Regulation of Thrombomodulin Expression by All-trans Retinoic Acid and Tumor Necrosis Factor-α: Differential Responses in Keratinocytes and Endothelial Cells

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Thrombomodulin is a cell-surface anticoagulant glycoprotein expressed by vascular endothelial cells and epidermal keratinocytes. Thrombomodulin expression in endothelial cells is regulated by retinoic acid and tumor necrosis factor-α (TNF), agents that also modulate epidermal differentiation. We examined thrombomodulin function and regulation of thrombomodulin expression by all-trans retinoic acid (ATRA) and TNF in human keratinocytes and endothelial cells. Untreated keratinocytes and endothelial cells expressed thrombomodulin of comparable activity and apparent thrombin affinity. Incubation of keratinocytes with 10 μmol/L ATRA for 24 hours increased thrombomodulin activity 5.4 ± 0.9-fold (mean ± SE), with equivalent increases observed in thrombomodulin protein (5.5 ± 2.1-fold) and mRNA (4.2 ± 1.2-fold). Incubation of keratinocytes with 1.0 nmol/L TNF markedly increased expression of keratinocyte transglutaminase, but had no effect on thrombomodulin activity, protein, or mRNA. In endothelial cells, ATRA produced a small increase in thrombomodulin activity (1.9 ± 0.1-fold), and incubation with TNF for 24 hours decreased thrombomodulin activity 83% ± 7%. The activity profile of keratinocyte thrombomodulin exhibited a distinct maximum near 1.0 mmol/L Ca²⁺. These results demonstrate that keratinocyte thrombomodulin is regulated by retinoids and Ca²⁺, but not by TNF, and that regulation of thrombomodulin expression differs in keratinocytes and endothelial cells.

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MATERIALS AND METHODS

Materials. ATRA was purchased from Sigma Chemical Co (St Louis, MO), and TNF was purchased from Genzyme Corp (Cambridge, MA). Human thrombin was purchased from Enzyme Research Laboratories (South Bend, IN). Human protein C and anti-
thrombin III were generous gifts of Dr Hans Peter Schwarz (Immuno AG, Vienna, Austria) and Dr Douglas Tollefson (Washington University, St Louis, MO), respectively. Rabbit lung thrombomodulin was purchased from American Diagnostica, Inc (Greenwich, CT). Recombinant TM456 was purified from tissue culture medium as described previously.\textsuperscript{22} Porcine intestinal heparin was obtained from Elkins-Sinn, Inc (Cherry Hill, NJ), and S-2366 was purchased from Kabi Pharmacia Hepar, Inc (Franklin, OH).

**Cell culture.** Human umbilical vein endothelial cells (HUVEC) and neonatal human foreskin keratinocytes were purchased from Clonetics Corporation (San Diego, CA). All cells were studied within 2 to 4 passages after primary culture. HUVEC were cultured in endothelial growth medium containing 2% fetal bovine serum (FBS) as described previously.\textsuperscript{4} Keratinocytes were cultured in serum-free keratinocyte growth medium (KGM) containing 0.07 mmol/L calcium chloride. When keratinocyte cultures reached 75% confluency, the medium was changed to KGM containing 1.4 mmol/L calcium chloride, and the cells were cultured for up to 96 hours in the presence of various concentrations of ATRA or TNF. Control cultures were incubated with either 0.1% dimethyl sulfoxide (DMSO; the vehicle for ATRA) or 0.3% phosphate-buffered saline (PBS; the vehicle for TNF).

**Protein C activation.** Protein C activation was measured using a two-stage assay described previously.\textsuperscript{4} Cells were washed three times with PBS and lysates were prepared in 20 mmol/L Tris-HCl, pH 8.0, 0.6% Triton X-100, and 100 mmol/L NaCl. The total protein concentration of cell lysates was measured by a modified Bradford assay (Bio-Rad Laboratories, Richmond, CA). For measurement of thrombomodulin cofactor activity, lysates were incubated for 30 minutes at 37°C with 2.6 nmol/L human thrombin, 0.84 μmol/L human protein C, and various concentrations of CaCl\textsubscript{2}. The reaction was stopped by addition of a mixture of antithrombin III (25 μg/mL) and heparin (25 U/mL), and the amidolytic activity of activated protein C was measured with the chromogenic substrate S-2366. All assays were performed in replicate (n = 3 to 6) and statistical comparisons were made using the two-tailed Student's t-test.

