Factor V Leiden and acquired activated protein C resistance among 1000 women with recurrent miscarriage

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Activated protein C (APC) resistance, both in its congenital form, due to the factor V Leiden mutation, and in its acquired form, are important risk factors for systemic venous thrombosis. In view of the suspected thrombotic aetiology of some cases of recurrent miscarriage, the prevalence of APC resistance was determined among 1111 consecutive Caucasian women with a history of either recurrent early miscarriage (three or more consecutive pregnancy losses at <12 weeks gestation; n = 904) or a history of at least one late miscarriage (>12 weeks gestation; n = 207). A control group of 150 parous Caucasian women with no previous history of adverse pregnancy outcome was also studied. Acquired APC resistance was significantly more common among both women with recurrent early miscarriage (8.8%: 80/904; P = 0.02) and those with late miscarriage (8.7%: 18/207; P = 0.04) compared with controls (3.3%: 5/150). In contrast, the frequency of the factor V Leiden allele was similar among (i) women with recurrent early miscarriage (3.3%:60/1808; 58 heterozygotes and one homozygote), (ii) those with late miscarriage (3.9%:16/414; 14 heterozygotes and one homozygote) and (iii) the control group (4.0%:12/300; 12 heterozygotes). Acquired but not congenital APC resistance (due to the factor V Leiden mutation) is associated with both early and late miscarriage.

Key words: activated protein C resistance/factor V Leiden/prevalence/recurrent miscarriage

Introduction

Pregnancy is a hypercoaguable state secondary to an increase in the concentrations of pro-coagulant factors, a reduction in the concentrations of the naturally occurring anticoagulant proteins and a decrease in fibrinolysis (Stirling et al., 1984; Clark et al., 1998). The hypothesis has been developed, supported by histological data, that recurrent miscarriage and later pregnancy complications are due to an exaggerated haemostatic response during pregnancy which results in thrombosis of the uteroplacental vasculature and subsequent fetal loss (Rushton, 1988; Preston et al., 1996; Rai et al., 1996b; Younis et al., 1997; Kupferminc et al., 1999). Such an exaggerated response may be due to a maternal thrombophilic defect such as activated protein C (APC) resistance.

A key component in the anticoagulant pathway is protein C, which when activated inhibits the actions of coagulation factors V and VIII. Resistance to the anticoagulant properties of activated protein C—APC resistance—was first reported in 1993 (Dahlback et al., 1993). It was later demonstrated that APC resistance may either be congenital or acquired. Congenital APC resistance is almost exclusively due to a single point mutation (G → A) at nucleotide position 1691 in the factor V gene, which results in a mutated form of factor V, known as factor V Leiden (Bertina et al., 1994). Mutated factor V is resistant to inactivation by APC, resulting in increased thrombin generation and a hypercoaguable state. Acquired APC resistance is associated with lupus anticoagulant and high concentrations of coagulation factor VIII (Laffan and Manning, 1996). Both factor V Leiden and acquired APC resistance are risk factors for systemic venous thrombosis (de Visser et al., 1999) and factor V Leiden has been reported in association with placental thrombosis (Rai et al., 1996; Dizon et al., 1997).

The prevalence of factor V Leiden and acquired APC resistance among women with recurrent miscarriage has been variably reported to be either similar to or increased compared to parous controls (Balasch et al., 1997; Brenner et al., 1997; Grandone et al., 1997; Dizon-Townsend et al., 1997; Metz et al., 1997; Ridker et al., 1998; Kutteh et al., 1998; Souza et al., 1999; Tal et al., 1999). Previous studies have included only small numbers of women, have been prone to selection bias and some have not divided women into those with recurrent first trimester miscarriage and those with later pregnancy loss.

