention of Arterial Thrombosis in a Rabbit Model


Abstract—Endothelial thrombomodulin plays a critical role in hemostasis by binding thrombin and subsequently converting protein C to its active form, a powerful anticoagulant. Thrombomodulin thus represents a central mechanism by which patency is maintained in normal vessels. However, thrombomodulin expression decreases in perturbed endothelial cells, predisposing to thrombotic occlusion. 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 in vivo thrombus formation was subsequently examined in a stasis/injury model of arterial thrombosis. The construct prevented arterial thrombosis formation in all animals, while viral and nonviral controls typically developed occluding thrombi. By histological analysis, nonviral controls exhibited intravascular thrombus occluding a mean of 70.52±3.72% of available lumen, while viral controls reached 86.85±2.82% thrombotic occlusion; in contrast, Adv/RSV-THM reduced thrombosis to 28.61±3.31% of lumen in cross section. No significant intima-to-media ratio was observed in the thrombomodulin group relative to controls. Local infiltration of granulocytes and macrophages significantly decreased in the Adv/RSV-THM group relative to controls, while neutrophilic infiltration increased in viral controls relative to nonviral controls. This construct thus offers a viable technique for promoting a locally thromboresistant small-caliber artery, without the inflammatory damage that has limited many other adenoviral applications. (Circ Res. 1999;84:84-92.)

Key Words: adenovirus | protein C | gene therapy

Despite extensive preventative efforts, cardiovascular disease remains the leading cause of death in the United States. Extensive research has thus been undertaken to better understand and manage the processes involved. Traditionally, most interest has been focused on the underlying vascular lesion, the atheroma. However, since the vast majority of acute myocardial infarctions and cerebrovascular accidents are precipitated by acute thrombotic events superimposed on underlying lesions, a great deal of attention has been focused recently on the process of thrombosis. Clinical research has shown that thrombolytic therapy improves survival in acute myocardial infarction and cerebrovascular accident patients, heparin improves survival in certain high-risk subpopulations, and chronic low-dose aspirin therapy may decrease the incidence of all such events. In addition, a number of new antithrombotic modalities, including tissue factor pathway inhibitors, have shown great promise in initial studies.

The surface glycoprotein thrombomodulin (THM), by binding thrombin on a 1:1 or a 2:1 basis and subsequently increasing its inactivation via antithrombin III, selectively removes a catalytically active procoagulant. Once bound, thrombin undergoes a conformational change, which alters its specificity and allows conversion of protein C to its active form, a powerful anticoagulant. In keeping with these essential functions, thrombotic disorders have been found to result from relative deficiency of THM or protein C/S, and homozygous deficiency of THM has even been shown to confer lethality in mice. Further, systemic infusion of recombinant soluble THM has shown promise as a useful anticoagulant therapy.

Endothelial cells reflect the local pathophysiological milieu and play active roles in determining, among other processes, the procoagulant/anticoagulant balance. A perturbed endothelial cell dramatically decreases expression of THM as a critical mechanism for shifting the local environment toward a procoagulant state. These observations provide further indirect evidence for the essential functions performed by THM. The question naturally arises whether, in the face of perturbation, local overexpression of THM might
be sufficient to maintain a thromboreistant state while all other procoagulant mechanisms remain intact.

Local gene overexpression can be readily achieved in defined locations by gene transfer to endothelial cells and vascular smooth muscle cells. Several laboratories have already reported retroviral, adenoviral, and direct DNA transfer to the vessel wall with varying efficiencies. However, most retrovirus-based protocols have encountered problems with relatively low gene expression, and direct DNA transfer has also been limited by low efficiencies. In contrast, our laboratory and others have found adenovirus-based models to be high-efficiency in vivo expression systems.