For determination of apparent equilibrium dissociation constant K\textsubscript{app}, the initial rate of protein C activation was measured in the presence of fixed concentrations of protein C (0.84 μmol/L) and CaCl\textsubscript{2} (2.5 mmol/L), and increasing concentrations of thrombin (0 to 50 nmol/L). K\textsubscript{app} was defined as the concentration of thrombin that produced half-maximal thrombomodulin cofactor activity, as determined by nonlinear regression analysis (SigmaPlot, Jandel Scientific, San Rafael, CA).

**Immunoblot analysis.** Cells were washed three times with PBS and lysates were prepared in 20 mmol/L Tris-HCl, pH 8.0, 0.6% Triton X-100, 100 mmol/L NaCl, 3.0 mmol/L CaCl\textsubscript{2}, and 10 mmol/L iodoacetamide. After centrifugation at 12,000g for 5 minutes, supernatant fractions containing 50 μg of total protein were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under nonreducing conditions. Immunoblot analysis was performed as described previously\textsuperscript{4} using mouse antihuman thrombomodulin monoclonal antibody TM1009 (Dako Corp, Carpinteria, CA) or mouse antihuman keratinocyte transglutaminase monoclonal antibody B.C1 (kindly provided by Dr Robert H. Rice, University of California, Davis, CA)\textsuperscript{3} with enhanced chemiluminescence detection.

**Nuclease S1 protection analysis.** Plasmids containing cDNA inserts for human thrombomodulin (pUC19TM12)\textsuperscript{5} and human γ actin (pHFYA-1, provided by Dr L. Kedes, University of Southern California, Los Angeles, CA)\textsuperscript{5} were linearized by digestion with M\textsubscript{Bol} and BglII, respectively. The linearized plasmids were treated with calf intestinal alkaline phosphatase, and end-labeled with \(^{32}P\)ATP and T4 polynucleotide kinase. Total cellular RNA was isolated from cultured cells by acid guanidinium thiocyanate-phenol-

chloroform extraction (Tri-reagent; Molecular Research, Inc, Cincinnati, OH). The end-labeled thrombomodulin and actin plasmids were hybridized overnight at 55°C with 50 μg or 5 μg of total cellular RNA, respectively, and analyzed by denaturing gel electrophoresis as described previously.\textsuperscript{14}

**Densitometry.** Laser densitometry of nuclease S1 protection autoradiographs and immunoblots was performed using a 300S computing densitometer with ImageQuant v5.0 software (Molecular Dynamics, Sunnyvale, CA).

**RESULTS**

Previous studies have shown that keratinocytes and endothelial cells contain thrombomodulin with similar apparent molecular mass and specific cofactor activity.\textsuperscript{4,6} To determine whether keratinocyte thrombomodulin and endothelial cell thrombomodulin differ in affinity for thrombin, we measured the apparent dissociation constant K\textsubscript{app} in a protein C activation assay. In three separate experiments, K\textsubscript{app} for thrombin ranged from 5.3 ± 0.3 to 8.4 ± 0.5 mmol/L with keratinocyte thrombomodulin (Fig 1A) and from 3.1 ± 0.4 mmol/L to 5.6 ± 1.3 mmol/L with HUVEC thrombomodulin (Fig 1B). These values, which are similar to those reported for other preparations of human thrombomodulin,\textsuperscript{29} indicate that thrombin binds to keratinocyte thrombomodulin and endothelial cell thrombomodulin with similar affinity.

**Effects of ATRA and TNF on thrombomodulin cofactor activity.** The effect of ATRA on the activity of keratinocyte thrombomodulin was measured in protein C activation assays with lysates prepared from keratinocytes incubated with various concentrations of ATRA for 24 hours (Fig 2A). ATRA produced a dose-dependent increase in thrombomodulin cofactor activity, with maximum activity after incubation with 20 μmol/L ATRA. Incubation with higher concentrations of ATRA caused cell toxicity. The time course of ATRA-induced increase in thrombomodulin activity is shown in Fig 2B. When keratinocytes were cultured for 96 hours in the presence of 10 μmol/L ATRA, thrombomodulin cofactor activity peaked at 24 hours, and then declined gradually. As reported previously,\textsuperscript{4} a more modest increase in thrombomodulin activity was observed when keratinocytes were incubated with 1.4 mmol/L calcium in the absence of ATRA.