The aim of this study was to address the limitations of existing data by determining the prevalence of both factor V Leiden and acquired APC resistance among more than 1000 consecutive women with recurrent miscarriage.
Table I. Demographic details of the study populations

<table>
<thead>
<tr>
<th></th>
<th>Recurrent early miscarriages only (n = 904)</th>
<th>At least one late miscarriage (n = 207)</th>
<th>Controls (n = 150)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years); median (range)</td>
<td>34 (23–46)</td>
<td>33 (20–45)</td>
<td>33 (20–41)</td>
</tr>
<tr>
<td>Number of previous miscarriages; median (range)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Early</td>
<td>4 (3–12)</td>
<td>3 (0–11)</td>
<td>0</td>
</tr>
<tr>
<td>Late</td>
<td>0</td>
<td>1 (1–3)</td>
<td>0</td>
</tr>
<tr>
<td>Number with a previous live birth</td>
<td>38 (41%)</td>
<td>91 (47%)</td>
<td>150</td>
</tr>
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</table>

Materials and methods

Subjects
The study population comprised 1111 consecutive Caucasian women with a history of either recurrent early miscarriage (three or more consecutive miscarriages at <12 weeks gestation; n = 904) or a history of at least one late miscarriage (>12 weeks gestation; n = 207). The demographic details and outcome of previous pregnancies of these women are shown in Table I.

A control population of 150 unrelated Caucasian women (median parity 1; range 1–3) with no previous history of miscarriage or late pregnancy complication was also studied (Table I). No woman in this study had a personal or family history of thrombo-embolic disease, was taking the combined oral contraceptive pill, had an abnormal peripheral blood karyotype or a partner with an abnormal karyotype. All women were investigated at least 12 weeks after their last pregnancy.

A variety of causes for recurrent miscarriage has been suggested over the decades. However, despite intensive investigation, only antiphospholipid antibodies (aPL)—lupus anticoagulant (LA) and anticardiolipin antibodies (aCL)—and parental karyotype abnormalities are established causes for recurrent miscarriage (Royal College of Obstetricians and Gynaecologists, 1998). The prevalence of aPL in the study cohort was 20.7% (230/1111).

Protocol for the investigation of recurrent miscarriage
All women attending the Recurrent Miscarriage Clinic are screened for the factor V Leiden mutation, APC resistance and for both LA and aCL prior to pregnancy. In addition, peripheral blood karyotyping is carried out on both partners.

Factor V Leiden
Genomic DNA was extracted from EDTA whole blood using standard techniques. The polymerase chain reaction (PCR) using known primers was used to amplify exon 10 of the factor V gene which contains the G → A mutation at nucleotide position 1691 (Bertina et al., 1994). Following amplification, a 20 μl aliquot of the product was digested overnight with 5 IU of the enzyme Mnl I (New England Biolabs, Hitchin, Herts, UK) at 37°C. Samples of the digested and undigested PCR product were separated electrophoretically in a 3% agarose gel and the bands visualized using ethium bromide. The undigested PCR product measures 223 base-pairs (bp) in size. Following cleavage with Mnl I, a normal allele produces bands of 37, 82 and 104 bp. A mutant allele produces bands of 82 and 141 bp due to loss of one Mnl I cleavage site. Controls on each gel included a known heterozygote, a normal control known not to possess the factor V Leiden mutation and a water blank containing no input DNA.

Activated protein C resistance
Venous blood samples were collected with minimal stasis using a 19-gauge butterfly needle into 0.109 mol/l trisodium citrate, nine parts blood to one part anticoagulant. Platelet poor plasma was prepared by double centrifugation of samples at 2700 g at room temperature for 20 min and stored at −70°C. APC resistance was assessed by measuring the anti-coagulant response in plasma on the addition of APC (Coatest®, Chromogenix, Epsom, Surrey, UK). A ratio of <2.26 for the clotting time in the presence of APC/clotting time in the absence of APC was taken to represent APC resistance (Rosen et al., 1994).

Definitions of congenital and acquired activated protein C resistance
Congenital APC resistance was defined as an APC ratio in the Coatest® of <2.26 in an individual who carried the factor V Leiden mutation and acquired APC resistance as a ratio of <2.26 in an individual with a normal factor V genotype.

