Article

Multiple thrombophilic gene mutations are risk factors for implantation failure

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Abstract

While the role of inherited thrombophilia has been accepted as a cause of recurrent late pregnancy complications, the contribution of mutated thrombophilic genes to implantation failure has not been studied. Proteins involved in fibrinolysis are necessary for trophoblast invasion into the endometrium. This study compared the prevalence of 10 thrombophilic gene mutations among 42 women with a history of recurrent implantation failure after IVF–embryo transfer with 20 fertile control women. Buccal swabs were taken from all of the women for DNA analyses. Women with a history of implantation failure displayed a higher prevalence of PAI-1 4G/5G mutations than controls (P = 0.007). No differences in the frequency of the other specific gene mutations were detected. However, the prevalence of total gene mutations among patients with implantation failure was significantly higher than among controls. More than three gene mutations among the 10 genes studied were observed in 74% of women with implantation failure and 20% of controls (P = 0.0004). It is concluded that inherited thrombophilias are associated with implantation failure. This association is manifest by total number of mutations as well as with PAI-1 mutations.

Keywords: embryo transfer, implantation failure, inherited thrombophilia, IVF, thrombophilic genes

Introduction

Implantation is the rate-limiting factor for the establishment of pregnancy after IVF and embryo transfer. Successful implantation requires a blastocyst to interact with the endometrium. This interaction includes a variety of molecules secreted by human trophoblastic as well as endometrial cells. The cross-talk between the implanting blastocyst and the endometrium involves either cell-to-cell or cell-to-extracellular matrix interactions, which are mediated by matrix metalloproteinases, cytokines and growth factors (Polan et al., 1995; Klempter, 1997; Salamonsen et al., 2000; Herrler et al., 2003; Nardo et al., 2003). Molecular interactions involving the coagulation and fibrinolytic systems at the embryo–maternal interface during the times of adhesion and invasion have been shown to play an important role in these communications (Axelrod, 1985; Feng et al., 2000, 2001; Chung et al., 2001; Whiteside et al., 2001; Solberg et al., 2003; Aflalo et al., 2004). These findings lead to the question of what influence, if any, inherited thrombophilic gene mutations would have on implantation. While the role of inherited thrombophilic genes has been accepted as a risk factor for difficulties in maintaining pregnancy (Ridker et al., 1998; Foka et al., 2000; Younis et al., 2000; Pihusch et al., 2001; Reznikoff-Etievan et al., 2001; Finan et al., 2002), their contribution to problems in establishing pregnancy is not known. The present study was undertaken to compare the prevalence of 10 thrombophilic gene mutations among women with a history of recurrent implantation failure after IVF–embryo transfer and fertile control women.

Materials and methods

Ten thrombophilic gene mutations, identified from the existing literature to be associated with adverse pregnancy outcomes,
were investigated. The thrombophilic markers were: factor V [G1691A; Leiden] (Casroldi et al., 2000; Ozcan et al., 2001; Buchholz and Thaler, 2003); factor V [H1299R (R2)] (Castoldi et al., 2000; Buchholz and Thaler, 2003); factor V [Y1702C] (Castoldi et al., 2000; Buchholz and Thaler, 2003); factor II prothrombin G20210A [Castoldi et al., 2000; Ozcan et al., 2001; Buchholz and Thaler, 2003]; factor XIII [V34L] (Pasrinen et al., 1999; Kohler et al., 1999; Kakko et al., 2002; Buchholz and Thaler, 2003); β-fibrinogen [–455G→A] (Behague, 1996; Kohler et al., 1999); plasminogen activator inhibitor-1 (PAI-1) [4G/5G] (Eriksson, 1995; Pasrinen et al., 1998; Sartori et al. 1998; Gluek et al., 2000b; Buchholz and Thaler, 2003); human platelet antigen 1 (HPA1) [a/b9L33P] (Pasrinen et al., 1998; Feng et al. 1999); methylenetetrahydrofolate reductase (MTHFR) [C677T] (Pasrinen et al., 1998; Ozcan et al. 2001; Bojesen et al., 2003; Buchholz and Thaler, 2003); MTHFR [A1298C] (Weisberg et al., 1998) (see Table 1).

