Polymorphisms in the endothelial protein C receptor gene and thrombophilia

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Summary
The protein C anticoagulant pathway plays a crucial role as a regulator of the blood clotting cascade. Protein C is activated on the vascular endothelial cell membrane by the thrombin-thrombomodulin complex. Once formed, activated protein C (APC) down-regulates thrombin formation by inactivating factors (F)Va and FVIIIa. Endothelial protein C receptor (EPCR) is able to bind protein C and increase the rate of protein C activation.

Keywords
Coagulation inhibitors, deep vein thrombosis, inherited coagulation disorders, polymorphisms, protein C/S pathway

Introduction
The protein C anticoagulant pathway plays a crucial role in the regulation of fibrin formation. Protein C is a vitamin K-dependent plasma glycoprotein that circulates as an inactive form (1). Thrombin activates protein C by cleavage at the N-terminus. This activation is slow and requires the presence of the transmembrane glycoprotein, thrombomodulin (TM). The thrombin-TM complex efficiently activates protein C to activated protein C (APC) (2). APC, with its cofactor protein S, down-regulates thrombin formation via proteolytic degradation of the procoagulant cofactors factor Va and VIIIa (3, 4). In vivo, APC is regulated by two major plasma inhibitors, protein C inhibitor and α1-antitrypsin (5–7).

Another endothelial cell-specific protein, which is involved in the activation of protein C anticoagulant pathway, is the endothelial cell protein C receptor (EPCR). EPCR is a type I transmembrane protein, which is highly expressed on the endothelium of large vessels while it is present at trace levels in most capillary beds (8). It binds protein C on the endothelial cell surface with high affinity and enhances the rate of protein C activation (9). The γ-carboxyglutamic acid domain of protein C is critical for the interaction between protein C and EPCR and the efficient activation of protein C (10). Once activated, APC may dissociate from EPCR, bind to protein S and exhibit its anticoagulant functions, or may remain bound to EPCR and display antiinflammatory, antiapoptotic and cytoprotective functions.

The clinical relevance of protein C activation by the thrombin-TM-EPCR complex is evident from reports showing a clear association between deficiencies of protein C (11–13) and protein S (14–16) or reduced APC levels (17,18) and thrombosis.

As indicated above, normal APC generation depends on the precise assemblage, on the surface of endothelial cells, of thrombin, thrombomodulin, protein C and EPCR. Therefore, any change in the efficiency of this assemblage may cause reduced/increased APC generation and modify the risk of thrombosis. This review highlights the different mutations/polymorphisms reported in the EPCR gene and their association with the risk of thrombosis.
Endothelial protein C receptor: Structure and function

EPCR is a type I transmembrane protein constitutively expressed by endothelial cells that shares homology with the major histocompatibility class I/CD1 family of proteins involved in the immune and inflammation responses (19) and is expressed on the luminal surface of endothelium. The human EPCR gene spans approximately 6 kb and is located on chromosome 20q11.2. It consists of four exons. Exon 1 (amino acids 1 to 24) encodes the 5’-untranslated region (UTR), the signal peptide, and seven additional residues. Exons 2 (amino acids 24 to 108) and 3 (amino acids 108 to 201) encode most of the extracellular region of EPCR. Exon 4 (amino acids 201 to 238) encodes an additional 10 residues of the extracellular region of EPCR, the transmembrane domain, the cytoplasmic tail, and the 3’-UTR (20). Transcription of the human EPCR gene starts from two major sites located at –79 and –82 bp relative to the translation initiation point (20, 21). Several potential regulatory elements have been identified in the EPCR gene (22, 23).

Protein C binds to EPCR on the endothelial cell membrane. This binding augments protein C activation by the thrombin-TM complex by five- to 20-fold (9), possibly by decreasing the $K_m$ of protein C for activation by the thrombin/thrombomodulin complex due to the high affinity between EPCR and protein C. TM is uniformly distributed on the endothelial cell surface of all blood vessels (24). Therefore, its relative concentration decreases in large vessels, which would result in an ineffective activation of protein C in these vessels, especially because the low affinity of the protein C for TM. This effect is counterbalanced by EPCR, which is expressed preferentially on the surface of large blood vessels. Its high affinity for protein C allows an effective localization and activation of protein C on the surface of this type of vessels (8, 25). Therefore, EPCR is seen as a complementary cofactor in protein C activation.

Recently, it has been reported that both factor VII and factor VIIa bind to EPCR on non-stimulated endothelial cells with a similar affinity to that of protein C/APC (26), suggesting that the interaction may play a role in factor VIIa endocytosis and could influence the activation of protein C and APC-mediated cell signaling. However, the pathophysiological consequences and relevance of this binding need further studies.