In agreement with previous studies,\textsuperscript{16,27} incubation of HUVEC with 10 μmol/L ATRA for 24 hours produced a small but significant increase in thrombomodulin activity (Fig 3A). However, the increase in thrombomodulin activity induced by this concentration of ATRA was considerably greater in keratinocytes (5.4 ± 0.9-fold) than in HUVEC (1.9 ± 0.1-fold). In contrast to ATRA, TNF produced a marked decrease in thrombomodulin activity in HUVEC, but had little effect on thrombomodulin activity in keratinocytes. Incubation of HUVEC with 1.0 mmol/L TNF for 24 hours decreased thrombomodulin activity by 83% ± 7% (Fig 3B). In several experiments, incubation of keratinocytes with TNF for up to 5 days resulted in no significant change in thrombomodulin expression compared with control keratinocytes incubated with PBS.

**Effects of ATRA and TNF on thrombomodulin protein and mRNA.** In endothelial cells, ATRA and TNF regulate thrombomodulin expression primarily at the level of tran-
DIFFERENTIAL REGULATION OF THROMBOMODULIN

Although cultured keratinocytes have been reported to express functional TNF receptors,18 the failure of TNF to alter thrombomodulin expression raised the possibility that keratinocytes may lack responsiveness to TNF under these culture conditions. To test this possibility, immunoblot analysis was performed using a monoclonal antibody to keratinocyte transglutaminase, a marker of keratinocyte differentiation that is synthesized in the granular layer of epidermis.24 In agreement with previous studies,24 we detected increased expression of transglutaminase after keratinocytes were cultured for 3 days in KGM containing 1.4 mmol/L Ca²⁺ and either PBS or DMSO (Fig 6). The increase in keratinocyte transglutaminase was inhibited by addition of 10 μmol/L ATRA, and enhanced by addition of 1.0 nmol/L TNF, confirming that keratinocytes respond to TNF under these cul-

Fig 1. Determination of Kₚᵥₑₚₑ for keratinocyte and endothelial cell thrombomodulin. The thrombin dependence of protein C activation was measured in the presence of (A) keratinocyte or (B) HUVEC lysates. Values ± SD for Kₚᵥₑₚₑ were 6.4 ± 0.5 for keratinocytes and 3.5 ± 0.5 nmol/L for HUVEC.

scription, producing equivalent changes in thrombomodulin protein and mRNA.13,15,27 To determine effects of ATRA and TNF on thrombomodulin protein and mRNA in keratinocytes, immunoblot and nuclease S1 protection analyses were performed. Similar amounts of thrombomodulin protein (Fig 4) and mRNA (Fig 5) were detected in keratinocytes incubated for 24 hours with 1.0 nmol/L TNF or vehicle (PBS or DMSO). Keratinocytes incubated for 24 hours with 10 μmol/L ATRA contained markedly increased amounts of thrombomodulin protein (Fig 4) and thrombomodulin mRNA (Fig 5) compared with keratinocytes incubated with TNF, PBS, or DMSO. No treatment-related changes in the level of actin mRNA were observed (Fig 5). Mean ATRA-induced increases in thrombomodulin protein (5.5 ± 2.1-fold in five experiments) and thrombomodulin mRNA (4.2 ± 1.2-fold in two experiments) were similar in magnitude to increases in thrombomodulin activity observed in ATRA-treated keratinocytes (Fig 3A).

![Graph A](image1)

**Fig 2.** Effect of ATRA on thrombomodulin activity in keratinocytes. (A) Keratinocytes were incubated for 24 hours in KGM containing 1.4 mmol/L Ca²⁺ and the indicated concentrations of ATRA. (B) Keratinocytes were incubated for the indicated times in KGM containing 1.4 mmol/L Ca²⁺ and either 10 μmol/L ATRA (○) or 0.1% DMSO (●). Thrombomodulin cofactor activity was measured in a two-stage protein C activation assay. Values represent the mean ± SE of three determinations.
ture conditions. In contrast, thrombomodulin expression was enhanced by ATRA and unaffected by TNF (Fig 6).

