REVIEW

Defects of genes encoding inhibitors of coagulation and their application in early miscarriage aetiology

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Abstract: Properly functioning coagulation in gravidity is not necessary only to provide the continuity of circulation in placenta. Today we recognize that proteins and cells of haemostasis in the maternal blood cooperate with the components of a coagulation cascade produced by embryonic trophoblast cells. Such coordination on the embroyomaternal interface is necessary for an intact embryogenesis. Other findings discuss the ability of coagulation components to act also outside the hemocoagulation process, especially as signal molecules, regulators of immune reactions, cell proliferation and others. Haemostasis is thus a complex system and we still do not know all of its pathways. This is perhaps also the reason that in the case of known procoagulant mutations (FV Leiden, gene mutation for prothrombine G20210A) we cannot explain why some carriers suffer recurrent miscarriages and others have uncomplicated pregnancies. The expert community believes that the phenotype manifestation of these mutations in terms of pregnancy losses could be connected to the simultaneous presence (synergistic effect) of other polymorphisms of gene-encoding proteins of haemostasis or the lack thereof (antagonistic effect) (Ref. 59). Text in PDF www.elis.sk.

Key words: endothelial protein C receptor, thrombomodulin, tissue factor pathway inhibitor, protein Z, recurrent pregnancy loss.


A balance between the coagulation and fibrinolytic system is necessary for an intact course of gravidity. Thus, impairment of the haemostatic system places a developing foetus at potential risk of gestational pathology. The first studies pointing out the effect of coagulation abnormalities on adverse pregnancy outcome were published in the mid-1990s. However, to date we cannot definitively state that carriers of the factor V Leiden (FVL) mutation or gene mutations for prothrombine (FII) G2020A are connected with miscarriage risks in the first trimester. This is particularly due to the controversial results of studies and the fact that recurrent miscarriages occur only with a minority of the carriers of the aforementioned procoagulant mutations. The expert public believes that the phenotype manifestation of these mutations in the sense of pregnancy losses can be affected by other, newly studied polymorphisms of genes encoding proteins acting as haemostasis components.

In an effort to find such candidates, the scientists study in animal models the genes necessary for proper embryonic development or they test the genome in Genome-Wide Association Studies where attention is focused on single nucleotide polymorphisms (SNP) with thrombophilic potential.

Protein C Pathway

Thrombin plays a crucial role in haemostasis. In addition to its important coagulation function and ability to stimulate fibrinolysis, it activates signal pathways of certain cells. It also leads to the activation of the protein C pathway, after which it finishes its own production. The protein C pathway consists of a thrombin bond on thrombomodulin and the subsequent activation of protein C (PC). Thrombomodulin is the cofactor of thrombin, which speeds up this process. Efficiency is also increased by the binding between PC and the endothelial protein C receptor. After its release, the activated protein C (APC) together with its cofactor, protein S, limits the amplification and progression of the coagulation cascade through factor Va and VIIIa degradation (1).
Protease-activated receptors

Thrombin produces its effect on cells (thrombocytes, endothelial cells, trophoblast, inflammatory cells, and others) through protease-activated receptors (PAR) (2, 3). By bonding on the thrombocytes PAR, thrombin stimulates their secretion and aggregation. Mammal genome encodes four types of PAR (PAR1-PAR4); PAR2 is the only type that is not thrombin-activated. Human thrombocytes express PAR1 and PAR4 and mice thrombocytes express PAR3 and PAR4. While a low thrombin level is sufficient for PAR1 activation, PAR4 requires a high concentration (3). It has been proved that activated protein C is able to activate PAR1 on endothelial cells (4), which is probably one of the pathways of APC-mediated protective effect on endothelium. The potency of APC for PAR1 cleavage is approximately 10^3-10^4-fold lower than thrombin; in other words, APC is a weak antagonist of PAR1 in comparison with thrombin (5). Thrombin itself is necessary for the presence of APC; therefore it was not clear why by the splitting of PAR1 elicits proinflammatory response, while by the activation of protein C has an opposed, protective effect on endothelium. It is assumed that in a physiologically quiescent state the high affinity of thrombin to thrombomodulin leads to a thrombin bond that is unable to activate PAR1. Simultaneously, the thrombin-TM complex activates the protein C pathway, which results in the APC splitting of PAR1 but with a potency that is distinctively lower than thrombin, by means of which the entire process has an ultimately protective effect. The basis for this is the presence of all three receptors adjacent in a cell membrane (6).

