Inherited Abnormalities in the protein C Activation Pathway

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**Key Words**
Thrombomodulin, endothelial protein C receptor, venous thrombosis, arterial thrombosis, myocardial infarction, protein C activation, mutations.

**Abstract**
The protein C (PC) anticoagulant pathway plays a crucial role in the regulation of fibrin formation via proteolytic degradation of the procoagulant cofactors factor Va and VIIIa by activated PC (APC). PC circulates in plasma as a zymogen, which is activated, on the surface of endothelial cells by the thrombin-thrombomodulin complex. Another endothelial cell-specific protein, the endothelial cell PC/APC receptor (EPCR), binds PC on the endothelial cell surface and further enhances the rate of PC activation. Normal APC generation depends on the precise assemblage, on the surface of endothelial cells, of at least four proteins: thrombin, thrombomodulin (TM), PC and EPCR. Therefore, any change in the efficiency of this assemblage may cause reduced APC generation and an increase in the risk of thrombosis. In the last years, several reports have suggested the association between mutations in TM and EPCR genes and venous and arterial thrombosis. Surprisingly, no studies have been reported linking mutations with levels of circulating APC, the final product of the interaction between thrombin, TM, PC and EPCR. Here, we describe the previously reported mutations in the TM and EPCR genes, and present the design and evaluation of a new strategy to investigate TM, EPCR, PC and prothrombin gene mutations in arterial and venous thrombosis.

**Introduction**
The protein C (PC) anticoagulant pathway plays a crucial role in the regulation of fibrin formation via proteolytic degradation of the procoagulant cofactors factor Va and VIIIa by activated PC (APC) (1-4). PC circulates in plasma as a zymogen, which is activated, on the surface of endothelial cells by the thrombin-thrombomodulin complex. Another endothelial cell-specific protein, the endothelial cell PC/APC receptor (EPCR), binds PC on the endothelial cell surface and further enhances the rate of PC activation. Normal APC generation depends on the precise assemblage, on the surface of endothelial cells, of at least four proteins: thrombin, thrombomodulin (TM), PC and EPCR. Therefore, any change in the efficiency of this assemblage may cause reduced APC generation and an increase in the risk of thrombosis. In the last years, several reports have suggested the association between mutations in TM and EPCR genes and
venous and arterial thrombosis (6-15). Many of the studies are case report or family studies, and others have been only of association between mutation and disease. Only in those studies that report consistent relationships linking polymorphisms, phenotype and clinical effect, there can be some confidence that the genetic variation is influencing disease. However, because TM and EPCR are integral membrane proteins, its deficiency is difficult to investigate in terms of phenotype. On the other hand, only a small number of mutations have been characterized in terms of expression and function studies. Surprisingly, no studies have been reported linking mutations with levels of circulating APC, the final product of the interaction between thrombin, TM, PC and EPCR.

Here, we will describe the previously reported mutations in the TM and EPCR genes, and will present the design and evaluation of a new strategy to investigate TM, EPCR, PC and prothrombin gene mutations in arterial and venous thrombosis.

**Thrombomodulin**

TM is an integral endothelial cell surface glycoprotein that functions as a receptor for thrombin. Following binding to thrombin, TM forms a high-affinity complex and increases the activation of PC (16). Therefore, any impaired function of the thrombin cofactor or reduced TM gene expression could also contribute to thrombotic disease. Direct gene screening of the TM gene in patients with thrombotic disease has showed the occurrence of several mutations (6-14), some of them associated with venous and arterial thrombosis, with varying results depending, in part, on the different population studied.