In this experiment (Fig 6), peak induction of thrombomodulin protein was observed 3 days after addition of ATRA (lane 8). In other experiments, peak induction of thrombomodulin was observed at various times between 1 and 3 days after addition of ATRA. More rapid induction of thrombomodulin expression was observed with keratinocyte monolayers that were more than ∼70% confluent, and slower induction was observed with less confluent cultures.

Calcium-dependence of thrombomodulin activity. To determine the calcium-dependence of keratinocyte thrombomodulin activity, protein C activation assays were performed with keratinocyte lysates in the presence of up to 10.0 mmol/L CaCl₂. The activity profile of keratinocyte thrombomodulin was compared with that of rabbit lung thrombomodulin and TM456, a soluble recombinant thrombomodulin fragment that comprises the fourth through sixth EGF-like domains of human thrombomodulin. Keratinocyte thrombomodulin demonstrated a biphasic activity profile, with activity increasing sharply as the CaCl₂ concentration was increased from 0 to 1.0 mmol/L, and activity decreasing at higher CaCl₂ concentrations (Fig 7A). TM456 also produced a biphasic activity profile, with peak activity at a slightly lower CaCl₂ concentration (0.5 mmol/L) than keratinocyte thrombomodulin (Fig 7B). Rabbit thrombomodulin exhibited a simple hyperbolic activity profile, with maximal activity at saturating concentrations of calcium ions (Fig 7C).

To determine whether stimulation with ATRA altered the calcium-dependence or thrombin affinity of keratinocyte thrombomodulin, keratinocytes were incubated with or without 10 μmol/L ATRA for 24 hours, and thrombomodulin activity was measured in the presence of either 1.0 or 4.0 mmol/L CaCl₂. The ratio of thrombomodulin activities at 1.0 mmol/L and 4.0 mmol/L CaCl₂ was 2.8 for ATRA-treated keratinocytes, compared with 3.8 for untreated keratinocytes, 7.1 for TM456, and 0.4 for rabbit thrombomodulin. The K_{app} for thrombin of thrombomodulin in ATRA-treated keratinocytes was 5.2 ± 1.8 mmol/L, which is similar to that of thrombomodulin in untreated keratinocytes (Fig 1A).
DISCUSSION

Thrombomodulin was identified 15 years ago as an endothelial cell cofactor for protein C activation. Recent evidence from several laboratories has established that thrombomodulin is expressed not only by vascular endothelial cells, but also by nonvascular cells such as keratinocytes. Although the function of thrombomodulin in nonvascular locations is poorly understood, its pattern of expression during embryogenesis suggests a possible role in cellular development and differentiation. This possibility is supported by the observation that homozygous deficiency of thrombomodulin produces an embryonic lethal phenotype in mice.

Previous studies have shown that thrombomodulin expression correlates strongly with epidermal differentiation in vivo, and with calcium-induced keratinocyte differentiation in vitro. This study demonstrates that keratinocyte thrombomodulin and endothelial cell thrombomodulin have similar cofactor activity and thrombin affinity, but differ in their responses to regulatory stimuli. Unlike thrombomodulin expression in endothelial cells, thrombomodulin expression in keratinocytes was stimulated markedly by ATRA, but was unaffected by TNF.

Retinoids regulate epidermal differentiation in a complex manner. Based on in vitro studies, as well as observations in animals with vitamin A deficiency (which produces epithelial changes such as hyperkeratosis and squamous metaplasia of transitional and columnar epithelium), it has been suggested that retinoic acid may inhibit epidermal differentiation. However, recent data from mice with targeted overexpression of dominant-negative retinoic acid receptor genes indicate that retinoids may be necessary for normal epidermal differentiation. The observation that ATRA is a potent stimulator of thrombomodulin expression in keratinocytes suggests that retinoids may play a physiological role in the regulation of thrombomodulin expression during squamous differentiation.