Patients

Forty-two women with a history of recurrent implantation failure after IVF–embryo transfer were included in the study. Recurrent implantation failure was defined as a study as a total of eight cleaved embryos transferred or four blastocysts transferred with human chorionic gonadotrophin (HCG) serum concentrations <5 mIU/ml 14 days after embryo transfer (Coulam, 1995). Both male and female partners of couples experiencing recurrent implantation failure were evaluated. Semen analysis and sperm DNA integrity assay were performed on all male partners. All women were examined by hysterosonography or hysterosalpingogram for detection of anatomic abnormalities of the uterine cavity. Records of previous IVF–embryo transfer cycles were reviewed.

Twenty fertile women served as controls. Buccal swabs were obtained from all women and analysed for 10 thrombophilic gene mutations. All women entered into the study were Caucasian.

Thrombophilia panel

DNA was extracted from the buccal swab samples using the Qiagen DNA Mini Kit (Qiagen, Crawley, UK), and followed by multiplex polymerase chain reaction (PCR) amplification according to established protocols (Sambrook and Russell, 2001). PCR products were analysed for the 10 respective genetic markers using standard methods, including reverse hybridization and agarose gel electrophoresis (with ethidium bromide staining).

Statistical analysis

The frequencies of homozygous and total number of thrombophilic gene mutations were compared between women experiencing recurrent implantation failures and controls using a 2 × 2 contingency table with Fisher’s exact test. In calculating the total number of gene mutations, a heterozygous mutation was considered as one gene mutation and a homozygous mutation was considered as two gene mutations. A two-tailed P-value < 0.05 was considered significant.

Results

Patients

The mean age of female partners experiencing recurrent implantation failure was 36.7 years and the number of previously failed IVF cycles was 4.3. All of the women had normal hysterosonographic findings and all had endometrial linings measuring >8 mm during previous IVF cycles. One of the study patients and none of the controls had a diagnosis of polycystic ovarian syndrome. All of the male partners had normal semen parameters on standard semen analysis, as well as normal DNA fragmentation indexes determined by the sperm DNA integrity assay. All couples had a total of at least eight cleaved embryos judged by the attending embryologist as ‘good quality’ or four viable blastocysts previously transferred.

Thrombophilia gene mutations

The frequencies of heterozygous mutations among 10 thrombophilic genes tested are shown in Figure 1. No differences in specific gene mutations were observed when patients experiencing recurrent implantation failure were compared with control women. Figure 2 illustrates the prevalence of homozygous mutations. PAI-1 was the only specific gene to show a significant difference between women with a history of recurrent implantation failure and controls. Of the 42 women with implantation failure, 16 (38%) were homozygous for PAI-1 4G/4G compared with two of 20 (10%) of control women (P = 0.03). In addition, women experiencing recurrent implantation failure displayed significantly more total homozygous mutations (31/42 or 74%) than control women (4/20 or 20%) (P = 0.007).

When the total number of mutations was compared counting a heterozygous mutation as one gene mutation and a homozygous mutation as two mutations, women experiencing recurrent implantation failure demonstrated significantly more total mutations than control women (74 versus 20%, P = 0.0004) (Figure 3).

Table 1. Summary of specific gene mutations and polymorphisms affecting haemostasis included in the study.

<table>
<thead>
<tr>
<th>Thrombophilic marker</th>
<th>Specific gene</th>
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<tr>
<td>Coagulation</td>
<td>Factor V</td>
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<tr>
<td></td>
<td>Factor V Y1702C</td>
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<tr>
<td></td>
<td>Factor V G1691A (Leiden)</td>
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<tr>
<td></td>
<td>Factor V H1299R (R2)</td>
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<td></td>
<td>Factor II</td>
</tr>
<tr>
<td></td>
<td>Prothrombin G20210A</td>
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<tr>
<td></td>
<td>β-Fibrinogen 0455G/A</td>
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<tr>
<td></td>
<td>Factor XIII V34L</td>
</tr>
<tr>
<td>Fibrinolysis</td>
<td>PAI 1 4G/5G</td>
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<tr>
<td>Thrombosis</td>
<td>HPA 1</td>
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Figure 1. Frequency of heterozygous mutations for factor V [G1691A; Leiden], factor V [H1299R (R2)], factor V [Y1702C], factor II prothrombin G20210A, factor XIII [V34L], β-fibrinogen [–455G→A], plasminogen activator inhibitor-1 (PAI-1) [4G/5G], human platelet antigen 1 (HPA1) [a/b9L33P], methylenetetrahydrofolate reductase (MTHFR) [C677T] and MTHFR [A1298C] among 42 women experiencing recurrent implantation failure (IF) compared with 20 fertile control women. No significant difference in prevalence of specific mutations was noted between the two groups.

