ENDOTHELIAL CELLS lining blood vessels possess anticoagulant functions, although they can rapidly become procoagulant when injured. The anticoagulant actions include production of prostacyclin, secretion of plasminogen activator, cell surface heparin containing proteoglycans that stimulate certain coagulation factor inhibitors, and thrombomodulin, an endothelial cell surface glycoprotein that promotes protein C activation. When thrombin binds to thrombomodulin, the procoagulant activity of thrombin is altered. Thrombin activates the anticoagulant protein C much more efficiently (greater than 1,000-fold), but no longer clots fibrinogen or activates platelets as effectively as unbound thrombin.

An anticoagulant activity in thrombin treated plasma was initially described in 1960 by Mammen et al., that later proved to be protein C, isolated by Stenflo in 1976. Protein C circulates in plasma at 4 to 5 μg/mL as an inactive zymogen, primarily as a two-chain protein held together by a single disulfide bond. It is activated by cleavage of a 12 amino acid peptide from the amino terminus of the heavy chain. Activated protein C, in the presence of a phospholipid surface, CA++, and a second vitamin K-dependent protein cofactor, protein S, inhibits coagulation by proteolytically degrading factors Va and VIIIa.

Protein C plays an important role in normal physiology as indicated by the occurrence of neonatal purpura fulminans in infants with severe or homozygous protein C deficiency. While some heterozygous protein C deficient subjects suffer thrombosis, most do not. There are decreased levels of protein C in patients with disseminated intravascular coagulation. Efforts to find an efficient protein activator of protein C were unsuccessful until 1981 when Esmon and Owen performed a clever experiment that led to the discovery of thrombomodulin. They reasoned that if an activator of protein C was not in blood, perhaps it was on the endothelial lining of blood vessels. They perfused an isolated rabbit heart with thrombin and protein C simultaneously and indicated by the occurrence of neonatal purpura fulminans in infants with severe or homozygous protein C deficiency. While some heterozygous protein C deficient subjects suffer thrombosis, most do not. There are decreased levels of protein C in patients with disseminated intravascular coagulation. Efforts to find an efficient protein activator of protein C were unsuccessful until 1981 when Esmon and Owen performed a clever experiment that led to the discovery of thrombomodulin. They reasoned that if an activator of protein C was not in blood, perhaps it was on the endothelial lining of blood vessels. They perfused an isolated rabbit heart with thrombin and protein C simultaneously and found an increase in activation of protein C compared with the effect of thrombin without the endothelium. The activity was confirmed to be on endothelial cells using cultured cells and was isolated as a detergent solubilized protein from rabbit lungs. Thrombomodulin has subsequently been isolated from human placenta, bovine lung, mouse lung, and rat lung.

Thrombin forms a 1:1 complex with thrombomodulin with a Kd of 0.5 nmol/L thrombin (Fig 1). The complex activates protein C with a K0.5 of 0.5 μmol/L protein C on cell surfaces or in phospholipid vesicles. When bound to thrombomodulin, thrombin is less efficient in catalyzing procoagulant reactions. Clotting of fibrinogen and activation of platelets, factor V and factor XIII, and inactivation of protein S by thrombin are all inhibited by binding of thrombin to thrombomodulin. Thrombomodulin also decreases the activation of prothrombin when complexed with factor Xa. Thus thrombomodulin provides a mechanism whereby thrombin found outside blood vessels, remote from the endothelium, promotes clot formation, while thrombin in proximity to endothelium generates the anticoagulant activated protein C to maintain blood fluidity.