Polymorphisms in the endothelial protein C receptor gene

Endothelial protein C receptor (EPCR) is a type I transmembrane receptor that is particularly present on endothelial cells of large blood vessels and the placenta. Structurally shares homology with the proteins of major histocompatibility class I (7, 8, 9). It is one of the inhibitors of coagulation in the protein C pathway. It amplifies PC activation by its ability to bind to the protein C, which helps to orient protein C to the thrombin-thrombomodulin activation complex (1). It has been demonstrated that EPCR also serves as a cellular binding site for FVII/FVIIa. The FVIIa-EPCR bond inhibits FVIIa coagulation activity and facilitates the elimination of FVII from the endothelium through endocytosis (10). In addition to the cellular form bound to the endothelium, EPCR is also present as a circulating, soluble form. Soluble EPCR (sEPCR) is released from the endothelium by the proteolytic cleavage of endothelial protein C receptor by metalloprotease with a loss of 4kDA part of molecule (11). It is naturally present in human plasma with an approximate concentration of 100ng/ml. Its level rises in the event of a systemic inflammation (12). It binds to PC and APC with the same affinity as EPCR (11), and it blocks the activation of protein C and the anticoagulation activity of APC (13). Thus, sEPCR has a predominantly procoagulant effect (14).

The human gene for EPCR, known as the PROCR gene, is localized on chromosome 20q11.2 (15). Its mutation, which causes a loss of function and drop of EPCR activity level on the membranes, should lead to a reduction of the anticoagulation effect with an acceleration of the risk of thrombosis and an impairment of the EPCR dependant processes. The disruption of the PROCR gene in murine embryonic stem cells resulted in the complete lethality of embryos with both defective alleles of PROCR (EPCR−/− embryos) prior to embryonic day 10.5. The absence of EPCR production by giant trophoblast cells probably determined the fibrin depositions in their surroundings, which indicated thrombosis on the embryomaternal interface as the cause of the uniform lethality of the embryos. If low-molecular heparin was delivered to heterozygous mother mice (EPCR−/+ ) the fibrin deposition did not develop around the embryos. In spite of this, only approximately 25 % of EPCR−/− embryos survived the stage, in which they originally used to die out. It means that thrombosis is not the only cause of death for embryos. No EPCR−/− embryo survived beyond embryonic day 15.5. If embryos were cultivated in vitro from embryonic day 7.5 without placenta in the course of four days; their development was uninterrupted at embryonic day 10.5. The study revealed the information on the necessity of EPCR for a correct placenta functioning and development, the prevention of thrombosis on the fetomaternal interface, the correct course of interaction between mother and embryo, probably the importance of EPCR also for the regulation of other processes (such as immune reactions), and in a later stage of embryogenesis the fact that its expression is irreplaceable in the foetus’ development of a vascular system. In the event of a reduced EPCR (EPCR<sup>−/−</sup>) activity, the development of embryos was normal and the mouse did not show any postnatal thrombotic complications (9).

Complete EPCR deficiency among humans has not been described. Several polymorphisms affecting EPCR activity were identified within the framework of human PROCR. Four haplotypes are most frequent in Caucasians (14). Three haplotypes (A1, A2, A4) are determined by the presence of rare alleles containing single nucleotide polymorphisms, which are haplotype specific. Haplotype A2 consists of common alleles of all haplotype specific SNP (16).

Haplotype 1 (A1), marked by the presence of allele G4678C, in homozgyous form leads to an increased activated protein C (APC) level, which is related to the described reduced risk of venous thromboembolism (17). The A4600G allele characterizes haplotype 3 (A3). This is associated with an increased plasmatic concentration of soluble EPCR (sEPCR) (18). In the case of this polymorphism, a higher share of sEPCR is probably determined by a conformation change in the EPCR molecule due to the amino acid change (Ser219Gly) and its subsequent enhanced susceptibility to metalloproteolytical splitting (16). Because sEPCR has a particularly procoagulant effect, we could expect an accelerated risk of venous thromboembolism (VTE) from increased rates of soluble EPCR in comparison to cellular EPCR. The thrombophilic potential of haplotype A3 however remains controversial. Medina et al (2004) confirmed an increased sEPCR load with the A3 haplotype, but they did not detect any increased risk of thrombosis (17). In the study population of Uitte de Willige et al (2004), the sEPCR level did not correlate with the level of thrombosis risk, but low levels were connected with a reduced incidence of VTE.
Polymorphism 4 (A4) with a G3811A allele was described as a factor that slightly increases the risk of VTE (16).