Our laboratory has thus chosen to pursue local vascular adenoviral delivery of THM to promote thromboreistance. In this investigation we assess the in vitro and in vivo function of a replication-deficient adenovirus expressing human THM under the control of a Rous sarcoma virus (RSV) promoter (Adv/RSV-THM). In cell culture lines expressing low-level endogenous THM, the Adv/RSV-THM construct consistently produces very high levels of THM antigen expression relative to controls. The increases in antigen correlate well with increases both in thrombin-binding capability and in quantitative activation of protein C. Comparable function of the construct in a rabbit model is confirmed by in vivo delivery to the common femoral artery (CFA) followed by assay of THM levels in the same vessel segment 3 days after infection. Finally, local overexpression of THM via the Adv/RSV-THM construct is shown to be sufficient for prevention of in vivo arterial thrombus formation.

Materials and Methods

Human thrombin, purified human protein C, and activated human protein C were obtained from Calbiochem (San Diego, Calif), and the chromogenic substrate Spectrozyme PCa (H-D-Lys[OAm]-PCa) was obtained from American Diagnostica (Greenwich, Conn). Texas red–conjugated rabbit anti-goat Fc IgG antibody in 2% horse serum for 4 hours was washed and incubated for 4 hours with a Texas red–conjugated goat anti-rabbit Fc IgG antibody for 4 hours. Control wells with an irrelevant primary antibody, no primary antibody, or no secondary antibody were examined to confirm the specificity of the immunofluorescence results. Images of all wells were acquired and digitized for analysis in the ImagePro Plus system. Briefly, density of fluorescence was calculated through automated processing of images and normalized to results from nonviral controls.

In Vitro THM Antigen Expression Levels

PLC and A549 cells were grown in DMEM supplemented with 10% FBS, penicillin 100 μg/mL, and streptomycin 100 μg/mL. Wells were then infected (at n=3 each) with Adv/RSV-THM, Adv/RSV-βgal, or PBS alone for 4.5 hours at multiplicity of infection (MOI) 300 and then washed with PBS and incubated in culture medium overnight. Wells were then fixed with 0.5% paraformaldehyde for 5 minutes and incubated at 4°C with a primary goat anti-human THM IgG antibody in 2% horse serum for 8 hours at 1:400 dilution. After incubation the wells were washed 3 times with 2% horse serum and then incubated with a Texas red–conjugated rabbit anti-goat Fc IgG antibody for 4 hours. Control wells with an irrelevant primary antibody, no primary antibody, or no secondary antibody were examined to confirm the specificity of the immunofluorescence results. Images of all wells were acquired and digitized for analysis in the ImagePro Plus system. Briefly, density of fluorescence was calculated through automated processing of images and normalized to results from nonviral controls.

In Vitro Thrombin Binding

PLC and A549 cells were infected (at n=3 each) with Adv/RSV-THM, Adv/RSV-βgal, or PBS for 4.5 hours at MOI 300 and then washed and incubated in culture medium overnight. Cells were incubated with 20 nmol/L human thrombin for 1 hour and then washed and incubated for 4 hours at 4°C with a rabbit anti-human thrombin IgG at 1:250 dilution. Wells were washed and incubated for 4 hours with a Texas red–conjugated goat anti-rabbit Fc IgG. Specificity of the immunofluorescence was confirmed as before. Results were assessed relative to one another through image analysis as before.

In Vitro Activation of Protein C

PLC cells were plated on 2-well slides coated with 1% gelatin and cultured for 48 hours. Human umbilical vein endothelial cells (HUVECs) were harvested by the method of Jaffe et al and grown on 2-well slides coated with 1% gelatin. Wells were then infected (at n=5 each) with MOI 300 Adv/RSV-THM, Adv/RSV-βgal, or PBS alone for 4.5 hours. After a 24-hour incubation, wells were washed and incubated with 200 μL of 20 nmol/L thrombin each for 30 minutes at 37°C. The supernatant was then discarded, and the cells were washed and incubated with 100 ng/mL protein C for 30 minutes. This supernatant was withdrawn and incubated in 20-μL aliquots and serial dilutions to 20-μL aliquots with the Spectrozyme PCa chromogenic substrate at 37°C for 15 minutes. Reactants were quenched with glacial acetic acid and optical density at 405 nm recorded for each. The results were compared with standard curves generated in the same assay using serial dilutions of commercially obtained activated protein C or background of protein C alone. In spite of washing, some residual thrombin without specific binding was likely to be present on all wells. Thus, only relative gains from 1 group to another can be directly evaluated in this assay.