STRUCTURE OF THROMBOMODULIN

The structure of thrombomodulin determined by a combination of protein structure analysis and cDNA cloning is shown in Fig 2. The human protein has an apparent molecular weight of 75,000 when analyzed on sodium dodecyl sulfate-polyacrylamide gels (SDS-PAGE). After reduction of disulfide bonds, the apparent molecular weight is 105,000, indicating secondary structure involving multiple cystine bridges. This secondary structure renders thrombomodulin stable under extremes of pH and in denaturing conditions. cDNAs for human and murine thrombomodulin, as well as a partial cDNA for bovine thrombomod...
modulin, have been isolated and sequenced. The structure of thrombomodulin resembles the low density lipoprotein (LDL) receptor (Fig 2). The amino terminal, initiator methionine, is followed by an 18 amino acid hydrophobic leader sequence. The mature protein starts with sequences distantly homologous to lectin-like regions in proteins such as the asialoglycoprotein receptor (residues 19 to 179). There are large areas of identity between the mouse and human cDNAs in the lectin domain; this conservation of sequence implies some yet unknown function for this region. Thrombomodulin contains six epidermal growth factor (EGF)-like repeats (residues 238 to 481). The last three of these contain the region required for thrombin binding and protein C activation (Fig 2). This has been demonstrated by isolation of proteolytic fragments of thrombomodulin that bind thrombin and promote protein C activation activity. Thrombin binding was localized to a peptide comprised of the fifth and sixth EGF repeats, although this fragment did not support protein C activation. Another peptide containing repeats 4 to 6 had both thrombin binding and protein C activation activities. The localization of protein C activation activity to the fourth through the sixth EGF repeats has been confirmed by evaluation of thrombomodulin function in cells transiently expressing truncated cDNA constructs of thrombomodulin. The EGF-like repeats may have an additional function as a signal for posttranslational modification of thrombomodulin. Beta-hydroxylation of aspartic acid and asparagine occurs on some coagulation factors with EGF repeats and on the LDL receptor. Beta-hydroxyl amino acids are thought to bind Ca2+. The consensus sequence that signals beta-hydroxylation is conserved in EGF repeats 3 and 6 in thrombomodulin from all species. Additionally, a beta-hydroxy aspartic acid residue has been found in bovine thrombomodulin. After the EGF repeats is the region that is most heterogeneous between species: a serine-threonine-rich region that is the site of O-linked glycosylation. Rabbit thrombomodulin has been reported to have heparin cofactor activity, as measured by accelerated anti-thrombin III inactivation of thrombin, although other investigators have not been able to confirm this finding. Heparin activity is not found in studies using human, bovine, or mouse thrombomodulin. Neither mouse nor bovine thrombomodulin contain the serine-glycine-X-glycine signal sequence required for heparin-like glycosaminoglycan attachment; human thrombomodulin has an unfavored site for such modification.

After the serine-threonine-rich region is the presumed transmembrane domain of 23 hydrophobic amino acids. This is the region of greatest amino acid conservation between species with 20 amino acids identical and the other three residues representing conservative substitutions. The LDL receptor, in contrast, has a heterogeneous transmembrane domain between species, while the platelet derived growth factor receptor displays strong conservation between species.

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**Fig 1.** Model of thrombin-thrombomodulin activation of protein C. APC indicates activated protein C.

**Fig 2.** Structure of thrombomodulin determined by a combination of protein structure analysis and cDNA cloning.
The fact that PDGF, but not LDL receptors, participate in transmembrane signaling infers that the conserved transmembrane domain of thrombomodulin may have a signaling function. The short cytoplasmic tail contains several potential sites for phosphorylation, and phosphorylation in cells has been associated with increased endocytosis and degradation of thrombomodulin. Thrombomodulin is glycosylated on asparagine residues. Conserved consensus sequences for asparagine-linked glycosylation in mouse and human thrombomodulin occur at human amino acid 115, and in mouse, human, and bovine thrombomodulin at human amino acid 420, which is at the beginning of EGF-like repeat five. Other potential sites for asparagine-linked glycosylation are not conserved between species.

There is a single copy of the gene for human thrombomodulin on chromosome 20. Both the human and mouse genes are intronless. Introns are uncommon and include beta-adrenergic receptors, alpha- and beta-interferon, Rhodopsin, angiogenin, some mammalian heat shock genes, and mitochondrial genes. No special function for intronless genes is known, although it has been postulated that lack of introns with decreased mRNA processing might allow more rapid increases in protein synthesis following increased transcription.

Analysis of thrombomodulin by Northern blot hybridization shows a single mRNA of approximately 3,700 bases, consistent with the size of cDNAs for human (3,693 bases) and mouse (3,658 bases) thrombomodulin. In humans the 5' untranslated region is 150 bases, the coding region is 1,725 bases encoding 575 amino acids, and the 3' untranslated region is 1,779 bases. The 3' untranslated region is highly conserved between species. The function of this region is not known but may determine message stability or translation rates. All three species contain the sequence TTATTTAT in the 3' untranslated region, which has been associated with short mRNA half-lives in some RNAs, c-myc, and interferon, for example. This sequence does not appear to have the same effect on thrombomodulin mRNA; the half-life of the mouse mRNA is about 9 hours. Several potential polyadenylation signals, AATAAA, are present in the cDNAs of all three species.