Antiphospholipid antibodies
All women were screened for aPL on at least two occasions more than 6 weeks apart prior to pregnancy. Lupus anticoagulant was detected using the dilute Russell’s viper venom time (dRVVT) together with a platelet neutralization procedure. Patient samples with a dRVVT ratio (test/control) of ≥1.1 were retested with a platelet neutralization procedure. A decrease of 10% or more in the ratio was considered to be positive for lupus anticoagulant (Lupus Anticoagulant Working Party on behalf of the BCSH Haemostasis and Thrombosis Taskforce, 1991). Anticardiolipin antibodies were identified using a standardized enzyme linked immunosorbent assay (ELISA). An immunoglobulin (Ig)G anticardiolipin value ≥5 GPL units and an IgM anticardiolipin value ≥3 MPL units was considered to be positive (Khamashata and Hughes, 1993). Women with a positive test for LA or a positive aCL titre had a confirmatory test performed on a second sample taken at least 8 weeks after the initial sample. Only women with persistently positive tests for either LA or aCL were considered to have the antiphospholipid syndrome.

Statistical analysis
Discrete variables were analysed using the χ² test and continuous variables analysed using the Mann–Whitney U-test. P values of < 0.05 were taken as statistically significant.

Results
Compared with parous controls, acquired APC resistance was significantly more common among both women with recurrent early miscarriage (80/904; 8.8%; P = 0.02) and those with late miscarriage (18/207; 8.7%; P = 0.04) (Table II). All women carrying the factor V Leiden mutation were APC resistant.

The frequency of the factor V Leiden allele was 3.3% (60/1808; 58 heterozygotes and one homozygote) among the 904 women with a history of recurrent early miscarriage. The
Activated protein C resistance and recurrent miscarriage

Table II. Prevalence of congenital and acquired activated protein C (APC) resistance

<table>
<thead>
<tr>
<th></th>
<th>Early miscarriages&lt;sup&gt;a&lt;/sup&gt; (n = 904)</th>
<th>Late miscarriage&lt;sup&gt;b&lt;/sup&gt; (n = 207)</th>
<th>Controls&lt;sup&gt;c&lt;/sup&gt; (n = 150)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number (%) with activated protein C resistance</td>
<td>139 (15.4)</td>
<td>33 (15.9)</td>
<td>17 (11.3)</td>
</tr>
<tr>
<td>Congenital due to factor V Leiden</td>
<td>59 (6.5)</td>
<td>15 (7.2)</td>
<td>12 (8.0)</td>
</tr>
<tr>
<td>Acquired</td>
<td>80 (8.8)</td>
<td>18 (8.7)</td>
<td>5 (3.3)</td>
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</table>

Acquired APC resistance: a versus c: P = 0.02; b versus c: P = 0.04.

Table III. Relationship between activated protein C resistance, previous live birth and antiphospholipid antibody (aPL) status

<table>
<thead>
<tr>
<th></th>
<th>Factor V Leiden (congenital APC resistance) (n = 74)</th>
<th>Acquired APC resistance&lt;sup&gt;a&lt;/sup&gt; (n = 98)</th>
<th>Normal APC resistance ratio&lt;sup&gt;b&lt;/sup&gt; (n = 972)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Previous live birth (%)</td>
<td>31 (41.9)</td>
<td>36 (37.5)</td>
<td>478 (49.2)</td>
</tr>
<tr>
<td>Positive aPL status (%)</td>
<td>13 (17.6)</td>
<td>18 (18.4)</td>
<td>199 (20.5)</td>
</tr>
</tbody>
</table>

Previous live birth: a versus b: P = 0.01.
APC = activated protein C.

allele frequency was similar, 3.9% (16/414; 14 heterozygotes and one homozygote), among the 207 women with a late miscarriage. There was no significant difference in the frequency of the factor V Leiden allele between women with recurrent miscarriage—early or late—and the parous control population (12 heterozygotes and no homozygotes; allele frequency = 4.0%).

Women with acquired APC resistance were significantly (P < 0.01) less likely to have had a previous live birth compared to those with a normal APC ratio (Table III). There was no association between APC resistance, congenital or acquired, and aPL positivity (Table III).