Local In Vivo Delivery of Recombinant Adenovirus for In Vivo THM Levels

New Zealand White rabbits were anesthetized with ketamine 42.8 mg, xylazine 8.6 mg, and acepromazine 1.4 mg at 0.5 to 0.7 mL/kg body weight. In vivo adenoviral delivery to an isolated vessel segment was performed as follows. Briefly, the left CFA of each rabbit was exposed. The left inferior epigastric artery (IEA) was ligated, cannulated, and divided, and a 3.0-cm segment of the left CFA beginning just proximal to the left IEA stump was then isolated from circulation viaatraumatic microvascular clamps. The contents of the lumen were aspirated via the IEA stump, and the lumen was gently washed with PBS before incubation of 200 μL of PBS alone, 200 μL of 5×10⁷ plaque-forming units (pfu)/mL Adv/RSV-βgal, or 200 μL of 5×10⁷ pfu/mL Adv/RSV-THM (n=4 each). The segment was allowed to stand for 15 minutes before incubation solutions were aspirated, and the lumen was washed before clamp removal.

Six days after viral delivery, the left CFA was excised and fixed in 10% neutral buffered formalin for 12 hours before sectioning and
immunostaining with a goat anti-rabbit THM primary antibody (a kind gift of K. Wright, M.D. Anderson Cancer Center, Houston, Tex) at 1:1000 dilution overnight. This primary antibody has been previously observed to recognize both human and rabbit THM. Antigenic sites were visualized with a secondary anti-goat IgG conjugated to alkaline phosphatase (Vector Laboratories). Alkaline phosphatase activity was visualized according to established methods, and high-resolution digital images were acquired for image analysis in the ImagePro plus system. Three sections per vessel were evaluated. Briefly, the image analysis protocol consisted of red channel extraction followed by $3 \times 3$ cross-closing filter $\times 1$, which was followed by determination of the number of positive pixels per cross section. The results were normalized to total cross-sectional area for each section. Automated processing results were manually confirmed for each section.

### In Vivo Thrombus Initiation After Adenoviral Delivery

Animals were anesthetized as before, and local in vivo delivery of recombinant adenovirus was accomplished, as described above for the following groups: (1) 200 $\mu$L of PBS, (2) 200 $\mu$L of Ad-RSV-$\beta$gal at a titer of $5 \times 10^8$ pfu/mL, and (3) 200 $\mu$L of Adv/RSV-THM at a titer of $5 \times 10^8$ pfu/mL (n=4 each). Three days after viral delivery, each animal was reexplored and a thrombus initiated. Briefly, at a point 1.0 cm distal to the IEA in the CFA segment previously isolated, the CFA was divided and reanastomosed using 10-0 nylon suture following standard microsurgical techniques. After the anastomosis was completed, 2 loose perpendicular loops of 8-0 silk suture were placed 0.5 cm distal to the anastomosis to introduce an element of stasis. Three days after thrombus initiation (6 days after viral delivery), the animals were assessed for degree of thrombus formation in the treated vessel segments as described below.

