Expression of Thrombomodulin by Smooth Muscle Cells in Culture: Different Effects of Tumor Necrosis Factor and Cyclic Adenosine Monophosphate on Thrombomodulin Expression by Endothelial Cells and Smooth Muscle Cells in Culture

By Gerald A. Soff, Robert W. Jackman, and Robert D. Rosenberg

Thrombomodulin (TM), a critical component of the protein C anticoagulant pathway, has previously been localized to endothelial cells (EC), but not smooth muscle cells (SMC) of the blood vessel wall. We demonstrate that cultured rat, bovine, as well as human SMC, but not blood vessel wall smooth muscle tissue, possess significant functional levels of TM and TM mRNA. Cyclic adenosine monophosphate stimulates TM expression in cultured SMC, but not EC, while tumor necrosis factor suppresses TM expression in EC but not cultured SMC. We postulate that following acute or chronic EC injury, luminal SMC can express TM, and are therefore able to protect the damaged blood vessel from thrombosis.

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Materials and Methods

Bovine aortic SMC (BASMC) were prepared by enzyme dispersion. BAEC were cloned from calf aortas as previously described. Human umbilical vein endothelial cells (HUVEC) were provided by Dennis Lynch (Boston, MA) and human saphenous vein SMC by Peter Libby (Boston, MA). Rat aortic SMC (RASM) were obtained from John Castellot (Boston, MA), or were isolated in our laboratory by enzyme dispersion. Cloning of BASMC was performed by enzyme dispersion of 1 g of calf aortic media and plating in a 100-mm plate. At 48 hours, the cells were trypsinized, counted, and replated at a dilution to yield one cell per well. Bovine gastric SMC were prepared by enzyme dispersion of a bovine stomach muscularis. These cells were morphologically indistinguishable from vascular SMC and also possessed α actin on Northern blots.

All primary cells were used within the first 10 passages, except the cloned BASMC. A427 (a human lung carcinoma line) and human skin fibroblasts (HSF) were from ATCC (Rockville, MD). The SMC, BAEC, HSF, and A427 were grown in Dulbecco’s modified Eagle’s media supplemented with 10% fetal bovine serum (FBS), penicillin 100 μg/mL, and streptomycin 100 μg/mL. HUVEC were grown on gelatin- or fibronectin-coated plates in M199 with 20% FBS, 0.1 mg/mL porcine heparin, 5 μg/mL “EC mitogen” (a crude bovine brain extract; Biomedical Technologies Inc, Stoughton, MA), penicillin 100 μg/mL, and streptomycin 100 μg/mL. The TM functional assay was based on the technique of Esmon and Owen as modified by Salem HH et al. One unit of TM is defined as a change of 1.0 in OD₅₇₀ per minute per 10⁶ cells. RNA isolation was performed by the method of Chirgwin et al. Northern blot analysis was performed by standard molecular biologic techniques. Human, bovine, and rat TM, human γ actin, and rat brain β-tubulin (Dr Matt Fenton, Cambridge, MA) cDNA probes were labeled by nick translation using a kit produced by BRL (Gaithersburg, MD). The signals from Northern blot analyses were quantitated either by autoradiography and densitometry or by a Betascope (Betagen, Waltham, MA).

Tumor necrosis factor (TNF) was supplied by Asahi Chemical Industry America (New York, NY). The specific activity was 2.2 x 10⁶ U/mg. TNF treatment consisted of supplementing complete medium with TNF for 16 to 18 hours. N₂,2'-O-dibutyryladenosine 3':5'-cyclic monophosphate (Dibutyryl cAMP), 8-bromoadenosine 3':5'-cyclic monophosphate (8-bromo cAMP), and theophylline were from Sigma (St Louis, MO).