The first mutation described was a frequent polymorphism at position 1418 consistent in a cytosine transition to thymidine resulting in an Ala to Val substitution at amino acid position 455 (Ala455Val) (6). The site is located in the region of TM responsible for thrombin binding, suggesting potential implications on PC activation but the authors found that, with respect to thrombophilia, the polymorphism is essentially neutral. In a study of 145 patients with a history of VTE and 162 controls, we found that the C1418T polymorphism is not a risk factor for VTE (17). However, there are conflicting results regarding its association with arterial thrombosis. Norlund et al. (18) found that the C allele was more frequent in myocardial infarction (MI) survivors (0.82) than in controls (0.72) (p=0.035), suggesting that the Ala/Val replacement may be functional. However, Ireland et al (10) screened the polymorphism in 104 patients with MI and 104 controls matched by sex, race, and age, and found 33 individuals in both groups carrying the T allele. Finally, Wu et al (19), within a large cohort study (ARIC), determined the TM-455 genotypes on 376 cases (23% black, 77% white) of coronary heart disease (CHD) and 461 controls. After controlling for age, sex, and other CHD risk factors, the V allele increased the risk of CHD by 6-fold (CI 1.7-22-9) in blacks but not in whites, concluding that the Ala455Val polymorphism predicts risk of developing CHD in blacks.

The second TM mutation described is a G1456T substitution that predicts an Asp468Tyr change in the Ser/Thr rich region, and was found in a 45-year-old Hispanic man that had suffered from pulmonary embolism (7). Constructs bearing the mutation were transiently transfected in COS-7 cells and the mutant TM showed a normal distribution around the cell surface membrane as well as normal cofactor activity (14). We have found that the mutation is not frequent in our population, since we did not detect any variant in 145 VTE patients and 162 controls (17).

In 1997, Öhlin et al. (9) described six TM mutations among unrelated patients and families with inherited thromboembolic disease. One of these TM mutations, the G127A mutation predicting an Ala25Thr substitution, has been associated with MI (11) but no abnormality in cell surface expression or TM cofactor function could be found (14).

Ireland et al. (14) found 3 mutations in the TM promoter region. One of these mutations, the G-33A, is frequent in Chinese population (20), and was significantly associated with coronary artery disease. However, there was no clear association between the G-33A mutation and gene expression (21).

Kunz et al (11) described a silent base substitution, G1686C, and a base insertion, insT1689, in one of the 104 patients with MI studied. The predicted mutant protein has normal sequences for its extracellular and transmembrane domains, but an elongated intracellular C terminal tail, with decreased surface expression of TM on the monocytes of carriers and decreased levels of soluble TM in plasma.

Franchi et al (13) screened the entire TM gene in 95 women with unexplained late fetal loss and in 236 controls women who gave birth to at least one healthy baby and had no history of late fetal death. In total, they found 4 mutations in 4 patients.

Le Flem et al (12) analyzed the distal promoter region of the TM gene (nucleotides ~300 to ~2052) in patients with VTE. Eight novel mutations were found, the more frequent being the G-1748C and the del-1208/-1209TT polymorphisms. One rare transition (G-1166A) might be functional as suggested by its position, but none of the 3 mutations were associated with thrombosis.

Recently, Kunz et al (14) described two new TM mutations. One is a deletion, del791-801, leading to STOP306, and a missense mutation, Arg385Ser. A construct with the deletion mutation was transiently expressed in COS-7 cells and showed mutated allele transcription. However, the mutated protein appeared to aggregate or to be associated with the cytoskeleton, suggesting that the expression and function of the TM on the endothelial cell surface of the patient will be 50%, and result solely from the normal allele. The TM Arg385Ser mutation also resulted in an approximate 50% reduction in expression and in a 4-fold increase in Km.
**Endothelial cell protein C receptor**

PC circulates in plasma as a zymogen that is activated on the surface of endothelial cells by the thrombin-TM complex. Another endothelial cell-specific protein, the endothelial cell PC receptor (EPCR), is a type I transmembrane protein that is highly expressed on the endothelium of large vessels while it is present at trace levels in most capillary beds (22), binds PC on the endothelial cell surface and further enhances the rate of PC activation (23), possibly by decreasing the Km for PC activation by the thrombin/TM complex due to the high affinity between EPCR and PC. EPCR is seen as a complementary cofactor in PC activation.

The human EPCR gene is located on chromosome 20, spans 6 kb and encodes 238 amino acids. The predicted protein structure of EPCR is that of a type I transmembrane glycoprotein of approximately 46 KDa. After removal of 17 residues, the mature protein consists of 221 amino acids containing a signal sequence at the amino-terminal, a transmembrane domain near the carboxyl-terminal, a short cytosolic domain, and four potential N-glycosylation sites.

