A possible role for activated protein C resistance in patients with first and second trimester pregnancy failure

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Thrombophilia was recently suggested as a possible factor in recurrent pregnancy losses. We studied prospectively 125 patients (mean age 31.4 ± 5.6 years) with one or more first or second trimester pregnancy losses for the prevalence of activated protein C resistance (APCR). Proteins C and S antigens, antithrombin III, anticardiolipin, and lupus anti-coagulant were also evaluated. Patients with uterine malformations, hormonal abnormalities, chromosomal translocations and infectious causes were excluded. A control group of 125 women with no past fetal loss were matched with the study group. Whenever the APC–sensitivity ratio (APC–SR) was ≤ 2.2, polymerase chain reaction for factor V mutation (Leiden) was performed. Heterozygosity for the mutation was found in 18 patients (14.4%) compared with seven heterozygous among 125 control group (5.6%; \( P < 0.05 \)). Acquired APCR (APC–SR 1.8 and Leiden negative) was revealed in seven patients (5.6%) in the study group and in three of the controls (2.4%; not significant). The rate of preclinical pregnancy losses (17/48) and second trimester miscarriages (10/48) in mutation carriers was significantly higher than in patients with no APCR (25/214) and (14/214) respectively (\( P < 0.001 \) and \( P < 0.01 \) respectively). Live birth rate was not different between the two groups. Occurrence of APCR with any kind of pregnancy loss calculated per patient, in our study group, was ~1/7, 1/4 and 1/5 with one, two and three or more pregnancy losses respectively. These findings suggest that assessment of APCR should be considered in a more extended evaluation of such patients.

Key words: activated protein C resistance/factor V mutation/repeated miscarriages/thrombophilia

Introduction

Recurrent miscarriages, defined as three or more spontaneous consecutive pregnancy losses prior to the 20th gestational week, occur in 1–2% of pregnancies. In spite of extensive work-up invested in finding reasons for this distressing event, identifiable causes could be detected in only 37–44% of cases (Stray-Pederson and Stray-Pederson, 1984). Suggested aetiologies for repeated miscarriages include hormonal abnorm-

alities, congenital and acquired uterine malformations, genetic, immunological and infectious causes.

Early development of a conception is a multifactorial process and involves among other things, formation of adequate vascular support. Ineffective blood flow caused by vascular impairment or thrombotic events in the trophoblast may have a deleterious effect on the developing pregnancy. Evidence for such a vascular insult resulting sometimes in pregnancy loss is found in patients with the antiphospholipid syndrome (Haywood and Brown, 1991). Recently, attention has been drawn to the possible association of early and late pregnancy loss and hypercoagulable states such as seen in different types of thrombophilia (Rai et al., 1995; Preston et al., 1996; Balasch et al., 1997; Brenner et al., 1997). Thus, a 20% activated protein C resistance (APCR) rate was found in habitual aborters if at least one of the miscarriages was in the second trimester and 5.7% if all the miscarriages were in the first trimester, which was comparable with the control group (4.3%) (Rai et al., 1995). A carrier state of Leiden mutation (factor V) was reported in 19 of 39 patients (48%) with repeated pregnancy loss ranging from the first to the third trimester (Brenner et al., 1997). Moreover, in nine of their patients acquired APC-resistance was demonstrated with no evidence for factor V mutation. The European multicentre study, EPCOT (Preston et al., 1996), revealed an odds ratio of 2.0 in women with factor V mutation who delivered a stillbirth but an odds ratio of 0.9 in women with the same mutation who miscarried.

The prevalence of the Leiden mutation in the European and American Caucasian population ranges from 3–5% (Preston et al., 1996) yet some ethnic groups have a higher carrier rate for the Leiden mutation, such as some population sectors in Israel in whom the Leiden mutation heterozygous carrier state is about 10% (U. Seligsohn, personal communication).

When should investigation for APC-resistance begin in patients with pregnancy failure? We planned a prospective case-controlled study to assess the prevalence of APC-resistance in patients referred to our infertility clinic with one or more first or second trimester pregnancy losses.