Figure 2. Frequency of homozygous mutations for factor V [G1691A; Leiden], factor V [H1299R (R2)], factor V [Y1702C], factor II prothrombin [G20210A], factor XIII [V34L], β-fibrinogen [–455G→A], plasminogen activator inhibitor-1 (PAI-1) [4G/5G], human platelet antigen 1 (HPA1) [a/b9L33P], methylenetetrahydrofolate reductase (MTHFR) [C677T] and MTHFR [A1298C] among 42 women with a history of recurrent implantation failure (IF) compared with 20 fertile control women. PAI-1 was the only specific gene to show a significant difference between women with a history of recurrent implantation failure and controls (P = 0.03). In addition, women with a history of recurrent implantation failure had a significantly greater total number of homozygous mutations compared with controls (P = 0.007).

Figure 3. Total number of mutations (counting a heterozygous mutation as one mutation and a homozygous mutation as two mutations) among 42 women experiencing recurrent implantation failure compared with 20 fertile control women. Women with a history of recurrent implantation failure (IF) demonstrated significantly more total mutations than control women (P = 0.0004).
Discussion

The data show that thrombophilic gene mutations are associated with recurrent implantation failure after in-vitro fertilized embryos are transferred into uteri of recipients. These results support the view that molecular abnormalities at the endometrial level and abnormal embryo-endometrium dialogue may be responsible for some cases of recurrent implantation failure (Urman et al., 2005a). As early as implantation, an accurate balance of coagulation and fibrinolysis is mandatory for trophoblastic invasion, secure fibrin polymerization and stabilization of the placental basal plate as well as to prevent excess fibrin deposition in forming intravillous spaces and placental vessels. The key element in regulating coagulation and fibrinolysis is the serine protease, thrombin. Thrombin is generated from the proenzyme prothrombin under the influence of prothrombinase complex that includes factor V in its composition. The crucial mechanism of feedback inhibition for the prothrombinase activity is the anticoagulant system involving protein C. Activated protein C acts as an anticoagulant by inactivating factor V, thus decreasing the production of thrombin. Once formed, thrombin initiates fibrin formation. Fibrin, in turn, stimulates conversion of plasminogen to plasmin, thus stimulating the process of fibrinolysis. This tightly regulated balance between, coagulation, anticoagulation and fibrinolysis at the implantation site can be disturbed by the presence of inherited thrombogenic gene mutations.

Previous reports of the association of specific gene mutations including factor V von Leiden, factor II prothrombin and MTHFR and adverse pregnancy outcome have been controversial. Some investigators have found a correlation between inherited thrombophilias and preimplantation pregnancy loss (Sarto et al., 2000; Grandone et al., 2001; Aflalo et al., 2004) and post-implantation pregnancy loss (Ridker et al., 1998; Foka et al., 2000; Younis et al., 2000; Reznikoff-Etievian et al., 2001; Pihusch et al., 2001; Finan et al., 2002), while others have not been able to confirm such a connection (Kutteh et al., 1999). It has previously been demonstrated that the association of inherited thrombophilias and recurrent miscarriage is manifest by total number of mutations rather than specific genes involved (Coulam et al., 2005). A similar finding was apparent in the present study of recurrent pregnancy loss characterized by recurrent implantation failure after IVF and embryo transfer in which inherited thrombophilias are related to total number of mutations studied as well as with PAI-1 mutations. This observation supports the concept that some coagulation factors have additional non-haemostatic functions during implantation.

Thrombophilic genes encode for proteins involved in blood clotting, inflammation, and tissue remodelling (Rawlins and Barrett, 1993). While the mechanism by which thrombophilic gene mutations impact the frequency of recurrent miscarriage are thought to be related to clotting of placental vessels (Many et al., 2001), the methods involved in recurrent implantation failure appear to involve the effects of hypofibrinolysis on trophoblast migration (Axelrod, 1885; Many et al., 2001). Trophoblastic invasion during implantation involves extracellular matrix degradation, which is facilitated by matrix metalloproteinases (MMP) (Chung et al., 2001; Aflalo et al., 2004). Expression of MMP at the implantation site is stimulated by the serine protease, plasmin. Thus trophoblastic implantation depends on the controlled production of plasmin from plasminogen, a process regulated by plasminogen activators (PA) and plasminogen activator inhibitors (PAI). PAI-1 is the main inhibitor of PA and hence fibrinolysis. Homozygosity for the 4G allele of the PAI gene is associated with increased transcription of PAI-1 gene with resultant enhanced gene expression (Sartori et al., 1998). Individuals homozygous for the 4G allele have the highest plasma PAI-1 concentrations, heterozygotes intermediate, and 5G homozygotes have the lowest concentrations of PAI-1 (Festa et al., 2003). High PAI-1 expression is associated with inhibition of conversion of plasminogen to plasmin and subsequent hypofibrinolysis. Hypofibrinolysis as a result of the 4G allele of the PAI-1 gene appears to be a risk factor for implantation failure by limiting trophoblastic invasion (Gris et al., 1997). 4G alleles are found in women with a diagnosis of polycystic ovarian syndrome with a statistically higher frequency compared with controls (Diamanti-Kandarakis et al., 2004), and have been suggested as a cause of implantation failure in these patients (Glueck et al., 2000a). Since only one patient in the current study had a diagnosis of polycystic ovarian syndrome, the association of recurrent implantation failure cannot be ascribed to polycystic ovaries.

