Thrombomodulin-Protein C-EPCR System
Integrated to Regulate Coagulation and Inflammation
Marlies Van de Wouwer, Désiré Collen, Edward M. Conway

Abstract—Late in the 18th century, William Hewson recognized that the formation of a clot is characteristic of many febrile, inflammatory diseases (Owen C. A History of Blood Coagulation. Rochester, Minnesota: Mayo Foundation; 2001). Since that time, there has been steady progress in our understanding of coagulation and inflammation, but it is only in the past few decades that the molecular mechanisms linking these 2 biologic systems have started to be delineated. Most of these can be traced to the vasculature, where the systems most intimately interact. Thrombomodulin (TM), a cell surface-expressed glycoprotein, predominantly synthesized by vascular endothelial cells, is a critical cofactor for thrombin-mediated activation of protein C (PC), an event further amplified by the endothelial cell protein C receptor (EPCR). Activated PC (APC), in turn, is best known for its natural anticoagulant properties. Recent evidence has revealed that TM, APC, and EPCR have activities that impact not only on coagulation but also on inflammation, fibrinolysis, and cell proliferation. This review highlights recent insights into the diverse functions of this complex multimolecular system and how its components are integrated to maintain homeostasis under hypercoagulable and/or proinflammatory stress conditions. Overall, the described advances underscore the usefulness of elucidating the relevant molecular pathways that link both systems for the development of novel therapeutic and diagnostic targets for a wide range of inflammatory diseases. (Arterioscler Thromb Vasc Biol. 2004;24:1-10.)

Key Words:

The coagulation system is characterized by the sequential activation of a series of serine proteases, culminating in the generation of thrombin, with subsequent conversion of fibrinogen into an insoluble clot. Beyond its central role in coagulation, thrombin has far-reaching effects on a variety of biological systems, including inflammation, cell survival, and cell proliferation.1 Consequently, modulation of the dynamic production of this important enzyme is essential to maintain homeostasis under a variety of pathophysiologic conditions. However, it is thrombin’s critical importance in coagulation that prompted intense studies to characterize those molecular pathways that regulate its production. Although several so-called natural anticoagulant systems are active in vivo, this review focuses on the protein C–thrombomodulin mechanism, highlighting the recently revealed clinically relevant molecular links that exist between inflammation and coagulation.

Protein C Is Activated by Thrombin–Thrombomodulin
Evidence for the existence of a circulating thrombin-activated protein, autoprotrombin II-A, and now referred to as APC, was first presented in the early 1960s2 and was followed by the discovery and isolation of its precursor, protein C (PC) in 1976.3–5 PC is a vitamin K-dependent plasma glycoprotein that is synthesized by the liver and circulates as a 2-chain biologically inactive species.6 It is transformed to its active form, APC, by thrombin-mediated cleavage of PC at the N-terminus. Effective activation of PC by thrombin requires the transmembrane glycoprotein, thrombomodulin (TM), as a cofactor for APC, protein S (PS). APC also may increase fibrinolytic activity by neutralizing plasminogen
The role of APC extends beyond hemostasis. APC has potent anti-inflammatory properties. Administration of APC protects baboons from Escherichia coli-induced sepsis. In mice, low levels of PC heighten the proinflammatory response to endotoxin, whereas in humans, low PC is implicated in the progression of interstitial lung disease. The most compelling evidence for its importance in inflammation comes from the PROWESS study, in which administration of recombinant human APC significantly decreased mortality in patients with severe sepsis.

Much effort has been expended to define the mechanisms by which APC exerts its anti-inflammatory properties (Table). By downregulating thrombin generation through its actions on factors Va and VIIIa, APC interferes with thrombin-induced proinflammatory activities that include platelet activation, cytokine-induced chemotaxis for monocytes and neutrophils, and upregulation of leukocyte adhesion molecules. However, APC also directly dampens inflammation by inhibiting monocyte/macrophage expression of tissue factor and tumor necrosis factor (TNF)-α, nuclear factor (NF)-κB translocation, cytokine signaling, TNF-α-induced upregulation of cell surface leukocyte adhesion molecules, and leukocyte–endothelial cell interactions. Many of these protective effects of APC are mediated by proteolytic cleavage of protease activated receptor 1 (PAR1). APC may also protect the vasculature by blocking p53-mediated apoptosis in ischemic cerebral vasculature. In some models, the anti-apoptotic function of APC is independent of its anticoagulant function, requires EPCR as a cofactor, and is mediated via PAR1.