Materials and methods

One hundred and twenty five patients referred to our clinic between June 1995 and May 1998 because of one or more first trimester or second trimester pregnancy losses were studied for thrombophilia as a possible reason for their pregnancy failure. First trimester pregnancy loss was either preclinical (a transient rise in serum HCG concentrations without ultrasonographic evidence for an intrauterine gestational sac and no positive ultrasonographic findings of an ectopic pregnancy), or clinical (cessation of pre-existing fetal heart activity in the first or second trimester diagnosed by ultrasonography). Since the aim of the
study was to assess the role of thrombophilia in pregnancy loss, we excluded from the study patients with miscarriages associated with fetal malformations detected by ultrasonography or by pathological examination and second trimester miscarriages as a result of cervical incompetence. Basic work-up for congenital or acquired uterine malformations, hormonal imbalance, abnormal parental karyotype and infectious causes was negative in all 125 patients.

A control group consisted of 125 women who had one or more live births but no pregnancy failures and were matched with the study group patients for age and ethnicity.

The age range of the 125 patients in the study group was 21–42 years with a mean of 31.4 ± 5.6 years, and of those in the control group 19–41 years with a mean of 30.7 ± 4.2 years.

Blood samples were collected by venipuncture into two plastic tubes, one containing 1/10 volume 3.8% sodium citrate for coagulation assays and the other containing 1/10 volume of 0.5 mol/l sodium EDTA for DNA extraction. None of the women in the study or control group was pregnant at the time of blood sampling and an interval of at least 2 months had passed between the last pregnancy and blood sampling. Women in the control group had coagulation assays only for activated protein C resistance and polymerase chain reaction (PCR) for detection of factor V mutation.

Coagulation assay

Protein C antigen was determined by enzyme immunoassay, using a specific Asserachrom® kit. Protein C activity was determined by a chromogenic assay, using the protein C Stachrom® kit. Total protein S antigen was determined by electro-immunoassay using Asseroplate® protein S kit. Antithrombin III activity was measured by chromogenic assay using the Antithrombin III Asserochrom® kit (all kits diagnostic, Stago, Asnières, France).

Lupus anticoagulant was detected by two different tests. Diluted Russell Viper Venom test (DRVVT) was performed using the Gradipore La Screen, Northryde, Australia (DRVVT)® kit. Values >1.2 were considered positive and in those cases, presence of lupus anticoagulant was verified by using the Gradipore La-confirm (DRVVT)® kit, which is a phospholipid-rich reagent for the specific correction of lupus anticoagulant.

A solid phase immunoassay technique was used to quantify anticardiolipin concentrations. IgG anticardiolipin concentrations were measured and a concentration of >16 U was considered positive.

Resistance to activated protein C caused by factor V Leiden mutation was determined using Coatest APC-resistance (chromorgenix® kit, Molendal, Sweden). About half of the tests were performed using the above kit and approximately half of the tests were performed using the more specific kit in which plasma samples are prediluted in factor V diluted plasma in order to avoid the influence of other plasma proteins on the assay (this kit was not available during the first year of the study). In both assays, response to APC was expressed by the sensitivity ratio: (SR) = clotting time + APC + CaCl2/clotting time + CaCl2. When the activated protein C sensitivity ratio (APC–SR) was less than 2.2 a PCR analysis for factor V mutation was done. According to our experience (unpublished data) the Leiden mutation was never found if the APC–SR was higher than 2.2.

Acquired APC-resistance (factor V Leiden negative) was defined when the APC–SR was less than 1.8. The inter-assay coefficient of variation for normal controls in our laboratory is 6.6%.

Molecular diagnosis of the Leiden mutation in factor V was made by PCR on DNA extracted from whole blood. The reaction was used to amplify exon 10 of the factor V gene, followed by allele-specific restriction with Mnl I for mutation detection as described elsewhere (Zoller et al., 1994).

Activated protein C resistance and miscarriage

Table I relates pregnancy outcome and results of APC-resistance studies. Preclinical pregnancy failure was significantly more frequent in Leiden mutation carriers, 17/48, than in patients with no APC-resistance, 25/214 (P < 0.001).

Likewise, second trimester pregnancy losses occurred more frequently in Leiden mutation carriers, 10/48, than in patients with no APC-resistance 14/214 (P < 0.01). First trimester clinical pregnancy losses occurred, however, significantly more in patients in whom APC-resistance was not found, 177/214 compared with patients with the Leiden mutation, 21/48 (P < 0.001). The live birth rate was not different between the mutation carriers and no APC-resistance subgroups: 20/71 and 104/319 respectively.