### Histological Evaluation of In Vivo Thrombus Formation

On day 6 after viral delivery, each treated vessel was harvested before euthanasia, fixed for 16 to 18 hours in neutral buffered formalin supplemented with zinc chloride (Antech Ltd), and divided into 5 equal segments as defined below, and serial histological sections were obtained from the proximal face of each. For all animals, zone A contained the IEA bifurcation, zone C contained the anastomotic line, zone D contained the perpendicular silk loops (stasis element), zone B was defined as the segment between zones A and C, and zone E was defined as that downstream of zone D. Hematoxylin and eosin staining and Verhoeff elastica staining were performed for each zone of each artery. Microscopic images were obtained and analyzed to determine the average cross-sectional area of thrombus in each zone. A Minolta RD-175 SLR digital camera was used to record images of each slide at high resolution. The resulting images were analyzed using the ImagePro analysis system to determine the average cross-sectional thrombus area. The results were normalized to the available total lumen for each section to eliminate variation due to section-to-section or animal-to-animal differences in vessel size. Briefly, thrombus margins were determined and defined as primary area of interest, and the number of pixels area contained in this region was tabulated. The length of the internal elastic lamina (by Verhoeff-von Gieson staining) was determined as a secondary area of interest. The internal elastic lamina was assumed to be nondistensible, and the circular “available lumen” was calculated from lamina length; this method was used to accurately assess available areas even with vessel collapse (fixation and sectioning artifacts) in segments with small thrombus percentages. The simplifying assumption used here is only inaccurate in segments that do not have a fully distending thrombus; in such segments, the degree of thrombus will be overestimated. Hence, this assumption tends to minimize demonstration of therapeutic effect by overestimating degree of thrombus only in low-thrombus cross sections, while accurately assessing full-thrombus controls. Each image was analyzed manually to confirm automated processing accuracy, and the results were tabulated as average cross-sectional percentage thrombus for each segment of each vessel. To avoid ambiguity in the presentation of this work, the average intima-to-media ratio was also determined for each image. In this manner, quantification of thrombus formation was afforded in each of the various zones of thrombus formation for each animal group. Statistical significance for these and subsequent analyses was analyzed using 1-factor ANOVA repeated measures, with significance determined at 95% (Bonferroni, Tukey-HSD, and Student-Newman-Keuls post hoc testing where applicable in SPSS 6.1) and with probability value as reported in the text. This system was also used to count the number of inflamatory cells after identification by immunohistochemical staining as detailed below.

### Evaluation of Local Granulocyte and Macrophage Infiltration

Three random cross sections (4 $\mu$m) from zone B (treated segment without foreign bodies or bifurcations) were obtained from each vessel and incubated with a primary monoclonal antibody to rabbit macrophage or subjected to cellular chloroacetate esterase staining directly to visualize neutrophilic granulocytes. Slides undergoing immunohistochemical evaluation were handled as follows. After decarcarion, antigen retrieval was performed on paraffin sections before immunohistochemistry. Slides (BioTek capillary gap slides, Ventana Medical Systems) were paired and bathed in a working solution of epitope recovery buffer (10X heat-induced epitope retrieval [HIER], HIER101, Ventana Medical Systems) in a steam environment for 20 minutes and then allowed to cool to room temperature. Slides were rinsed in distilled water, immersed in PBS with 0.2% Tween (Sigma), and blocked with serum. Automated immunostaining was performed on an automated capillary immunostaining device (TekMate 500, Ventana Medical Systems). Slides were incubated overnight with primary monoclonal antibody to rabbit macrophage (clone RAM-11, 1:800, Dako) at a 1/800 dilution or diluted negative control. After washes, the slides were incubated with biotinylated anti-mouse IgG (Vector Laboratories) at 2.25 $\mu$g/mL for 45 minutes at room temperature. Endogenous peroxidase activity was blocked using 3% hydrogen peroxide in methanol for 15 minutes at room temperature, and the sections were incubated with avidin-peroxidase (Vector Laboratories) for 45 minutes at room temperature. Antigenic sites were visualized using diaminobenzidine enhanced with 1% nickel chloride as the chromogen. Identification of neutrophilic granulocytes was based on cellular chloroacetate esterase activity as previously described. Briefly, after decarcarion, sections were incubated at room temperature for 30 minutes in a phosphate buffer containing naphthol AS-D chloroacetate (Sigma) as the substrate and hexazoned new fuchsin as the chromogen. Sections were rinsed well in distilled water, lightly counterstained with Mayer’s hematoxylin, and blued in tap water.

Images of each section were again acquired as before to determine the number of positive cells per cross section. These analyses were performed both including and excluding cells present within the thrombus, to avoid ambiguity in the direct contribution of inflammatory infiltrate within the thrombus to this evaluation. Briefly, RAM-11 and esterase images each underwent red channel extraction, with probability value as reported in the text. This system was also used to count the number of inflammatory cells after identification by immunohistochemical staining as detailed below.