Activation of TAFI by Thrombin–Thrombomodulin

TM is also a cofactor for thrombin-mediated activation of the thrombin-activatable fibrinolysis inhibitor (TAFI). TAFI is a plasma procarboxypeptidase B that, when activated to TAFIa, catalyzes the removal of the C-terminal basic amino acid residues Lys and Arg. Inhibition of fibrinolysis is accomplished by removal of Lys residues from modified fibrinogen, which impedes the conversion of plasminogen to plasmin. Although the in vivo significance of TAFIa as a regulator of fibrinolysis has not been clearly established, its potential role as a natural anti-inflammatory molecule is currently being explored, with recognition of its ability to inactivate the potent anaphylatoxins C3a and C5a and the proinflammatory mediators bradykinin and osteopontin.

Thrombomodulin: Structure–Function

It is just >20 years ago that Esmon and Owen identified and isolated TM. Since that time, steady progress has been made in elucidating the molecular mechanisms by which this single molecule regulates coagulation, inflammation, fibrinolysis, and cellular proliferation. Although originally described as a vascular endothelial cell receptor, TM has since been detected in a variety of cells and tissues in adults and during development, including astrocytes, keratinocytes, mesothelial cells, neutrophils, monocytes, and platelets. Consequently, it is no surprise that it has functions beyond coagulation (Table).

Encoded by an intronless gene, the mature single-chain glycoprotein in the human is 557 amino acids long, structu-
ally organized into 5 distinct domains (Figure 1). From the intracellular C-terminus, TM has a short cytoplasmic tail, deletion of which in mice has no effect on development, survival, coagulation, or inflammation. After a well-conserved membrane-spanning region, there is a serine/threonine-rich domain with potential sites for O-linked glycosylation, which support the attachment of a chondroitin sulfate (CS). Biochemical studies, yet to be confirmed in vivo, indicate that the CS of TM enhances the PC cofactor activity of TM, accelerates the neutralization of thrombin by heparin–antithrombin and by the protein C inhibitor, and facilitates binding of PF4 to PC to increase its activation.

Adjacent to the serine/threonine-rich region is the best-characterized domain, which comprises 6 epidermal growth factor (EGF)-like repeats. This domain has mitogenic effects on cultured fibroblasts and vascular smooth muscle cells, mediated via activation of protein kinase C and mitogen-activated protein kinases (MAPK). The clinical significance of these findings has not been established, but they suggest a possible role in cellular proliferation and atherogenesis. EGFR-like repeats 3, 4, 5, and 6 (EGF3 to 6) have been studied in detail by several groups and are essential for activation of PC and TAFI by thrombin. Additional antifibrinolytic activity is supported by the EGF-like repeats of TM, because they also accelerate thrombin-mediated conversion of single-chain urokinase-type plasminogen activator (scu-PA) to thrombin-cleaved 2-chain urokinase-type plasminogen activator (tcu-PA/T), thereby interfering with the generation of plasmin.

At the N-terminus of the molecule and joined to the first EGF-like repeat by a 72-amino acid residue hydrophobic stretch, there is a 154-amino acid residue module with homology to other C-type lectins. Electron microscopy and computer models indicate that the lectin-like domain of TM is globular and situated furthest away from the plasma membrane, such that it might effectively and easily interact with other molecules. Although lacking in anticoagulant function, this domain plays a major role in inflammation and cell survival (see later).

**EPCR: Another cofactor for PC Activation**

EPCR, constitutively expressed by endothelial cells, is structurally similar to the major histocompatibility complex class 1/CDI family of proteins, which are commonly involved in immunity/inflammation. EPCR accelerates thrombin-mediated activation of PC while concentrating it near the surface of the vessel wall. In contrast to TM, EPCR is more

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### Functions of TM, EPCR, and APC in Coagulation, Inflammation, and Cell Proliferation