While hypofibrinolysis associated with PAI-1 4G gene mutation leads to decreased trophoblast migration and thus decreased implantation rates, increased fibrinolysis would be expected to increase implantation rates. Increased fibrinolysis occurs following enhanced thrombin production as a result of increased prothrombinase activity caused by decreased effect of activated protein C. Since activated protein C degrades activated factor V, activated protein C resistance leads to increased thrombin production via persisting prothrombin activity (Dahlenback et al., 1993). The most common inherited thrombophilia associated with activated protein C resistance is factor V von Leiden (Bertine et al., 1994). This mutation is found in a heterozygous state is 5–10% of Caucasians. Individuals carrying this mutation are at increased risk of thrombosis and consequent pregnancy wastage (Ridker et al., 1998; Younis et al., 2000), but why then is the gene so prevalent in the population? A study of patients undergoing IVF and embryo transfer showed the first embryo transfer to be successful in 90% (9 of 10) of factor V von Leiden carriers compared with 49% in non-carriers (P = 0.018) (Gopel et al., 2001). The overall rate of unsuccessful embryo transfers was also lower in factor V von Leiden carriers (P = 0.02) (Gopel et al., 2001). These observations suggest that improved implantation rate is an important genetic advantage of the factor V von Leiden mutation, providing an explanation for the high prevalence in the general population even though it has significant detrimental effects on reproduction (Ridker et al., 1998; Younis et al., 2000).

Another thrombogenic gene mutation has been shown to have an effect on human reproduction that is not manifest as placental vessel thrombosis. MTHFR is an enzyme that catalyses remethylation of homocysteine to methionine and is involved in folic acid and vitamin B metabolism (Nelen et al., 1998). Individuals homozygous for the C677T mutation in the MTHFR gene are 2.3 times less likely to carry a twin pregnancy than non-carriers of the mutation (P = 0.0008) (Nelen et al., 1998). This effect on dizygotic twinning was postulated to be related to the influence of folic acid on the proliferation of rapidly dividing embryonic and maternal cells (Hasbargen,
2000). Indeed, an increased risk of dizygotic twinning has been associated with folic acid supplementation (Kallen, 2005).

Whereas a shift of balance towards coagulation results in thrombosis intravascularly, a shift of balance leading to hypofibrinogenesis at the implantation site results in decreased trophoblast invasion and implantation failure. It follows that a shift in balance leading to increased fibrinolysis would be associated with increased trophoblast invasion mediated through MMP and consequent increased implantation rates. Increased fibrinolysis could result from increased coagulation mediated through increased thrombin generation as a consequence of decreased anticoagulation activity mediated by APC (activated protein C). High prevalence of fibrinolytic abnormalities observed in women with repeated failure of implantation after embryo transfer suggests that hypofibrinolysis could be a parameter of early reproductive failure.

Thus, it appears that during implantation, proteins involved in coagulation and fibrinolysis have a non-haemostatic function. Genes that encode for thrombogenic proteins have been shown to be involved not only in coagulation and fibrinolysis, but also in fertilization, embryonic development and tissue remodelling (Rawlins et al., 1993). A tightly regulated balance between thrombogenic gene expression of coagulation and fibrinolytic proteins at the implantation site may control the beginning of adequate trophoblast invasion and also limit this invasion to a tolerable extent ensuring a biologically healthy haemochorial placenta. Effective treatment for imbalances in expression of haemostatic proteins at the time of implantation need to be established (Urman et al., 2005b).

References


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