LOCATION OF THROMBOMODULIN

Thrombomodulin was initially described as an endothelial cell surface protein, and it is there that its function is most easily understood. Most of the vascular thrombomodulin is contained in capillaries which comprise greater than 99% of endothelial surface area. Immunohistochemical analysis of human tissues demonstrated thrombomodulin on endothelia of blood vessels and lymphatics except for in the central nervous system (CNS). Thrombomodulin is also absent from the CNS of the mouse, but is present in the brain of rabbits. The syncytiotrophoblast of the placenta contains thrombomodulin, while hepatic sinusoids and the postcapillary venules of lymph nodes do not. Recently thrombomodulin was identified by immunohistochemistry on the squamous epithelium of the epidermis. Thrombomodulin has also been found in human platelets where there are about 60 molecules per platelet, compared with 50,000 to 100,000 per endothelial cell. A smaller form of thrombomodulin, which is soluble in the absence of detergents, has been isolated from human blood and urine. There is about 20 ng thrombomodulin/mL in serum as determined by immunnoassay. The isolated serum protein has a Kₐ for protein C about twice that of tissue thrombomodulin. Soluble thrombomodulin does function in clotting blood in vitro as demonstrated by adding antibodies to thrombomodulin to nonanticoagulated blood or recalcified plasma, which delays the inactivation of factor Va. The structure of soluble thrombomodulin is not known but presumably represents a cleaved form of tissue thrombomodulin with loss of part of the serine-threonine-rich region, the transmembrane domain, and the cytoplasmic tail. It is possible that circulating thrombomodulin has some physiologic importance. Several other circulating mediators including macrophage colony-stimulating factor (M-CSF), epidermal growth factor, and transforming growth factor α are synthesized as transmembrane glycoproteins that are proteolytically cleaved. Thrombomodulin has been found in a variety of cultured cells, including NIH 3T3 cells, A549 small cell lung cancer cells, and Chinese hamster ovary cells. Some endothelial neoplasms express thrombomodulin. Choriocarcinoma but not choriocarcinoma cell lines have been found to express thrombomodulin.

INTERACTION OF THROMBOMODULIN WITH OTHER FACTORS

The sites on thrombin involved in interactions with thrombomodulin are not clearly delineated. Thrombin is a disulfide-bonded two-chain protease with the active site serine on the larger B chain. The active site of thrombin is not required for thrombin binding to thrombomodulin; active site inactivated diisopropylphospho-thrombin competes equally with intact thrombin for thrombomodulin binding. Additionally, the active site of thrombin bound to thrombomodulin is available for inhibition by antithrombin III. Thrombomodulin competes with fibrinogen and hirudin for thrombin binding, suggesting either a common site or steric interference between sites. The binding site appears to be on the B chain of thrombin since thrombin derivatives with cleavages in this chain do not bind well. Antipeptide antibodies to thrombin amino acids 62 to 73 of the B chain inhibit thrombin interaction with thrombomodulin, hirudin, and fibrinogen, suggesting a common binding site near these amino acids. That thrombomodulin alters thrombin conformation is suggested from changes in electron spin resonance spectra of the active site of thrombin when free versus when bound to thrombomodulin.

The thrombin:thrombomodulin complex also binds protein C. Unlike the thrombin-thrombomodulin interaction, protein C binding is Ca²⁺ dependent. Although the gamma-carboxyglutamic acid residues (gla domains) in protein C bind Ca²⁺, they are not required for activation of protein C by thrombomodulin, since protein C lacking the gla domain (by proteolysis) is activated equally as well as natural protein C in vitro. Ca²⁺ binding to gla domain-deleted protein C induces a conformation that reduces activation by thrombin,
but enhances activation by the thrombin: thrombomodulin complex. This Ca\textsuperscript{2+} binding site appears to be the beta-hydroxylated aspartic acid 71 of the first EGF repeat of the light chain of protein C.\textsuperscript{79} Ca\textsuperscript{2+} binding to this site results in conformational changes in protein C, as determined by evaluation with Ca\textsuperscript{2+} dependent antibodies,\textsuperscript{80} and alterations in susceptibility to disulfide bond reduction.\textsuperscript{81} The thrombin: thrombomodulin complex, in solution, has the same K\textsubscript{m} for native protein C as for gla-domainless protein C. However, the affinity of protein C for thrombomodulin on endothelial surfaces is tenfold less with gla domainless protein, compared with intact protein. This suggests that the gla-domains on protein C interact with either some component distinct from thrombomodulin, or that a site for interactions with the gla-domains is available when thrombomodulin is on the cell surface but not when solubilized in detergents. Evidence for the latter hypothesis comes from studies involving a fragment of thrombomodulin formed after treatment with elastase. This fragment is soluble in the absence of detergents, presumably from loss of the transmembrane domain, and will bind thrombin and activate protein C. Like cell surface thrombomodulin, this molecule activates intact protein C better than gla-domainless protein C.\textsuperscript{82} Thrombomodulin incorporated into phospholipid vesicles also activates protein C better than gla-domainless protein C either by reducing the K\textsubscript{m} for protein C or increasing the velocity of the reaction.\textsuperscript{83,84}