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<th>Structure</th>
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<th>Coagulation/Fibrinolysis</th>
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<td>Interferes with monocyte activation and TF expression</td>
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<td>Inhibits endothelial cell apoptosis—via APC</td>
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prominently expressed in large vessel endothelial cells \cite{61,62} but is also detected in neutrophils. \cite{27} When APC is generated, it remains bound to EPCR for a short time before associating with protein S on the surface of platelets or endothelium, whereon it cleaves its substrates, factors Va/VIIa, after which it is inactivated by α1-antitrypsin, the protein C inhibitor, \cite{63} and/or α2-macroglobulin. \cite{64} In addition to its role in amplifying activation of PC, EPCR switches the substrate specificity of APC, analogous to TM and thrombin. When APC is released from EPCR, it has anticoagulant properties, yet when transiently complexed with EPCR, APC cleaves PAR1, initiating intracellular signaling that provides anti-apoptotic protection. \cite{28,29}

**TM and Inflammation**

TM functions as an anti-inflammatory molecule at several levels. First, as a critical cofactor in the activation of PC, TM has an obligate role in regulating the anti-inflammatory properties of APC. Thus, high levels of anti-inflammatory/anticoagulant/vasculoprotective APC would be generated locally in the presence of adequate or excess functional TM and thrombin. Indeed, in a vascular restenosis model in rabbits, administration of TM via adenovirus prevented restenosis and dampened the inflammatory response. \cite{65} Conversely, downregulation of TM would be expected to yield low APC levels and a proinflammatory procoagulant diathesis. In this respect, Weiler et al demonstrated that mice with low APC levels (TM<sub>n/LD</sub> mice) display a heightened inflammatory response to systemic endotoxemia. \cite{66} However, the story is more complicated, because the TM<sub>n/LD</sub> mice, when exposed to respiratory bacterial pathogens, did not generate a proinflammatory response, despite enhanced fibrin/fibrinogen deposition. \cite{67}

There are additional indirect mechanisms by which TM may provide anti-inflammatory protection. For example, the putative role that TAFIa plays in suppressing complement activation also requires an intact thrombin–TM complex. Recombinant soluble TM prevented leukocyte infiltration into the kidney in a rat model of glomerulonephritis, an effect that was at least partly mediated through an increase in TAFIa and subsequent complement inactivation. \cite{68} Furthermore, when associated with TM, the proinflammatory properties of thrombin are abrogated, and indeed reversed; thus, TM, a "sink" for thrombin," once again behaves effectively, albeit indirectly, as an anti-inflammatory molecule. When TM expression is downregulated by, for example, cytokines such as TNFα or IL-1β, thrombin would then be available to promote coagulation and inflammation.

It has long been recognized that C-type lectins, through interactions between their carbohydrate recognition domains and carbohydrates attached to proteins, often participate in innate immune functions, including complement activation, leukocyte trafficking, and regulation of apoptosis. \cite{69,70} This observation prompted us to explore the possibility that the C-type lectin-like domain of TM might play a direct role in modulating inflammation. For this reason, transgenic mice that lack the N-terminal lectin-like domain of TM (TM<sub>L/LD</sub>) were generated. \cite{71} Although appearing normal under baseline conditions, further phenotypic analysis revealed that they have reduced survival after endotoxin exposure, accumulate more neutrophils in their lungs, respond with larger infarcts after myocardial ischemia/reperfusion, and develop worse arthrogenic-induced arthritis than their wild-type counterparts. \cite{72} Notably, deletion of the lectin-like domain of TM did not interfere with in vivo activation of PC, indicating that the apparent proinflammatory effect seen in the TM<sub>L/LD</sub> mice was not caused by suppression of APC. Rather, the lectin-like domain of TM was demonstrated to have direct anti-inflammatory properties, conferring protection by interfering with neutrophil adhesion to endothelial cells. \cite{71} Increased leukocyte adhesion to TM<sub>L/LD</sub> endothelium was at least partially explained by enhanced expression of leukocyte adhesion molecules (intercellular adhesion molecule-1, vascular cell adhesion molecule-1), mediated by increased phosphorylation of MAPK (extracellular signal-regulated kinase [ERK], ERK1/2), and activation of NF-κB. Recent studies further suggest that the lectin-like domain of TM may be important to maintain the integrity of cell–cell interactions, and thus might also prevent leukocyte transmigration. \cite{73} Overall, the lectin-like domain of TM dampens the response of the vascular endothelium to proinflammatory stimuli by suppressing activation of well-conserved intracellular signaling pathways. Notably, the mechanisms by which APC and the lectin-like domain of TM exert their anti-inflammatory effects are similar, indicating the close coordination and importance of these apparently redundant protective biologic systems.