Discussion

The haemostatic system plays an important role in three crucial stages of pregnancy—ovulation, implantation and placentation. Attention has recently focused on the potential role that thrombophilic defects, which predispose to the development of thrombosis, may play in the aetiology not only of recurrent miscarriage but also of later pregnancy complications (Preston et al., 1996; Rai et al., 1996b; Younis et al., 1997; Brenner et al., 1999; Kupferminc et al., 1999). This large study reports that the frequency of acquired APC resistance is significantly higher among women with either recurrent early miscarriage or a previous late miscarriage compared to parous controls. In contrast, the frequency of congenital APC resistance is similar to that of appropriately matched controls. The factor V Leiden allele frequency in the control group is in agreement with that previously reported in two studies among 381 asymptomatic, unrelated Caucasians (Beauchamp et al., 1994; Rees, 1996).

In a previous much smaller study, in which APC resistance was assessed among women with recurrent miscarriage but no differentiation was made between congenital and acquired causes, it was reported that the frequency of APC resistance was similar among women with early miscarriage compared with controls (Rai et al., 1996a). This is also the case in the present study, which emphasizes the importance of discriminating between congenital and acquired causes of APC resistance (Table II).

Previous studies reporting an increased frequency of the factor V Leiden allele among women with recurrent miscarriage have been based on small numbers of women, and may have been prone to acquisition bias. For example, the study reporting the highest frequency of the factor V Leiden allele among women with recurrent miscarriage (15%) is based on those referred for investigation to a specialist haemostasis unit (Brenner et al., 1997). Similarly, prevalence studies of acquired APC resistance among women with recurrent miscarriage have also been based on small numbers of patients and may also have been prone to acquisition bias (Balasch et al., 1997; Brenner et al., 1997; Tal et al., 1999).

Although both factor V Cambridge (Williamson et al., 1998) and the HR2 haplotype (Bernardi et al., 1997) have been reported to be associated with APC resistance, these are both rare and no common genetic cause for APC resistance apart from the factor V Leiden mutation has been reported.

In contrast, high concentrations of coagulation factor VIII (Laffan and Manning, 1996), antiphospholipid antibodies (Aznar et al., 1997), pregnancy (Cumming et al., 1995) and the combined oral contraceptive pill (Olivieri et al., 1995) are associated with acquired APC resistance. In this study, no woman was taking the contraceptive pill and all were tested at least 12 weeks after their last pregnancy. No association
was found between aPL and APC resistance among this miscarriage population. This is not surprising as aPL are a heterogeneous family of auto-antibodies. Indeed, attention is shifting away from the concept of aPL associated pregnancy loss being purely thrombotic in aetiology towards emphasizing the adverse non-thrombotic effects of aPL on the trophoblast and the decidua (Rai, 2000).

As women were tested after their miscarriages, an important caveat when interpreting the coagulation data is that it does not establish a temporal relationship between the development of APC resistance and pregnancy loss. This caveat, of course, applies to all non-genetic epidemiological studies of miscarriage. Despite this limitation, these data suggest that APC resistance may be an important cause of pregnancy loss, the mechanism of which is likely to be thrombosis of the placental vasculature. APC resistance has been demonstrated to develop during normal pregnancy (Cumming et al., 1995; Clark et al., 1998) and it is possible that among women who are APC resistant prior to pregnancy, this effect is amplified. The placental vasculature would be particularly prone to thrombosis as during pregnancy there is a relative increase in intravascular coagulation in the placental bed compared with haemostatic changes in the systemic circulation (Bonnar et al., 1970). An interesting finding in the current study is that the prevalence of acquired APC resistance was significantly higher among those with recurrent first-trimester miscarriage compared with controls. If maternal thrombophilic defects are a cause of recurrent first-trimester miscarriage and the mechanism of pregnancy loss is placental vasculature thrombosis, it is important to determine at what gestational age a placental intervillous circulation is established. Recent historical data suggest that while before 8 weeks gestation, the maternal arterial connections with the intervillous space are common in women with fetal loss without apparent cause.

We are currently examining the prospective outcome of untreated pregnancies among women with recurrent miscarriage and acquired APC resistance in order to determine the significance of our findings.

Acknowledgements
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