A soluble form of EPCR (sEPCR) circulates in plasma (27), which is generated in vitro through proteolytic cleavage by metalloprotease activity inducible by thrombin and other inflammatory mediators (28), a process called shedding. Recently, tumor necrosis factor (TNF)-α converting enzyme/ADAM17 has been shown to be responsible for EPCR shedding (29). sEPCR is increased in patients with sepsis and systemic lupus erythematosus (30). However, the physiological significance of sEPCR in vivo is not well known. It binds protein C and APC with similar affinity as the membrane form of EPCR, and may inhibit both protein C activation and APC anticoagulant activity (31, 32), suggesting that it may be procoagulant. On the other hand, sEPCR is able to bind to activated neutrophils (33), and this interaction may reduce leukocyte-endothelial cell interactions, thus modulating inflammation and preventing endothelial damage (33–35).

EPCR expression is tightly regulated (36). Lipopolysaccharide, IL-1β, TNFα and thrombin all negatively regulate EPCR expression (37). Additionally, transient reduction in EPCR and/or TM expression is suggested by the variation in APC levels seen in some individuals over time (17).

Animal studies evidenced that reduced EPCR might contribute to increased risk of thrombosis (38) and exacerbated coagulopathic and inflammatory response to *Escherichia coli*, suggesting that EPCR provides an additional critical step in the host defense against *E. coli* (39). EPCR is essential for normal embryonic development and plays a key role in preventing thrombosis at the maternal-embryonic interface (40). EPCR (-/-) embryos die on or before embryonic day 10.5, showing greatly increased fibrin deposition around trophoblast giant cells (40). However, minimal levels of EPCR are able to support embryonic development, as mice with a severe, but not complete, deficiency of EPCR gene develop, survive, and reproduce normally, and do not present with enhanced arterial thrombosis after challenge, showing that it supports all these functions (41). On the other hand, Li et al. (42) have shown that overexpression of EPCR results in resistance to thrombin formation in response to a procoagulant stimulus, and decreased susceptibility to endotoxin induced septic shock. These authors generated transgenic mice that expressed massive amounts of EPCR in all tissues examined, ranging from nine-fold higher levels in kidney to 150-fold higher levels in lung. Furthermore, EPCR was overexpressed in the endothelium of both large and small blood vessels. The animals did not exhibit gross hemorrhagic abnormalities, but they exhibited about eight-fold increase in APC generation in response to infusion of thrombin and were resistant to a lethal dose of bacterial lipopolysaccharide (42).

Acquired protein C pathway defects have been described in patients with autoimmune diseases. Thus, it has been described the presence of antibodies against EPCR in antiphospholipid syndrome (43), suggesting that they may be a risk factor for fetal death. In addition, antibodies against EPCR had been identified in young women with acute myocardial infarction (44).

There is evidence that EPCR is expressed in cells other than endothelial cells. Thus, EPCR is also expressed on the surface of monocytes, neutrophils and eosinophils (45, 46), brain endothelial cells (47) and embryonic giant trophoblast cells (48). More recently, EPCR was reported to be expressed in murine hematopoietic stem cells (49) and in human vascular smooth muscle cells (50). The specific role of this non-endothelial EPCR as well as its differential expression need to be clarified.

Besides its anticoagulant role in protein C activation, there is evidence that membrane-bound EPCR may play an important role in the anti-inflammatory, anti-apoptotic and cytoprotective functions of APC (34, 51–53). Many, if not all, of these effects are mediated through EPCR-dependent cleavage of protease-activated receptor 1 (PAR-1) (47, 50, 54–56). These cellular responses triggered by PAR-1 engagement via the APC-EPCR complex are similar to those elicited by thrombin or other specific PAR-1 agonists. For example, in a model of stauroporine-induced apoptosis using EA.hy926 endothelial cells, inhibition of apoptosis by APC was dose-dependent and required the active site of the enzyme. In this model, both PAR-1 and EPCR were required for the anti-apoptotic effects of APC (55) and for the direct effects of APC on endothelial cells (54).
The cytoprotective effect of endogenous APC generated by the thrombin-TM complex in EA.hy926 endothelial cells was significantly higher than that of the exogenous APC, suggesting that protective signalling by APC is mechanistically linked to protein activation (57). A recent report provided one possible explanation of why the endogenous APC can exert a more efficient cytoprotective effect (58). These authors showed evidence that the receptors required for both protein C activation (TM and EPCR) and APC cellular signalling (EPCR and PAR-1) pathways are colocalized in the membrane lipid rafts in endothelial cells. The colocalization of TM with these receptors on the same membrane microdomain can also allow to recruit thrombin to activate the EPCR-bound protein C, initiating PAR-1 signalling events that are involved in the APC protective pathways (58).