**Soluble TM**

Thrombomodulin expression is not restricted to the cell membrane and also exists in a soluble form in the plasma, generated by enzymatic cleavage of the intact protein. \cite{74,75} Under normal conditions, levels of soluble TM in the plasma range from 3 to 50 ng/mL. \cite{76} During disorders associated with vascular damage, including a variety of infections, sepsis, and inflammation, soluble TM levels are increased, presumably cleaved from endothelial cells by neutrophil-derived enzymes, and possibly also by rhomboids, a recently described family of intramembranous proteases. \cite{78} Although controversial, recent studies suggest that plasma TM levels may be inversely correlated with the development of coronary heart disease, implying that soluble forms of TM may be vascular-protective. \cite{77,79} It is not known which proteolytic fragments of TM provide protection, although likely candidates include EGF1–6 and/or the lectin-like domain.

From in vitro studies, we established that soluble recombinant lectin-like domain of TM suppresses cytokine-induced neutrophil adhesion to vascular endothelial cells via intercellular adhesion molecule-1-dependent and independent pathways through suppression of ERK1/2 activation. It was further observed that this domain of TM has prosurvival/anti-apoptotic activity. \cite{71,72} These findings are consistent with the elegant gene inactivation studies performed by Weiler et al, showing that during development TM has a critical role, unrelated to coagulation, in controlling the growth and survival of trophoblasts in the placenta. \cite{80,81} and with earlier reports linking low levels of TM expression with increased proliferation of tumors. \cite{82}
Regulation of Expression of TM and EPCR
The complex regulation of TM underlines its importance in a wide variety of pathophysiologic conditions and biological systems. TM is transcriptionally upregulated by thrombin, vascular endothelial growth factor, histamine, dibutyryl cAMP, retinoic acid, theophylline, heat shock, and statins, whereas shear stress, hypoxia, oxidized low-density lipoprotein, and transforming growth factor-β will suppress TM gene expression. Although tumor TNFα and IL-1β upregulate macrophage expression of TM, these cytokines suppress TM in endothelial cells at transcriptional and posttranscriptional levels. TM PC-cofactor activity can be abrogated by oxidation of a methionine in the EGF-like repeat, likely to occur during inflammation as a result of neutrophil activation.

EPCR expression is similarly tightly regulated. Transcription is suppressed by lipopolysaccharide, IL-1β, TNFα, and thrombin. Moreover, EPCR can be cleaved from the cell surface by matrix metalloproteinases that are activated by IL-1β, thrombin, or phosphor myristate acetate. Soluble EPCR (sEPCR) retains its ability to bind to PC and APC and inhibits APC anticoagulant function by blocking phospholipid interaction and altering the active site of APC. sEPCR release increases in Gram-negative sepsis whereon it complexes with the elastase-like protein, proteinase-3 (PR3), that is released from activated neutrophils. The sEPCR–PR3 complexes then may bind to and interfere with the function of neutrophil integrins, such as CD11b/CD18 (Mac-1), which otherwise facilitate neutrophil adhesion to activated endothelial cells and extravasation into the extravascular space in response to endotoxin.

Finally, crystallization of EPCR confirms its structural and potentially functional relationship to the CD1/MHC class I family of molecules, which are directly involved in host defense against several bacterial pathogens, thereby linking the TM-PC-EPCR system with innate immunity. Overall, although the functions of EPCR and sEPCR are not yet fully elucidated, like TM and APC, they modulate inflammation via complex regulatory pathways.

Integrating the Functions of APC, EPCR, and TM
From this discussion, it is apparent that TM, APC, and EPCR have diverse yet distinct regulatory, structural, and functional motifs regulating multiple biological functions, including coagulation, fibrinolysis, inflammation, and apoptosis. In health and disease, these appear to be well-integrated to maintain homeostasis. Under normal conditions or in response to minor injury, the vascular endothelium remains protected, as TM sequesters thrombin, generating adequate local levels of APC to protect the vasculature from inflammatory, procoagulant, and pro-apoptotic forces (Figure 2A). Signals mediated directly by APC, the APC-EPCR complex via PAR1, and the lectin-like domain of TM help to suppress cytokine release and tissue factor expression by circulating leukocytes, interfere with endothelial cell apoptosis, damen endothelial cell activation of MAPKs, and prevent expression of leukocyte adhesion molecules, impeding local accumulation of neutrophils and monocytes.