### Results

#### In Vitro THM Antigen Expression

Multiple cell lines were screened for THM expression using primary antibodies to THM and a peroxidase-conjugated secondary antibody system. The PLC cell line was identified as having relatively little detectable endogenous expression...
of THM, while A549 cells and HUVECs were found to express high levels of endogenous antigen.

As described, near-confluent wells of PLC cells or A549 cells were infected with Adv/RSV-THM, Adv/RSV-βgal, or PBS alone. Representative results for the PLC cell experiments are shown in Figure 1a. THM is expressed at very high levels in the Adv/RSV-THM–treated cells and at low baseline levels in the 2 groups of control cells. Representative results for the A549 cell experiments are presented in Figure 1b. Although this cell line exhibits high levels of endogenous THM, cells infected with Adv/RSV-THM expressed significantly higher amounts of the antigen in this assay. Quantitative data confirm significant differences between treatment and nonviral or viral controls (P<0.01 for each comparison).

In Vitro Thrombin-Binding Assays
In PLC cell experiments, thrombin binding, a measure of THM function, was markedly elevated in the Adv/RSV-THM group relative to mock virus and PBS controls (Figure 2a), each of which exhibited minimal binding in this cell line by average relative intensity (Table 1). Figure 2b presents the results obtained from the A549 cell experiments. Again, thrombin binding increased significantly in the Adv/RSV-

**TABLE 1.** Average Fluorescence Intensity Relative to Nonviral Controls

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<tr>
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<th>PLC</th>
<th>A549</th>
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<tr>
<td>THM</td>
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<tr>
<td>Buffer</td>
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<td>Thrombin</td>
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<tr>
<td>Buffer</td>
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<td>1.00±0.22</td>
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<tr>
<td>Adv/RSV-βgal</td>
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<td>1.12±0.17</td>
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<tr>
<td>Adv/RSV-THM</td>
<td>2.93±0.19</td>
<td>2.04±0.30</td>
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Table depicts the mean±SE of relative fluorescence intensity for THM antigen as in Figure 1 and for thrombin antigen after thrombin binding as in Figure 2.

**In Vivo Activation of Protein C**
In an attempt to quantify expression of functional THM more precisely, activation of protein C, the most complete measure of THM function, was examined in PLC cells and HUVECs (Figure 3). Although, in our hands, A549 and HUVECs were found to have comparable levels of endogenous THM expression, primary endothelial cultures were used here to more closely simulate in vivo responses and to confirm function in these cells before initiation of true in vivo experiments.

As in previous experiments, PLC controls were found to posses minimal THM function (Figure 3a). A 400% increase over nonviral control values was observed in Adv/RSV-THM–treated PLC cells (P<0.001). Cells infected with Adv/RSV-βgal did not statistically differ from those of PBS controls at 95% confidence. As expected, buffer-treated HUVECs activate high levels of protein C in this assay (Figure 3b). Viral controls exhibited no significant increases or decreases relative to nonviral controls; thus, use of adenovirus as used here did not seem to perturb the level of functional THM expression in this in vitro experiment. Adv/RSV-THM–treated HUVECs exhibited significantly elevated protein C activation even relative to viral and nonviral controls. Interestingly, Adv/RSV-THM–treated PLC cells also demonstrated levels of protein C activation higher than those of HUVEC controls (120% of nonviral HUVEC controls with P<0.01). Thus, the Adv/RSV-THM construct was able to produce levels of THM function significantly above those of uninfected endothelial cells in this assay.