Thus, no definite clinical effect of the abovementioned EPCR gene polymorphisms on haemostasis has been proved. However, their importance probably lies in their ability to modulate the phenotype expression of known thrombophilic mutations (FV Leiden, FII G20210A). In the study by Medina et al (2005), the presence of the A1 allele in FVL carriers significantly reduced the VTE risk (p = 0.002) (19). Navarro et al (2008) proved that the polymorphism of the A3 gene for EPCR was associated with an elevated VTE risk among carriers of the G20210A allele for prothrombin. Moreover, FII 620210A carriers with the A3 allele developed the first episode of VTE at an earlier age than carriers of the unaffected allele (18).

Neither the isolated haplotype A1 nor A3 showed an association with recurrent miscarriages in the study of similarly affected couples (20). However, it was demonstrated that the A1 haplotype reduced the risk by more than a half of recurrent miscarriage in the first trimester among patients with FVL (21).

Hurtado et al (2004) investigated the presence of antibodies against EPCR (anti-EPCR) among patients with antiphospholipid syndrome who had overcome venous and/or arterial thrombosis and patients with pregnancy loss after the 10th week of gestation. Both groups showed significantly increased levels of anti-EPCR in comparison with the control population (22).

**Defects in the thrombomodulin gene**

Thrombomodulin (TM) is a transmembrane receptor that is located mainly on the luminal surface of endothelial cells of blood vessels (23). The thrombomodulin effect primarily occurs in the process of haemostasis as a protein C pathway anticoagulant, in which it functions as a thrombin receptor. Thus, through the bond on TM, thrombin loses its procoagulant activity and acquires a significantly increased ability to activate protein C (1.24).

The predisposition for arterial thrombosis was more often seen in cases of polymorphisms of thrombomodulin gene than in a similar disorder of the venous system (25–30).

Severe thrombomodulin deficiencies are incompatible with life (31). Healy et al (1995) studied the impact of the disruption of the thrombomodulin mice gene in embryonic stem cells producing TM deficiency. The outcome of the heterozygous genotype (TM+/−) was a 50 % reduction of thrombomodulin activity, which however was not manifested through thrombotic phenotype. Homozygous embryos (TM−/−) died out before embryonic day 9.5 (32). Isermann et al (2001) proved that the early lethality of TM−/− mice embryos is caused by impairment of the function of the early placenta (yolk sac placenta) due to a deficit of thrombomodulin production by giant trophoblastic cells and parietal endoderm. If they allowed its limited expression only in these non-endothelial extraembryonic cells, embryos survived day 8.5 post coitum (p.c.) and continued to develop normally. However, the TM deficit in the endothelium of embryonic blood vessels caused an excessive activation of the embryonic coagulation system and the development of a lethal consumptive coagulopathy, which was incompatible with a survival beyond day 16 p.c. Vascularization was not altered. Thus, thrombomodulin is not necessary for the morphogenesis of blood vessels, but for haemostasis control. Whereas in non-endothelial extraembryonic tissue, it is probably necessary for the limitation of thrombin production and thus the regulation of signal pathways (see further) and in the embryonic endothelium of blood vessels in an effort to prevent excessive blood coagulation (33).

The essence of the necessity of thrombomodulin production is the permanent activation of the coagulation cascade by tissue factor at the fetomaternal interface. Opposed to a mature vascular system, where the tissue factor production is limited to vessel damage, trophoblast constitutively express this initiator of coagulation (34, 35). Thus, without inhibitors of coagulation, the unregulated activation of the coagulation cascade at the fetomaternal interface would occur. Isermann et al (2003) found that the thrombomodulin deficit (TM−/−) in trophoblast cells of an embryo led to damage of the placental function in two ways. On one hand, the excessive triggering of blood coagulation caused a pathological increase in the amount of fibrin degradation products inducing the extinction of trophoblast cells. In the second pathomechanism, they anticipated that the thrombomodulin-protein C pathway protected the placenta by the controlled activation of protease-activated receptors (PAR) (34).