In the context of a more profound injury or inflammatory stimulus (Figure 2B), cytokines from activated leukocytes suppress cell surface expression of TM and EPCR via transcriptional and endocytotic pathways, resulting in reduced levels of APC. The “sink” effect of TM on thrombin is jeopardized, and excess thrombin accumulates locally, free to exercise its proinflammatory and procoagulant effects on surrounding substrates, including fibrinogen and platelets, endothelial cells, and monocytes. Diminished levels of APC and EPCR imply less APC–EPCR complex-induced signaling via PAR1, rendering the endothelium less protected from pro-apoptotic and proinflammatory factors. Leukocytes migrate to the injury site, and their releasates oxidize the Met388 residue of the remaining membranous TM, abrogating its anticoagulant function and PC-cofactor activity, reducing additional generation of APC, and enhancing local clot formation. TAFI activation may persist despite oxidation of Met388 and thus prevent fibrinolysis of newly laid-down fibrin clot, thereby enhancing localization of the injury while downregulating inflammation by cleaving anaphylatoxins, bradykinin, and osteopontin. Remaining TM is cleaved by leukocyte proteases, thus releasing soluble fragments of TM, including those with intact lectin-like and EGF-containing domains. Loss of the lectin-like domain from intact TM results in local endothelial activation of MAPKs, and upregulation of vascular adhesion/migration molecules with decreased anti-apoptotic protection, facilitating further leukocyte accumulation and extravasation at the site of injury. Similarly, remaining endothelial cell EPCR is cleaved from the activated endothelium by cytokine- or thrombin-induced activation of metalloproteinases, further diminishing the capacity to generate activated PC. Overall, the stage is set for the development of a vicious circle promoting inflammation, endothelial dysfunction, and tissue destruction.

To prevent the damaging inflammatory process from spreading to unaffected adjacent tissues, ie, localizing the injury, protective mechanisms must be recruited (Figure 2C). These may include upregulation of TM by stresses, such as heat shock, with consequent augmented local production of APC, switching thrombin’s activity away from its otherwise proinflammatory, procoagulant, and antifibrinolytic function. APC can furthermore indirectly increase the fibrinolytic response by inhibiting PAI-1. Other agents, including EPCR, PF4, protein C inhibitor, and heparin–antithrombin serve to further dampen the proinflammatory process. The soluble fragments of TM and EPCR, released from the injury site, may “float” to adjacent regions, where they together with locally generated APC can provide protection by suppressing inflammation, inducing prosurvival pathways, interfering with leukocyte–endothelial cell interactions, and promoting endothelial proliferation so that healing may proceed.

Therapeutic Prospects
Reduced levels of PC are found in the majority of patients with sepsis and are associated with increased morbidity and mortality. Treatment with recombinant human APC reduces plasma levels of D-dimer and IL-6, and in the PROWESS study resulted in a significant reduction in mortality in patients with severe sepsis. Despite the reported
increased risk of bleeding with APC, and some controversy regarding its use over PC. 

Furthermore, the safety and efficacy of these approaches will most certainly be enhanced as assays are improved to measure circulating levels of APC and other relevant markers of inflammation, including soluble TM fragments and sEPCR.

Administration of recombinant forms of TM that encompass EGF1 through EGF6 are protective in a variety of animal models of tissue factor- or endotoxin-induced disseminated intravascular coagulation or lung injury. Although these forms of TM may be effective as anticoagulants, their clinical use for sepsis or inflammatory disorders is likely to be complicated by bleeding, similar to APC. Might the lectin-like domain of TM be efficacious as a therapeutic agent in sepsis, thereby sparing the bleeding side effect? Our studies suggest that this nonanticoagulant form of soluble TM may modulate inflammation by attenuating MAPK pathways and interfere with neutrophil–endothelial cell interactions. The clinical usefulness of this fragment of TM is being evaluated using in vivo models of sepsis, ischemia/reperfusion, arthritis, and atherosclerosis.