Table II shows the occurrence of APC-resistance after one, two and three or more pregnancy losses per patient. Of 47 patients with one pregnancy loss, APC-resistance (due to the Leiden mutation or acquired) was revealed in seven (14.9%), two with the mutation and five acquired. Forty three patients

<table>
<thead>
<tr>
<th>Pregnancy outcome</th>
<th>Leiden mutation carriers</th>
<th>Acquired APC-resistance subgroup</th>
<th>No APC-resistance subgroup</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patients</td>
<td>18/7</td>
<td>100</td>
<td>125</td>
<td></td>
</tr>
<tr>
<td>Preclinical loss</td>
<td>17/2</td>
<td>25</td>
<td>44</td>
<td></td>
</tr>
<tr>
<td>First trimester loss (clinical)</td>
<td>21/7</td>
<td>177</td>
<td>205</td>
<td></td>
</tr>
<tr>
<td>Second trimester loss</td>
<td>10/1</td>
<td>14</td>
<td>25</td>
<td></td>
</tr>
<tr>
<td>No. of pregnancy losses</td>
<td>48/10</td>
<td>214</td>
<td>272</td>
<td></td>
</tr>
<tr>
<td>Stillbirth</td>
<td>3/–</td>
<td>1</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>Live birth</td>
<td>20/6</td>
<td>104</td>
<td>130</td>
<td></td>
</tr>
<tr>
<td>Number of pregnancies</td>
<td>71/16</td>
<td>319</td>
<td>406</td>
<td></td>
</tr>
</tbody>
</table>

| a P < 0.001 between Leiden mutation carriers and no APC-resistant patients. |
| b P < 0.01 between no APC-resistant patients and Leiden mutation carriers. |
| c P < 0.01 between Leiden mutation carriers and no APC-resistant patients. |
| d Up to 26 weeks of gestation. |

Chromosomal analysis

Chromosomal analysis of fetal material was performed after two or more miscarriages whenever possible. Preclinical pregnancies were not analysed for chromosomal abnormalities because of the paucity of material available for examination in these cases. Cytogenetic analysis was performed as described previously (Eiben et al., 1990).

The χ² and t-tests were used for statistical analysis of discrete and continuous variables respectively.

Results

Of the 125 patients in the study group the Leiden mutation was detected in 18 patients (14.4%) compared with seven (5.6%) of the control group (P < 0.05). All 18 patients in the study group and the seven patients in the control group carrying the mutation were heterozygous. Acquired APC-resistance was revealed in seven patients of the study group (5.6%) and in three (2.4%) of the control group (NS). The other 100 patients of the study group and 115 patients in the control group did not have APC-resistance. The mean ± SD APC–SR of the acquired APC-resistance subgroup (1.59 ± 0.2) and the Leiden mutation carriers subgroup (1.75 ± 0.2) were significantly lower than of patients with no APC-resistance (2.57 ± 0.4); P < 0.01 and P < 0.01 respectively.

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Likewise, second trimester pregnancy losses occurred more frequently in Leiden mutation carriers, 10/48, than in patients with no APC-resistance 14/214 (P < 0.01). First trimester clinical pregnancy losses occurred, however, significantly more in patients in whom APC-resistance was not found, 177/214 compared with patients with the Leiden mutation, 21/48 (P < 0.001). The live birth rate was not different between the mutation carriers and no APC-resistance subgroups: 20/71 and 104/319 respectively.

Table II shows the occurrence of APC-resistance after one, two and three or more pregnancy losses per patient. Of 47 patients with one pregnancy loss, APC-resistance (due to the Leiden mutation or acquired) was revealed in seven (14.9%), two with the mutation and five acquired. Forty three patients
had two pregnancy losses and 11 of them (25.6%) had APC-resistance, 10 with the mutation and one acquired. Of 35 patients with three or more abortions, seven (20%) had APC-resistance, six with the mutation and one acquired. Therefore, in our study group APC-resistance occurred in ~1/7 patients with only one pregnancy loss, in 1/4 after two pregnancy losses and in 1/5 after three or more pregnancy losses.

Concentrations of antithrombin III, protein C and protein S were in the normal range for all study group patients. Elevated titres of anticardiolipin IgG antibodies were detected in nine patients of the study group (three in those with APC-resistance and six in those with no resistance). Lupus anticoagulant was found in only one patient with acquired APCR who had also high titres of anticardiolipin antibodies.