**In Vivo THM Expression**
To confirm function of the construct in a true in vivo system, 3.0-cm segments of New Zealand White rabbit CFAs were infected in vivo with 200 μL of 5×10⁹ pfu/mL Adv/RSV-THM, 5×10⁹ pfu/mL Adv/RSV-βgal, or PBS alone. At 6 days after infection, in vivo THM levels were examined as

![Figure 1. Immunofluorescence for THM antigen. a to c, Low-level expression line, PLC, treated with buffer alone (a), MOI 300 of Adv/RSV-βgal (b), or MOI 300 of Adv/RSV-THM (c). d to f, High-level expression line, A549, treated with buffer alone (d), MOI 300 of Adv/RSV-βgal (e), or MOI 300 of Adv/RSV-THM (f).](http://circres.ahajournals.org/)

![Figure 2. Thrombin binding visualized by immunofluorescence for thrombin antigen. a to c, Low-level expression line, PLC, treated with buffer alone (a), MOI 300 of Adv/RSV-βgal (b), or MOI 300 of Adv/RSV-THM (c). d to f, High-level expression line, A549, treated with buffer alone (d), MOI 300 of Adv/RSV-βgal (e), or MOI 300 of Adv/RSV-THM (f).](http://circres.ahajournals.org/)
described. At this dose, shown in prior work to yield under 35% to 40% infection rates (by β-galactosidase staining), the Adv/RSV-THM construct was able to produce 153±12% of nonviral control levels. Further, at this dose, no significant reductions were observed in THM function in Adv/RSV-βgal groups relative to nonviral controls (92±17%). Although this approach was used to confirm the general function of the construct in vivo, precise evaluation of total THM level was not possible, since epitope selectivity of the anti-rabbit primary antibody could have yielded artificially low results in the Adv/RSV-THM treatment group. As a result, an assay to evaluate the function of the construct in a quantifiable functional in vivo system was devised. Specifically, the construct was tested in a model of thrombus formation to determine whether expression levels were sufficient to prevent thrombus formation.

Prevention of In Vivo Thrombus Formation

To examine the impact of local in vivo overexpression of THM on thrombus formation, rabbit CFAs were incubated in vivo with 200 μL of 5×10⁶ pfu/mL Adv/RSV-THM, 5×10⁶ pfu/mL Adv/RSV-βgal, or PBS alone. Based on prior β-galactosidase work, the 5×10⁶ pfu/mL dose results in infection of 35% to 40% of cells. Three days after infection, a thrombus was initiated using a combination of a nondenuding injury and a stasis element as described in Materials and Methods. This model of thrombus induction was selected because the endothelium remains relatively preserved despite both injury and stasis elements. Six days after infection, the vessels were reexposed.

To quantitatively examine these effects and to better define the spatial distribution of any alterations in thrombus formation within the vessel segments, each segment was subdivided into 5 roughly equal zones as described (Figure 4a). Serial sections were performed in each zone of each artery. Microscopic images were obtained and analyzed to determine the average cross-sectional area of thrombus in each zone. These results were then normalized to the available lumen in each corresponding section, with the results reported as average cross-sectional percentage thrombus for each zone (Figure 4).

Representative histological findings for each treatment group are presented in Figure 5. The mean of sections for all zones was also compiled for each group. Overall, nonviral controls exhibited intravascular thrombus occluding a mean of 70.52±3.72% of available lumen, while viral controls reached 86.85±2.82% thrombotic occlusion; in contrast, Adv/RSV-THM reduced thrombosis to 28.61±3.31% of lumen in cross section. Thus, statistically significant reductions in overall cross-sectional thrombus were achieved in Adv/RSV-THM treatments relative to controls (P<0.01 for each). No statistically significant overall increases in extent of thrombus formation were present in viral controls relative to nonviral controls, in spite of apparent trending (P=0.058).

Intima-to-media ratios were also calculated as the ratios of the appropriate areas for each section; no significant changes in intima-to-media ratios were observed in the viral controls or THM animals relative to nonviral controls (mean±SEM values: 0.131±0.027, 0.105±0.010, and 0.103±0.010 for viral controls, Adv/RSV-THM, and nonviral controls, respectively; P>0.05 for all comparisons).