Results

The levels of TM biologic function were quantitated in cultures of vascular SMC and EC (Table 1). BASMC exhibited 70% of the TM activity per cell as compared with BAEC, and human saphenous vein SMC possessed 240% of receptor activity per cell associated with HUVEC. RASM are also endowed with significant levels of TM functional activity, although cultures of rat EC were unavail-

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TM is defined as a change of 1.0 in OD, per minute per 10^6 cells. Owen" as modified by Salem et al. [13]. Data are presented as mean TM units compared with BASMC. Human skin fibroblasts and the TM expression was because of a subset of the cells. Of 20 tric muscularis) exhibited 85% of the TM activity per cell as able for comparison. Nonvascular SMC (from bovine gas-
activity, contained less than 3% of receptor activity per cell known to possess undetectable levels of TM biologic human lung carcinoma line A427, cell types previously
initial colonies, 14 survived expansion to 1-cm wells (approx-
1.8- to 4.0-fold increase in the levels of TM receptor as well as TM mRNA in the α isoform, while cultured bovine SMC possessed 70% of actin mRNA in the β/γ doublet. The rat aortic media SMC exhibited 85% of actin mRNA in the α isoform while the cultured rat SMC possessed 89% of actin mRNA in the β/γ doublet.

TNF significantly reduces TM expression of EC, [16] and cAMP analogues greatly enhance TM expression of mega-
karyoblastic leukemia cell lines. [17] Therefore, we evaluated the effects of the two agents on cultured SMC (Table 2). TNF (400 U/mL) produced a minimal decrease (10% to 20%) in the levels of TM receptor as well as TM mRNA in cultured BASMC whereas no significant alteration in either parameter was observed with rat SMC. These results are in contrast to the marked cytokine-dependent suppression of the levels of TM receptor and TM mRNA (80% to 90%) in bovine and human EC. The expression of TM by vascular SMC and EC was also differentially effected by cAMP analogues. Treatment of rat and bovine SMC with dibutylryl cAMP (500 μmol/L), and theophylline (500 μmol/L) for 18 hours, produced a 1.8- to 4.0-fold increase in the levels of TM receptor and TM mRNA. Treatment of rat SMC with 1 mmol/L 8-bromo cAMP and 500 μmol/L theophylline for a similar period of time, resulted in a 2.8-fold increase in TM activity. These results show that the cAMP moiety enhances

Table 1. TM Functional Levels

<table>
<thead>
<tr>
<th>Sample</th>
<th>TM Function</th>
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<tbody>
<tr>
<td>BAEC</td>
<td>0.37 ± 0.02 (n = 3)</td>
</tr>
<tr>
<td>Bovine SMC</td>
<td>0.26 ± 0.05 (n = 3)</td>
</tr>
<tr>
<td>HUVEC</td>
<td>0.71 ± 0.18 (n = 3)</td>
</tr>
<tr>
<td>Human saphenous vein SMC</td>
<td>1.70 ± 0.50 (n = 4)</td>
</tr>
<tr>
<td>RASMC</td>
<td>0.55 ± 0.14 (n = 4)</td>
</tr>
<tr>
<td>Bovine gastric SMC</td>
<td>0.22 ± 0.02 (n = 2)</td>
</tr>
<tr>
<td>HSF</td>
<td>0.005 ± 0.001 (n = 3)</td>
</tr>
<tr>
<td>A427</td>
<td>0.004 ± 0.001 (n = 3)</td>
</tr>
</tbody>
</table>

The TM functional assay was based on the assay of Esmon and Owen [12] as modified by Salem et al. [13]. Data are presented as mean TM units ± standard deviation for (n) number of experiments. One unit of TM is defined as a change of 1.0 in OD₆₅₀ per minute per 10⁶ cells.

Northern blot analyses of SMC and RASMC were performed to confirm the results of the TM functional studies. Significant amounts of TM mRNA were demonstrated in cultured RASMC and BASMC. The TM mRNA signals from both rat and bovine cultured SMC were fourfold above the background counts per minute (Fig 1). In contrast, TM mRNA was not detectable in smooth muscle tissue from rat and bovine aortic media. The counts in the TM mRNA regions were within 3% of background levels. The blots were then probed with human γ actin cDNA, which cross-hybridizes with rat and bovine α, β, and γ actin mRNA. The intensity of the actin bands in the various lanes confirmed the comparable sample loading of RNA. The actin probing also showed the change in the predominant actin isoform between the cultured and media SMC. Bovine aortic medial SMC exhibited 55% of actin mRNA in the α isoform, while cultured bovine SMC possessed 70% of actin mRNA in the β/γ doublet. The rat aortic media SMC exhibited 85% of actin mRNA in the α isoform while the cultured rat SMC possessed 89% of actin mRNA in the β/γ doublet.