The potential therapeutic use of soluble fragments of EPCR to prevent and/or treat inflammation remains an open question. A simplified model is presented to describe the possible functions of TM, PC/APC, and EPCR in response to vascular injury. A, With minor vascular injury induced by relatively low levels of endotoxin, thrombin (IIa) is generated locally as tissue factor is exposed by monocytes. Adequate vasculoprotection to prevent fulminant tissue damage is mediated by production of APC, which prevents further thrombin generation, interferes with leukocyte activation/adhesion and cytokine production, and protects the endothelium in concert with EPCR via PAR1 signaling effects. Dampening of MAPK pathways and NFκB translocation is accomplished by APC/EPCR signaling via PAR1 and via the lectin-like domain of TM, both of which serve to prevent endothelial cell activation, adhesion molecule expression, and endothelial cell apoptosis, thereby maintaining the vasculature in a quiescent, anticoagulant state. B, With exposure of the vasculature to more significant injury (eg, higher levels of endotoxin), the locally injured vasculature is exposed to cytokines (eg, TNF, IL-1), and consequently both TM and EPCR expression are suppressed by transcriptional and posttranscriptional mechanisms. Cytokines also promote the recruitment and activation of neutrophils, which release reactive oxygen species that render TM nonfunctional, by oxidizing the critical methionine (M-O) within the EGF-like domain of TM. Metalloproteinases, neutrophil elastases, and endothelial cell rhomboids cause the release of soluble EPCR (sEPCR) and TM fragments in the form of the lectin-like domain and the EGF-like domain. Overall, with the diminished levels of cell surface functional TM and EPCR, the production of APC decreases and, with that, cofactors Va and VIIIa are not cleaved, allowing thrombin (IIa) generation to be enhanced. Signals normally transmitted via the lectin domain of TM and via APC/EPCR through PAR1 that otherwise suppress MAPK activation and NFκB translocation, are lost, and adhesion molecule (AdMs) expression is thus augmented. Neutrophils can therefore adhere to the vascular endothelium, promoting thrombosis and endothelial cell damage, and transmigrate into the tissues, causing further damage. With that, thrombin generation is further promoted, and the inflammatory process tends to perpetuate. The capacity of the organism to rapidly and effectively contain the injury and/or to respond by recruiting protective mechanisms determines the outcome. C, In the vascular beds outside the site of injury, normal functional TM and EPCR expression are maintained by upregulatory forces, thereby maintaining the region free of clot. sEPCR and soluble TM fragments from regions of injury accumulate and interfere with leukocyte–endothelial cell interactions. These fragments, and any APC that is generated, further promote endothelial cell survival, partly via PAR1 signaling, and dampen activation of MAPK and translocation of NFκB, thereby preventing leukocyte adhesion molecule expression and interfering with neutrophil trafficking into the tissue. Overall, these mechanisms help to restrict the damage to a local site and allow healing to occur.
question. Although current data support the concept that sEPCR interferes with neutrophil adhesion and extravasation, elevated levels of sEPCR have recently been associated with an increased risk of thrombosis.\(^2\)\(^2\) Undoubtedly, as the molecular mechanisms by which sEPCR and partner proteins interact with leukocytes and the vascular endothelium are delineated, novel new therapeutic approaches will be uncovered.

**Beginnings**

Despite advances in characterizing the mechanistic links between coagulation, inflammation, and cell survival, a predisposition to developing inflammatory, infectious, or proliferative disorders in humans has yet to be definitively connected to mutations in the TM, PC, or EPCR genes. Nonetheless, the described progress in understanding the PC-TM-EPCR system should stimulate the scientific community to consider the important impact of the so-called coagulation system in modifying the risk of onset and altering the progression of these disorders. Numerous questions remain to be answered. For example, which specific structures of TM and EPCR modulate inflammation and cellular proliferation? What other soluble and/or membrane-associated protein/carbohydrate structures are involved? What intracellular signaling pathways mediate the biological effects of soluble TM and soluble EPCR? What genetic factors that might alter the risk of disease modify expression and function of those proteins involved in the PC-TM-EPCR pathways? As the complexity of the PC-TM-EPCR system is further elucidated, novel therapeutic targets will likely be revealed, and early identification of individuals genetically predisposed to the development of a variety of illnesses will be facilitated.

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