Mutations in the EPCR gene and thrombosis

### Point mutations

As indicated before, normal APC generation depends on the precise coupling, on the surface of endothelial cells, of thrombin and protein C to their respective receptors, TM and EPCR. Any change in the efficiency of this coupling may cause altered APC generation and a modification in the risk of thrombosis. In fact, several mutations have been reported in the EPCR gene, some of them associated with the risk of thrombosis. The first EPCR gene mutation described was a 23bp insertion in exon 3, at position 4031 (numbering according to Simmonds and Lane [20], with the nucleotides of the gene being numbered relative to the first nucleotide of the translation initiation codon, Met). This mutation duplicates the preceding 23 bases and results in a STOP codon downstream from the insertion point (59). Although statistical analysis did not reveal a significant association between the mutation and the risk of thrombosis, expression studies in 293T 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. However, given its low population frequency (<1%) (60, 61), it will be difficult to assess the effect of this mutation on the risk of venous and arterial thrombosis. In fact, after combining data from seven studies that genotyped a total of 2,508 VTE patients and 2,617 controls (59–65), the prevalences of the mutation were 0.48% and 0.38%, respectively (OR=1.2, 95% CI 0.5–2.9, p=0.754). A similar combination of the three studies that genotyped a total of 669 patients with myocardial infarction and 372 controls (59, 62, 66) revealed prevalences of 1.20% and 0.27% (OR=4.4, p=0.170). Franchi et al. (67) have identified one mutation in the promoter region of the EPCR gene, a T-318G substitution present in 1/95 women with late fetal loss and 0/236 controls. Biguzzi et al. (68) reported four point mutations in the EPCR gene promoter. However, they were rare in patients with venous thromboembolism (VTE) or myocardial infarction, and the in-vitro characterization did not reveal any decreased activity of the reporter gene in basal conditions. A heterozygous C to T variation at position 2769 was identified, which predicts a substitution of Arg to Cys at position 98 in the mature protein (69). However, its prevalence was 0.44% in patients (1/217) and 0.83% in controls (2/230), suggesting no role for this mutation in VTE. The in-vitro expression and characterization of the EPCR R98C variant revealed that it does not seem to alter molecular stability of the protein, supporting no role of this mutation in VTE (69).

### Polymorphisms

Simmonds and Lane (20) 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. In the last years, up to four haplotypes of EPCR have been reported (70–72), H1, H2, H3 and H4, three of which contain one or more single nucleotide polymorphisms (SNPs) that are haplotype specific (Fig. 1). H3 haplotype, tagged by the rare allele of 4600A/G (rs 867186), is associated with increased plasma levels of sEPCR, but its association with risk of VTE is controversial (70–72). Thus, three different groups have reported that H3 haplotype is strongly associated with increased sEPCR (Table 1). However, Saposnik et al. (70) reported that carriers of the H3 haplotype have an increased risk of venous thrombosis (OR=1.8, p=0.004), whereas Medina et al. (71) (OR=0.90, P=0.562) and Uitte de Weijler et al. (72) (OR=1.2, P=0.712) did not find significant association between H3 haplotype and the risk of thrombosis. H1 haplotype, tagged by the rare allele of 4678G/C (rs 9574), was reported to be associated with a reduced risk of VTE (Odds ratio = 0.59, 95% CI=0.41–0.84) (71). H1 haplotype also reduced the risk of thrombosis in carriers of FV Leiden (OR=0.31; 95% CI=0.16–0.83) (73). In patients with the factor V Leiden mutation, the mean age at the first thrombosis was significantly higher in propositi carrying the 4678CC (H1H1) than in those not carrying the H1 haplotype (p=0.046). In addition, the probability of being free of thrombosis at age 40 was significantly higher in the 66 carriers of the H1H1 genotype (94%) than in the 138 carrying the GC (72%) or in the 72 with the 4678GG genotype (60%) (p<0.001) (73). In contrast, two other groups found no association of the H1 haplotype with the risk of thrombosis (70) or a slight reduction of the risk (72). Finally, H4 haplotype was reported to be associated with a slight increase in the risk of VTE (72).

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 inci-
Table 1: Levels of soluble EPCR (sEPCR) in patients with a history of venous thrombosis and in controls, according to the presence or absence of the EPCR H3 haplotype.