Factor V also interacts with the thrombomodulin-protein C system. Activated factor V and isolated factor Va light chain directly accelerate the activation of protein C by thrombin,\textsuperscript{85,86} although about 20 times less efficiently than thrombomodulin.\textsuperscript{87} Unlike the detergent isolated thrombomodulin-protein C interaction, the factor Va-protein C interaction is dependent on the gla domains of protein C; gla-domainless protein C is activated about 5\% as well as native protein C by factor Va. There is species variation in the reaction; while human factor Va acts as a cofactor for protein C activation, bovine factor Va does not. Factor Va also affects protein C activation by thrombomodulin in humans. At low concentrations (<50 nmol/L), factor Va and Va light chain stimulate the activation of protein C by thrombin:thrombomodulin complexes on cell surfaces.\textsuperscript{88} At higher concentrations, however, factor Va light chain inhibits the activation of protein C by thrombin:thrombomodulin. This may represent a "dampening" process on coagulation; since activated protein C inactivates factor Va by cleaving the heavy chain, free factor Va light chain may be formed after inactivation of factor Va. The free light chain of factor Va may inhibit further activation of protein C by the thrombin:thrombomodulin complex.

REGULATION OF THROMBOMODULIN EXPRESSION

Thrombomodulin expression appears to be regulated by a variety of mechanisms. Thrombomodulin is limited to a small number of cell types in vivo; this tissue specific expression is presumably directed by unique promoters of transcription in the thrombomodulin gene. Alterations in the rates of transcription and subsequent translation of thrombomodulin also control expression, as do changes in the amount of thrombomodulin expressed at the cell surface. Cultured endothelial cells appear to have different properties when isolated from different sources within the vascular system\textsuperscript{89} or from different species. These differences either reflect differences in function among various endothelia in the body, or are artifacts of tissue culture.

Thrombomodulin is subject to regulation by internalization of the cell-surface molecule, with loss of protein C activating activity. Thrombin binding to thrombomodulin can induce internalization of thrombin:thrombomodulin complexes, with transport to lysosomes, release and degradation of thrombin, and subsequent return of thrombomodulin to the cell surface.\textsuperscript{72} This endocytosis is inhibited by protein C but not by activated protein C, suggesting that the endocytosis will not occur until protein C activation is complete.\textsuperscript{86} Endocytosis may not be a function of all endothelial cells; human saphenous vein endothelial cells as well as an endothelial cell line, EA.hy 926, do not display internalization of thrombin:thrombomodulin complexes in vitro.\textsuperscript{81} Phorbol myristate acetate (PMA), a potent activator of protein kinase C, also induces endocytosis of thrombomodulin, with subsequent increased degradation of thrombomodulin.\textsuperscript{83} The PMA effect may be indirect since thrombomodulin is not phosphorylated by protein kinase C in vitro, yet in vivo thrombomodulin is phosphorylated on serine residues, presumably by some other kinase activated by protein kinase C. This phosphorylation may represent a signal for endocytosis and/or degradation of thrombomodulin.

Cytokines, mediators of inflammatory reactions, also affect the expression of thrombomodulin. Tumor necrosis factor (TNF) rapidly alters the anticoagulant properties of the endothelial cell surface by inducing expression of the procoagulant, tissue factor, and reducing thrombomodulin activity.\textsuperscript{90} The reduction in activity is associated with endocytosis and lysosomal degradation of thrombomodulin.\textsuperscript{91} The long half-life of thrombomodulin (19 hours),\textsuperscript{92} implies that one effect of TNF is to increase the rate of degradation of thrombomodulin. TNF also inhibits transcription of thrombomodulin mRNA,\textsuperscript{84} and the subsequent decrease in thrombomodulin protein synthesis may also contribute to the decline in thrombomodulin activity. Other cytokines, interleukin-1,\textsuperscript{93} and endotoxin\textsuperscript{94} also cause decreased thrombomodulin activity on endothelial cell surfaces. The mechanism of the reduction in response to these cytokines is not known, but presumably represents increased endocytosis and degradation as is seen with PMA and TNF.