Number of inflammatory cells per cross section was quantified for esterase-bearing granulocytes and RAM-11–positive rabbit macrophages as detailed above, with the results presented in Table 2. There were no significant increases in granulocyte or macrophage infiltration in Adv/RSV-THM–treated vessels relative to nonviral and viral controls. Statistically significant reductions in granulocyte infiltration of the vessel wall were actually achieved in treated vessels relative to viral controls (P=0.0214) and in macrophage infiltration relative to both viral (P=0.0010) and nonviral controls (P=0.0001). Viral controls exhibited significant increases in granulocyte infiltration of the vessel wall.
(and total cross section) relative to nonviral controls ($P=0.0096$ and $P=0.0001$, respectively) but did not exhibit statistically significant elevations in macrophage infiltration ($P=0.8129$ for wall and $P=0.1869$ for overall cross section).

**Discussion**

Efficient in vivo adenovirus-mediated gene delivery to the vessel wall has been accomplished in our laboratory and others. In this work, a recombinant adenovirus expressing human THM was constructed, shown to be functional both in vitro and in vivo, and demonstrated to be a novel effective approach for prevention of in vivo intravascular thrombus formation in a targeted vascular segment. When in vitro THM levels were examined in cell lines with low or high endogenous THM expression, high levels of Adv/RSV-THM–produced THM expression were found. Since the increase in THM epitope availability could be due to differential antibody reactivity, the functional ability of the Adv/RSV-THM–produced THM to bind thrombin was also examined in vitro with results paralleling the increases in antigen level observed in the initial experiments. Since activation of protein C requires an entirely normally functioning THM-thrombin complex and allows signal amplification, an assay was constructed to quantify this process. In vitro experiments demonstrated significant increases in cells infected with Adv/RSV-THM.

Although function of the construct had been evaluated in vitro, an experiment was performed to confirm elevation of THM levels after in vivo delivery. As detailed, the potential for epitope selectivity of the primary antibody precludes rigorous determination of total THM levels in the Adv/RSV-THM group, since levels observed through this method may thus be spuriously low in this group. Although the absolute value of these effects cannot be conservatively ascertained from these results, the increase in the Adv/RSV-THM treatments relative to viral and nonviral controls demonstrates high-level THM production in vivo as expected from the in vitro experiments. In these experiments, the low-dose adenovirus used did not sufficiently damage or perturb the endothelium to alter endogenous THM levels in mock-virus treatment groups relative to controls. These results validate the use of low-dose adenovirus as a delivery system that can be used with minimal adverse impact on THM expression levels. THM function elicited in this manner might thus

**Figure 4.** Cross-sectional percentage thrombus by zone after delivery of buffer alone, 200 μL of $5 \times 10^9$ pfu/mL Adv/RSV-βgal (βgal), or 200 μL of $5 \times 10^9$ pfu/mL Adv/RSV-THM (THM) with thrombus initiation as described in text.

(a) An illustration of anatomic segments corresponding to zones A through E. Arrow indicates direction of blood flow, and stitches depict stasis elements as described in Materials and Methods.

(b) Cross-sectional thrombus in zone A.

(c) Cross-sectional thrombus in zone B.

(d) Cross-sectional thrombus in zone C.

(e) Cross-sectional thrombus in zone D.

(f) Cross-sectional thrombus in zone E. Values represent mean ± SE as depicted.
remain at sustained elevated levels even in perturbed or damaged vessels. To test whether this system could be used to locally alter vascular responses to one such perturbation, the impact of in vivo gene delivery on intravascular thrombus formation was examined at a higher viral dose.

The Adv/RSV-THM construct prevented the formation of an occluding intravascular thrombus. Histological analysis confirmed that the Adv/RSV-THM group maintained significant patency in all treated and adjacent vessel segments, while occluding thrombi formed in the viral and nonviral controls. Thus, effective thrombus prevention was demonstrated in spite of the complex, extensive local perturbation deliberately used to initiate thrombus formation. In contrast, the mock virus treatment groups actually exhibited increases in degree of thrombus formation in some zones relative to nonviral controls, although no statistically significant differences were present between viral and nonviral controls overall. Examination of the treated vessels by defined zones thus confirmed effective thrombus prevention in Adv/RSV-THM groups throughout, including both the site of anastomosis and the site of stasis. This work thus demonstrates that maintenance of high THM levels is sufficient to maintain a thromboresistant state in spite of a complex, potent impetus for thrombosis. These results, taken with the prior evidence of Figure 5. Representative low-magnification (×6.25; left) and high-magnification (×62.5; right) photographs of CFA cross sections 3 days after thrombus initiation for animals treated with buffer alone (a), 200 μL of 5×10⁵ pfu/mL Adv/RSV-βgal (b), or 200 μL of 5×10⁶ pfu/mL Adv/RSV-THM (c).