In a subsequent study, the team of Sood et al (2008) proved that PAR4 deficiency in the mother or the absence of maternal thrombocytes enabled approximately one third of TM−/− embryos to develop to term. This is probably based on the locally increased production of thrombin on the surface of the trophoblast due to the absence of TM, which through activation of PAR4 stimulates maternal thrombocytes. It is unclear how subsequently altered maternal plates caused the impairment of the placental function (35). There are hypotheses regarding the possible effect on the placental development by factors released from activated thrombocytes that affect angiogenesis (36) or modulating immune reactions. These studies demonstrate the ability of the fetal prothrombic state to influence the function of maternal thrombocytes to enable it to damage the placenta and the possibility of such mechanism being more essential in establishing embryonic lethality with TM−/− embryos as a fibrin formation. However, the activation of maternal thrombocytes only partially explains foetal losses in TM−/− embryos. Unfortunately, even the induced absence of PAR1 or PAR2 on the surface of foetal trophoblastic cells did not bring any explanation; it did not protect TM−/− embryos from loss (35). In all probability, there are several mechanisms that determine the loss of embryos due to the unregulated activation of coagulation. In addition to the production of fibrin degrading products and the ability of thrombin to activate its own or maternal PAR, there are other factors that must be identified.

Stortoni et al (2010) measured the expression of the gene for TM (thrombomodulin mRNA) in human placental tissue acquired from spontaneous recurrent miscarriages and voluntary abortions as a controls. The expression of thrombomodulin in placental tissue from spontaneous miscarriages was reduced by 45 % when compared to the control group (37).
Defects in the tissue factor pathway inhibitor gene

Tissue factor pathway inhibitor (TFPI) is another inhibitor of blood coagulation. It directly inhibits factor Xa and through FXa the complex initiating blood coagulation, tissue factor (TF)-factor VIIa (FVIIa) (38). TFPI is mainly produced by endothelium (2, 39) and predominantly remains bound to its surface (40). A small amount of TFPI is bound to thrombocytes; the rest circulates free in plasma or in 80 % as a complex with plasma lipoproteins. It seems that TFPI connected with lipoproteins has a weaker anticoagulant effect than free TFPI (41, 42). All of the aforementioned inhibitors of coagulation (EPCR, TM and TFPI) are also produced by human trophoblast cells (2).

Huang et al (1987), who examined the effect of the disruption of the exon 4 of TFPI gene encodes Kunitz domain-1 required for factor VIIa/tissue factor inhibition in mouse embryonic stem cells, demonstrated the participation of TFPI in embryogenesis. The complete deficiency of functional TFPI determined by the homozygous disruption of the TFPI gene (TFPI−/−) led to embryonic lethality between embryonic days 9.5 and 11.5 with the yolk sac haemorrhage in 60 % of cases; the rest died in a later gestation period, probably due to the uncontrolled origin of the FVIIa/TF complex with the development of consumptive coagulopathy and subsequent bleeding. Organogenesis was intact, which is evidence of the fact that TFPI is not necessary for the development of a specific tissue (43).

The theory regarding the uncontrolled FVIIa/TF activation as the cause of embryonic lethality in the case of TFPI deficiency is confirmed by the outcomes of the genetically induced VII factor (44) and tissue factor deficiency (45). Reduced FVII activity determined by its homozygous or homozygous deficiency prevented TFPI− mice embryos from intrauterine lethality. The same result was achieved in the case of reduced TF expression.

Repeatedly, the elevated risk of thrombosis was expected due to TFPI reduction as the inhibitor of coagulation. However, the heterozygous state of TFPI (TFPI−/+) deficiency among mice revealed that even despite the 50 % reduction of TFPI activity no prothrombotic phenotype was observed (43). Yet, the simultaneous incidence of the heterozygous genotype for TFPI deficiency and the homozygous carriage of murine homologue of the FV Leiden mutation (FvLQ/Q/TFPI+−) in mouse embryo resulted in the almost complete postnatal mortality of such mice with an extensive deposition of fibrin in the liver, lungs and kidneys indicating disseminated thrombosis. Individuals with isolated homozygosity for FV Leiden showed normally with a rare incidence of spontaneous thrombosis. Based on the above facts, the reduction of TFPI activity does not have to be manifested in terms of thrombotic phenotype, but combined with other thrombophilic states it can accelerate the procoagulation potential in mice (46).

The combination of reduced TFPI activity and the reduction of expression and activity of another anticoagulation protein, thrombomodulin (TM), led to partial intrauterine lethality among mice embryos (TFPI−−/TM−−−−). The mortality rate among embryos was significantly increased if the mother was a carrier of the TFPI−−/TM−−−− or TFPI−/TM−−−− genotype. This proves the possibility of the synergism of the maternal and embryonic prothrombotic genotype at embryomaternal interface, which consequently could be the cause of embryonic lethality in mice. An increased venous prothrombotic potential among TFPI−−−−/TM−−−−−− individuals surviving into adulthood was recorded (47).