While inflammatory mediators reduce thrombomodulin activity, a potentially more desirable change would be increased protein C activation. Cycloheximide, an inhibitor of protein synthesis, increases thrombomodulin mRNA levels two- to fourfold.\textsuperscript{94,97} This results from increased rates of mRNA transcription, and not from message stabilization, as has been demonstrated for some other RNAs.\textsuperscript{98} An increased rate of transcription in response to protein synthesis inhibition suggests the existence of a labile inhibitor of thrombomodulin mRNA transcription. If such an inhibitor exists, a physiologic response of the endothelial cell may include increased expression of thrombomodulin. Good candidates as mediators of this response are elements of the coagulation
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pathway. In fact, thrombin increases thrombomodulin transcription rates, mRNA levels, and total thrombomodulin protein in cultured mouse hemangioma cells.97

Infusion of TNF into humans increases the production of fibrin, indicating activation of coagulation.99 Protein C activation was also increased in contrast to what might have been predicted by the in vitro effect of TNF on thrombomodulin activity and protein C activation. This observation is consistent with additional regulation of thrombomodulin in vivo by components of the hemostatic system, perhaps by thrombin as discussed above.

Fetomodulin is a murine glycoprotein, expressed in embryos, for which expression is increased by cyclic adenosine monophosphate (cAMP).100 Isolation of a cDNA clone for fetomodulin found it to be identical to mouse thrombomodulin.103 The role of cAMP in the control of thrombomodulin expression is not clear. Inhibitors of cAMP phosphodiesterase, which elevate cellular levels of cAMP, have diverse effects, including an antithrombotic effect perhaps mediated through increased expression of thrombomodulin.

CLINICAL PROSPECTS

Variation in thrombomodulin levels may have clinical effects, either increasing thrombosis in deficient states, or preventing thrombosis when present in large amounts. Neither circulating nor endothelial thrombomodulin levels have been measured in large numbers of normal subjects or patients with various thrombotic disorders. Such measurements should now be made using plasma or even tissue samples to evaluate the possibility that varying thrombomodulin levels may determine the risk of thrombosis. We have found very high levels of circulating thrombomodulin antigen in some patients with thrombotic thrombocytopenic purpura. Whether these levels are in any way pathogenic or reflect endothelial injury is uncertain. Protein C activation by thrombomodulin in vitro is inhibited by some plasmas from patients with lupus anticoagulants.102,105 Some of these patients have thromboses and/or recurrent abortions that could be caused by abnormalities in thrombomodulin function.

Strategies to alter thrombomodulin in patients may also provide a mechanism for decreasing the tendency for thrombosis or for therapeutic anticoagulation. It is possible to increase thrombomodulin levels and activity in cultured cells with agents such as cycloheximide, thrombin, or cAMP. Investigation of other pharmacologic agents may result in clinically useful medications that could result in systemic anticoagulation by increasing vascular thrombomodulin expression. Such a strategy might avoid the major toxicity of other antithrombotic agents, ie, bleeding. This is because other agents anticoagulate blood or dissolve fibrin clots in both intra- and extravascular locales, while increasing vascular expression of thrombomodulin would restrict the effects to the desired intravascular sites, assuming that protein C only acts locally. Alternatively, infusions of thrombomodulin or thrombomodulin derivatives could result in therapeutic anticoagulation. Infusion of activated protein C into baboons before injection with E. coli decreases attendant coagulopathy and mortality.106 Activated protein C infusions also reduce fibrin and platelet deposition in a baboon thrombosis model.107 Infused thrombomodulin might have a similar therapeutic effect with less toxicity, since thrombomodulin will only generate activated protein C in the presence of thrombin and could therefore restrict the anticoagulant effect to the region of thrombosis. Infusions of detergent solubilized thrombomodulin into mice does reduce the lethal thrombotic effects of injected thrombin, and therefore, soluble forms of thrombomodulin may be useful as therapeutic agents.

In summary, thrombomodulin is a potent activator of protein C that acts by a novel mechanism whereby the protease thrombin is converted from a procoagulant to an anticoagulant. Further studies are required to establish the importance of thrombomodulin in pathogenesis, therapy, or prevention of thrombotic disorders.

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