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<thead>
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<th>Granulocytes</th>
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<td>Vessel Wall</td>
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<tr>
<td>Buffer</td>
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<tr>
<td>Adv/RSV-βgal</td>
<td>520.50 ± 142.51</td>
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<tr>
<td>Adv/RSV-THM</td>
<td>110.00 ± 23.94</td>
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Table depicts the mean values ± SE for number of esterase-bearing cells (neutrophilic granulocytes) and RAM-11–positive cells (rabbit macrophages) per cross section. Results are presented for total cross section including thrombus (total) and for vessel wall alone, with intravascular thrombus excluded from calculation (vessel wall).
the essential functions performed by THM, help to define THM as a central factor in the antithrombotic/prothrombotic balance.

Some histological evidence of inflammation was present in each group because of the injury and foreign bodies used in thrombus induction. Significant inflammation was present in many sections and zones of the βgal vessels. Previously published experiments have demonstrated that adenoviral doses substantially higher than those presented here result in vigorous local inflammatory responses, with higher-dose virus groups developing more extensive inflammatory infiltrates than lower-dose groups.35,36 These same experiments suggested that doses high enough to achieve certain therapeutic effects would thus be expected to exhibit excessive inflammation. As demonstrated, we are able to achieve our desired functional effect at a lower dose than that suggested by these prior studies, so we may be able to use this system without the high rates of inflammation that have limited other applications. In the present work, we demonstrate that 5×10^10 pfu/mL Adv/RSV-βgal results in no statistically significant elevations in local macrophage infiltration relative to nonviral controls, although local granulocyte infiltration does significantly increase at this dose. Interestingly, Adv/RSV-THM achieved significant reductions in vessel wall (and total) infiltration by both macrophages and granulocytes relative to viral controls at the same dose. No significant increases in either type of inflammation were observed in Adv/RSV-THM–treated animals relative to nonviral controls; remarkably, significant decreases in macrophage infiltration of the vessel wall were observed in the Adv/RSV-THM group even relative to nonviral controls. Many factors may contribute to the reductions in inflammation observed in the THM group, since thrombin binding to thrombin receptors (inhibited by THM binding) has been shown to enhance local inflammation through alteration of endothelial adhesion molecule expression, and protein C itself has been shown to possess some direct anti-inflammatory properties. However, it is likely that a different extent of intravascular thrombus has also altered local inflammation in the vessel wall of the control groups relative to THM-treated animals, which exhibited significantly less thrombus bulk. Given the complex nature of the present study, evaluation of local inflammation and alterations in ICAM-1 and VCAM-1 must be undertaken in a system without thrombus initiation to better characterize these promising effects. However, for the application presented here, the Adv/RSV-THM group achieves significant thromboreistance while simultaneously decreasing local inflammation relative to controls.

The Adv/RSV-THM construct thus offers a viable strategy for promoting a locally thromboresistant small-caliber artery, even at a dose that avoids the inflammatory damage limiting many other adenoviral applications. Since arterial thrombosis in atherosclerotic arteries remains the leading cause of mortality in Western nations, experiments have also been initiated to confirm the impact of this construct in atherosclerotic vessels. The principal limitations of current systemic anticoagulant/thrombolytic therapies are hemorrhagic complications and rebound thrombosis within 10 days after discontinuation of therapy. The strategy presented in this work offers the novel potential to avoid both of these problems. Since delivery of this construct is local, systemic hemorrhagic complications should not occur. Further, current adenoviral vectors have been shown to have a 2- to 4-week expression period, which extends beyond the highest risk window for rebound thrombosis. On a basic level, this work suggests that THM may play a more critical role in these processes than has been previously appreciated.

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