Because the EPCR seems to play an important role in the PC anticoagulant pathway, it is conceivable to hypothesize that any functional mutation will lead to increased risk of thrombosis. The first EPCR gene mutation described was a 23bp insertion in exon 3 which duplicates the preceding 23 bases and results in a STOP codon downstream from the insertion point (15).

Although statistical analysis did not reveal a significant association between the diseases and the mutation, expression studies in 293 T cells showed that the truncated protein is not localized on the cell surface, cannot be secreted in the culture medium, and does not bind APC, suggesting that the insertion is a risk factor for arterial and venous thrombosis.

Simmons and Lane (24) reported the organization and nucleotide sequence of the human EPCR gene and suggested two potential polymorphic sites, the T2532C substitution in intron 1 and the A4600G substitution that predicts a Ser219Gly change.

Franchi et al (13) have described one mutation in the promoter region of the EPCR gene, a T-318G substitution present in 1/95 women with late fetal loss and in 0/236 controls.

We have studied two mutations in the EPCR gene (25). One is located in exon 4, an A4600G substitution which predicts a Ser219Gly change in the transmembrane region of the EPCR molecule, and the other is a new polymorphism, a G4678C substitution located in the 3’UTR region. In a study of 184 VTE patients and 160 controls, the AG genotype of the A4600G polymorphism was present in 18% of the patients and 15% of the controls, suggesting that it is not associated with VTE.

However, the G4678C polymorphism seemed to reduce the risk of thrombosis. The presence of the CC genotype reduced the risk of VTE 2.6-fold, probably due to the higher APC levels observed in individuals carrying the CC genotype.

**New approach to the search for TM and EPCR gene mutations**

Because TM and EPCR are membrane proteins, their level in plasma cannot be used as a general detection method for deficiency. Therefore, the availability of a screening assay which assesses overall activation of the PC anticoagulant pathway would be of interest to select patients with a history of thrombosis and an alteration in the mechanism of PC activation that could reflect functional mutations in the TM and EPCR genes. It is evident that if mutations in TM or EPCR are to be functional, they will affect the rate of activation of PC and, therefore, the levels of circulating APC. We have reported a simple assay for measuring the concentration of circulating APC in blood and hypothesized that low APC levels in plasma could be associated with a higher incidence of thromboembolic events (26). More recently, we measured the free APC levels of patients with VTE who did not bear any of the more usual thrombophilic defects associated with thrombophilia, and in a control group of healthy individuals, in order to determine whether a low level of APC is an independent risk factor for VTE. The results showed that a reduced concentration of APC is a strong, prevalent, independent risk factor for VTE (27). Reduced APC levels (below 0.69 ng/ml, i.e. 5th percentile of controls) was found in 24% of patients and increased the risk of VTE 4.7-fold. A preliminary familial study revealed that in some instances the low APC phenotype is hereditary.

Reduced APC levels may be the result of various pathophysiological mechanisms, either acquired or hereditary. For example, a qualitative or quantitative dysfunction in any of the proteins involved in PC activation may cause a reduction in APC generation and in plasma APC levels. This means that defects in the genes coding for these proteins and/or a transient, acquired reduction in the expression of thrombomodulin and/or endothelial PC receptor could account for the reduced levels of APC found in patients with VTE. Hence, a previous selection of patients with venous or arterial thrombosis who have reduced circulating APC levels should increase the probability to find functional mutations in the components of the PC activation pathway, including PC, prothrombin, TM and EPCR.

Using this approach, we selected 26 VTE patients with persistent low APC levels and sequenced the codifying sequences and their flanking regions of the PC, prothrombin, TM and EPCR genes. Preliminary results have shown a total of 15 new mutations or polymorphisms that need further studies to assess the prevalence and their association with thrombosis. These results encourage the use of this approach to search for mutations involved in the PC activation pathway.
Acknowledgements

This work was supported in part by research grants from the Fundación Ramón Areces and Fondo de Investigación Sanitaria (PI020125).

References