The effect of reduced TFPI level in humans has yet to be precisely defined. The complete absence of TFPI was not observed (47), which can indicate an embryonic lethal phenotype as with mice. Human foetal cells (epithelial and endothelial tissue in particular) produce TFPI from the 8th week of gestation, the placenta (syncytiotrophoblast, cytotrophoblast, vascular endothelium and extravillous trophoblast) from the 10th week (48). White et al (2010) proved that TFPI fraction produced by endothelium and myelomonocytic cells has no effect on embryogenesis (49). Probably it is the trophoblast playing a crucial role by production of inhibitors of coagulation, by which it acquires abilities similar to endothelial cells in the sense of regulation of haemostasis at foetomaternal interface (2).

Impact of the combination of a prothrombotic genotype and gene defect of inhibitor of coagulation (thrombomodulin) in mother and foetus

Sood et al (2007) studied the co-participation of the maternal and embryonic defects of haemostasis on the embryomaternal interface in the mouse model, and their impact on the fate of the embryo. Neither homozygosity for murine homologue of the FV Leiden (FvQ), nor the isolated mutation of the gene for thrombomodulin leading to the reduction of its expression and ability to activate protein C (TMpro) affected fertility. However, their combination could lead to embryonic lethality depending on genotypes of the embryo and mother. For example, all FvQ/Q/TMpropro or FvQ+/ TMpropro embryos in FvQ/TMpro mothers died out. Yet both embryonic genotypes were compatible with the embryo survival in mother suffering from FvQ/TMpromutations. This demonstrates that the phenotype expression of genetically determined defects of haemostasis can be determined by both, maternal and foetal genotype.

Embryonic lethality was not attributed to placental thrombosis, as could be expected based on the unregulated coagulation due to the absence of an inhibitor. Again, it was based on the impairment of placental morphogenesis mediated by the PAR4 receptor-dependent activation of maternal thrombocytes. As it has been proved, platelet depletion at embryonic day 7.5 or elimination of PAR4 receptor from the mother was able to reverse the unfavourable impact on the development of the embryo (36).

Defects in protein Z gene

Protein Z (PZ) is a K vitamin-dependant glycoprotein produced by the liver. As a cofactor, it has an anticoagulant effect in complex with a protein Z-dependent protease inhibitor (PZI), which binds and deactivates the Xa factor. PZI is able to inhibit FXa on its own, but the cooperation with PZ accelerates the inhibition by a thousand times (50, 51).
In addition to the relation to the increased risk of thrombosis in the arterial or venous system (52–54), we can also find studies that demonstrate the connection between the defects of protein Z gene or the presence of anti-Z antibodies with recurrent miscarriages.

Yin et al (2000) studied the results of protein Z deficiency in the case of the disruption of the PZ gene among mice. Isolated PZ deficiency (PZ−/−) did not manifest phenotypically and was compatible with normal survival. However, when combined with the homozygous genotype for murine homologue for the FV Leiden, it caused intrauterine and perinatal thrombosis with consumptive coagulopathy, which led to almost total mortality. The extent of vascular thrombosis was reduced if only one Z protein allele was impaired (PZ+/−/FvQ/Q), or with the parallel presence of homozygous deficiency of protein Z and the heterozygous state for FV Leiden (PZ−/−/FvQ/Q) (55).

When studying the influence of protein Z deficiency on recurrent early miscarriages or foetal losses, a significant number of PZ deficiencies were observed in women with early pregnancy losses (p < 0.001) (56). It has been proven that antibodies against protein Z are also connected with recurrent miscarriages. Sater et al (2011) detected their significant increase among patients affected in such way in comparison with the control group (p < 0.001) (51).


Conclusion

It is probable that the haemostatic aetiology of recurrent miscarriages is complex and its expression requires a combination of specific defects with a synergic effect or conversely, the antagonistic effect of certain polymorphism may lead to phenotypic masking of other mutation. By revealing the ability of trophoblast cells to even produce the regulators of coagulation, the embryonic/foetal genotype also acts as an object of interest. The study of the genes encoding proteins of coagulant and fibrinolytic cascade and the mutual relations of their defects could be helpful in understanding how the congenital defects of haemostasis affect the early stage of pregnancy.

References


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