Fig 1. Composite Northern blot analyses of RNA prepared from bovine and rat aortic med-
dial SMC and cultures of SMC. Blots were probed with bovine or mouse TM cDNA and re-
probed with human γ actin cDNA. The counts per minute (CPM) of the TM signals in both medial samples were within 3% of back-
ground, while the CPM of the tissue culture TM samples were fourfold of background. The ac-
tin reprobing confirmed compa-
rable loading of RNA between the medial and tissue culture samples and also indicated the switch from the α actin isoform, predominant in the media to the β/γ actin isoforms in the cultured SMC.
expression of the receptor. Exposure of SMC to theophylline alone is insufficient to increase the levels of TM. These data are in contrast to the lack of any significant effect by cAMP analogues on receptor expression in bovine and human EC.

**DISCUSSION**

Prior investigators have observed that TM expression in the blood vessel wall is confined to the endothelium with medial SMC possessing no detectable receptor. In our current study, we have extended these observations by demonstrating with Northern analyses no detectable TM mRNA in medial SMC. In contrast to the above data, SMC in tissue culture express TM mRNA and receptor. This expression was observed in primary-passage SMC, and is independent of passage number. The analyses of BASMC clones show that TM expression is a durable phenotypic trait and that the presence of receptor is not because of a small subpopulation of expressing cells. Our results also show that cultured bovine gastric SMC possess TM, which shows that nonvascular SMC can express the receptor.

The expression of TM appears to be regulated differently in cultured SMC as compared with EC. Firstly, TNF treatment of cultured SMC does not inhibit TM gene transcription as observed for EC. The explanation for the absence of this inhibition is unknown, but may be related to reduced numbers of TNF receptors or alterations in the intracellular components required for postreceptor signaling such as the recently defined cytokine-responsive trans-acting factors NF-κB and NF-κMa. Secondly, cAMP analogues significantly enhance TM expression in cultured rat and bovine SMC, but exert no effect on the levels of receptor in cultured EC. It is known that cAMP acts as an intracellular second messenger via the protein kinase A pathway, and influences mRNA levels either by stimulating gene transcription, or by increasing or decreasing mRNA stability. The mechanism by which cAMP augments TM expression in MEG-01 and human erythroleukemia cells has not been elucidated nor has the agonist(s) been defined that stimulates receptor level via cAMP. Our results show that cAMP elevates expression of TM in cultured SMC by increasing receptor mRNA levels, but it has yet to be determined if this phenomenon is because of an enhanced rate of gene transcription or an increased survival of mRNA.

Numerous investigations have shown that medial SMC migrate to the blood vessel lumen following EC injury, and form a stable nonthrombogenic neointima. Prior studies have also documented phenotypic similarities between neointimal SMC and cultured SMC. These shared characteristics include a switch from the α actin isoform, predominant in the normal media, to the β and γ isoforms, predominant in neointimal SMC and cultured SMC. We postulate that after acute or chronic intimal injury, neointimal SMC express TM, and are therefore able to protect the damaged blood vessel from thrombosis. The cAMP-dependent mechanism may be required to function in concert with changes initiated by phenotypic modulation to generate full in vivo expression of the receptor. The relative levels of neointimal SMC TM could be relevant to the maintenance of coronary artery patency after angioplasty. The neointimal SMC are also believed to play a role in the early stages of atherosclerosis. Thus, investigations of TM gene expression by neointimal SMC may serve as a marker for defining the transition from medial to neointimal SMC, providing insight into the intracellular pathways responsible for regulating this event.

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Expression of thrombomodulin by smooth muscle cells in culture: different effects of tumor necrosis factor and cyclic adenosine monophosphate on thrombomodulin expression by endothelial cells and smooth muscle cells in culture

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