Dittman et al have identified functional retinoic acid response elements within the 5'-flanking region of the human thrombomodulin gene. These investigators suggested that the thrombomodulin promoter may be more responsive to retinoids in cells with low basal levels of thrombomodulin expression than in cells with high basal levels of thrombomodulin expression. Our observations in keratinocytes are consistent with transcriptional regulation of the thrombomodulin gene by ATRA, in that increased thrombomodulin
activity in ATRA-treated keratinocytes was associated with increased levels of thrombomodulin mRNA. However, despite similar levels of basal thrombomodulin expression, we observed a greater response to ATRA in keratinocytes than in HUVEC. This suggests that responsiveness of the thrombomodulin promoter to retinoic acid is regulated independently of the basal transcription rate.

The failure of TNF to alter expression of keratinocyte thrombomodulin while markedly stimulating expression of keratinocyte transglutaminase also suggests differential responsiveness of the thrombomodulin promoter. Recent studies indicate that an Ets-like element within the proximal thrombomodulin promoter may be involved in TNF-mediated transcriptional inhibition in endothelial cells. This sequence appears to recognize a novel nuclear factor that is present in endothelial cells. Whether this factor is responsible for TNF-mediated inhibition of thrombomodulin transcription has not been determined, and it is not known if this factor is present in keratinocytes.

Protein C contains a high-affinity Ca$^{2+}$ site that must be occupied for efficient activation by the thrombin/thrombomodulin complex. The effect of Ca$^{2+}$ on protein C activation is also influenced by the glycosylation state of thrombomodulin. Isoforms of thrombomodulin that lack chondroitin sulfate (CS) exhibit peak cofactor activity at [Ca$^{2+}$] below 1.0 mmol/L, whereas isoforms containing CS are most active at saturating [Ca$^{2+}$]. Studies with cultured endothelial cells indicate that a variable fraction of human thrombomodulin is devoid of CS, implying paradoxically that protein C activation may be suboptimal at plasma [Ca$^{2+}$]. With both untreated and ATRA-treated keratinocytes, we observed maximal thrombomodulin activity at 1.0 mmol/L Ca$^{2+}$, which suggests that keratinocyte thrombomodulin does not contain large amounts of CS. However, the possibility that CS may have been lost or modified during preparation of cell lysates cannot be excluded.

Unlike endothelial cell thrombomodulin, keratinocyte thrombomodulin may be exposed to extracellular [Ca$^{2+}$] lower than that found in plasma. In vivo studies in mice suggest that an extracellular [Ca$^{2+}$] gradient exists within the stratified squamous epithelium of the epidermis, with lower [Ca$^{2+}$] in the basal and spinous layers, and higher [Ca$^{2+}$] in the granular layer. Thrombomodulin is selectively expressed in the spinous layer, where relatively low extracellular [Ca$^{2+}$] may support optimal cofactor activity.

In summary, we have shown that the thrombin affinity and basal level of expression of thrombomodulin are similar in cultured keratinocytes and endothelial cells, but that regulation of thrombomodulin expression differs in these two cell types. Keratinocyte thrombomodulin is upregulated strongly by ATRA, but is unaffected by TNF. In contrast, endothelial cell thrombomodulin is upregulated weakly by ATRA, and downregulated strongly by TNF. Therefore, clinical settings associated with the production or administration of retinoids or inflammatory cytokines may produce differential effects on thrombomodulin expression in blood vessels and epidermis. These data provide additional support for the hypothesis that thrombomodulin has distinct biological functions in vascular and nonvascular cells.

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REFERENCES

1. Esmon CT: Thrombomodulin as a model of molecular mechanisms that modulate protease specificity and function at the vessel surface. FASEB J 9:946, 1995
4. Raife TJ, Lager DJ, Madison KC, Piette WW, Howard EJ, Sturm MT, Chen Y, Lentz SR: Thrombomodulin expression by hu-
26. Lentz SR, Sadler JE: The molecular basis of thrombomodulin function, in Giddings JC (ed): Thrombin, Thrombomodulin, and the Control of Hemostasis, Austin, TX, RR Landes, 1994, p 91
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