<table>
<thead>
<tr>
<th>Reference</th>
<th>Carriers of H3 haplotype</th>
<th>Non-carriers of H3 haplotype</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Patients</td>
<td>Controls</td>
</tr>
<tr>
<td>Saposnik et al. (70)†</td>
<td>ND</td>
<td>264 ± 174 (138–854)</td>
</tr>
<tr>
<td>Medina et al. (71)‡</td>
<td>231</td>
<td>70–770</td>
</tr>
<tr>
<td></td>
<td>55</td>
<td>74</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Utte de Willige et al. (72)†</td>
<td>ND</td>
<td>274 (129–513)</td>
</tr>
<tr>
<td></td>
<td>109</td>
<td></td>
</tr>
</tbody>
</table>

Values are expressed as: *mean ± SD, (range) and N; ¶median, (range) and N. ND = not determined.

dence of thromboembolic events (74). Using this APC assay, we measured circulating APC levels in VTE patients 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 (17). Reduced APC levels (below 0.69 ng/ml, i.e. 5th percentile of controls) were 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. Therefore, we measured APC levels in patients and controls and assessed the correlation between APC levels and an EPCR haplotype. Both in patients and in controls, APC levels were significantly higher in carriers of the H1 haplotype than in those not carrying the H1 haplotype (71). These results show that the EPCR H1 haplotype confers a protective effect against VTE, both in patients with no thrombophilic mutations and in carriers of FV Leiden, probably due to the higher APC levels observed in individuals carrying the H1 haplotype.

Presently it is unknown which SNP in H1 haplotype is responsible for the reported effects. H1 haplotype contains eight specific alleles, the 1451T, 1541A, 1880C, 2322C, 2897A, 3424C, 3997C and 4678C (Fig. 1). Therefore, any of these nucleotides may be responsible for the observed association of H1 with increased levels of plasma APC and reduced risk of VTE (71, 73). Further studies are needed to identify which polymorphism is responsible for the observed associations.

Regarding the H3 haplotype, the 4600G allele is the more obvious candidate responsible for the association of H3 haplotype with increased sEPCR levels, because the EPCR cleavage to form sEPCR occurs within the region of EPCR encoded by exon 4, near the 4600 position. The 4600G change predicts a conformational change in the protein due to the Ser219 to Gly substitution, which could render EPCR more susceptible to metalloprotease cleavage (70, 71). This hypothesis has been supported by two independent studies (75, 76). In-vitro studies of EPCR-transfected cells showed increased basal release of sEPCR from cells expressing the 219Gly EPCR phenotype (75). Qu et al. (76) established stable cell lines expressing either the EPCR Ser219 or the Gly219 variant and showed that both constitutive and stimulated shedding were several fold higher in the Gly219 cell line (H3 haplotype) than in the Ser219 cell line (H1 haplotype). They also isolated human umbilical vein endothelial cells from H1/H1 or H1/H3 origins, showing that the H3 haplotype promoted cellular shedding compared with the H1 haplotype. These data strongly support the concept that the Ser219Gly change is responsible for the higher sEPCR levels observed in individuals carrying the H3 haplotype. Nevertheless, the molecular mechanism by which the increased sEPCR levels observed in individuals bearing the H3 haplotype could increase the risk of venous thrombosis remains to be determined. The mean physiological sEPCR concentration is about 3 nM, well below the concentration of circulating protein C, about 70 nM and the Kd of the EPCR-protein C/APC interactions, about 30 nM. It has been hypothesized that in subjects with markedly increased sEPCR due to the H3 haplotype, with levels between 6 to 20 nM, the local concentration at the endothelial surface may approach or exceed the Kd of the protein C interaction and result in decreased APC generation and inhibition of generated APC.

Conclusion

EPCR plays an in vivo role in the regulation of coagulation in humans. Functional polymorphisms in the EPCR gene may increase/decrease the risk of thrombosis, especially in carriers of prothrombotic mutations, as VTE is a multifactorial disease (77, 78). Many thrombosis patients have more than one predisposing genetic and/or environmental risk factor, and there is convincing evidence that risk increases in proportion to the number of predisposing factors (79–81). However, other polymorphisms in the natural anticoagulant pathways may induce a gain of function and, therefore, induce a reduction in the risk of VTE. Our results in the EPCR gene (71, 73) suggest that the H1 haplotype may induce increased EPCR synthesis and protein C activation. The result of such an increase in the plasma concentration of APC would be a decrease in the risk of VTE or a delay in the age of onset.

Although the direct evaluation of risk situations associated with EPCR haplotypes could be of clinical significance in the near future, the insights gained from these studies on the mechanisms of haemostasis are producing their impact already now in the way we understand the contribution of EPCR (82).
Medina et al. EPCR polymorphisms and thrombotic risk

References


Thrombophilia and anticoagulant pathway

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