Fetal material analysis was performed in 79 miscarriages in the group of patients with no APCR and 35 had chromosomal abnormalities (44.3%). In the group of patients with APCR due to the factor Leiden mutation, five cases of chromosomal abnormalities were found in 22 miscarriages analysed (22.7%). Of three trophoblastic materials analysed in patients with acquired APCR, one had a chromosomal abnormality.

**Discussion**

Pregnancy failure is a distressing event for the couple who desire pregnancy and the question regarding the fate of a future conception is almost always brought up. Accepting an independent risk of miscarriage occurrence to be 15%, a second loss could be calculated to occur at a rate of 2.3% and a third loss in 0.34% of women (Cunningham et al., 1997). Thus, non-invasive or minimal invasive investigations may be suggested to the couple, tailored to the individual case, in order to assess a possible reason for the repeated miscarriages.

Our study group was comprised of patients with 1–8 pregnancy losses (mean 2.9 ± 1.8) in whom a routine work-up had not revealed a cause for the pregnancy failure. We therefore postulated that a thrombotic event due to thrombophilia could be a possible reason for these patients’ pregnancy losses, as recently suggested (Rai et al., 1995; Preston et al., 1996; Balasch et al., 1997; Brenner et al., 1997) and that was the basis for our study.

Pregnancy losses were divided into preclinical, first trimester clinical and second trimester. We found a significantly increased rate of preclinical pregnancy failure in Leiden mutation carriers than in no APCR patients (P < 0.001). To the best of our knowledge this is the first report to show this difference.

Whether hypercoagulability (due to the Leiden mutation) may lead to such an early pregnancy loss is merely speculative. However, development of the fetal arterial supply to the placenta begins as early as 18–20 days after conception and soon thereafter the fetoplacental villous circulation becomes established (King, 1987; Demir et al., 1989). During this early period of conception, detection of a pregnancy is made by serum β-HCG measurement because it is too early for imaging by transvaginal ultrasonography. Either way, this was the period when our patients’ preclinical pregnancy losses took place.

We found in our study a decreased rate of clinical first trimester miscarriages in patients with hereditary APCR compared with patients with no APCR. Similarly in a group of 19 patients with APCR (Brenner et al., 1997) no increase in the rate of first trimester abortions was found. Rai et al. (1995) took a different approach in their analysis but also did not find a difference in the prevalence of APCR between first trimester aborters and a control group.

The increased rate of second trimester abortion in Leiden mutation carriers revealed in our study group is in accordance with the study of Brenner et al. (1997) who reported an increased risk for second trimester abortions in their patients with APC-resistance. Rai et al. (1995), looking specifically at second trimester pregnancy losses, found a 20% rate of Leiden mutation carriers in that group of patients, significantly more than in the control group (4.3%). Grandone et al. (1997) found seven cases with the Leiden mutation in a sample of 43 Caucasian women (16.8%) with a history of two or more unexplained fetal losses compared with five carriers among 118 controls with uneventful pregnancies (4.2%; P = 0.011). This association was stronger for late events than for first trimester abortions. Ridker et al. (1998) have recently evaluated 113 women with recurrent pregnancy losses for the factor V mutation and found a 2.2-fold increase in the prevalence of the mutation among the aborters compared with controls (P = 0.026).

In contrast, other reports do not support an association of APCR with miscarriage.

The EPCOT study (Preston et al., 1996), defining a miscarriage as a pregnancy loss before 28 weeks of gestation, could not detect an increased risk for such a fetal loss among carriers of the factor V mutation (odds ratio 0.9). Balasch et al. (1997) compared a group of 55 patients with unexplained first trimester repeated abortions with 50 healthy control women who had at least one child but no previous abortion. In each group they detected one heterozygous Leiden mutation.

<table>
<thead>
<tr>
<th>Number of pregnancy loss(es)</th>
<th>Leiden mutation carriers</th>
<th>Patients with acquired APC-resistance</th>
<th>Patients with no APC-resistance</th>
<th>Patients with APC-resistance/all (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2</td>
<td>5</td>
<td>40</td>
<td>7/47 (14.9)</td>
</tr>
<tr>
<td>2</td>
<td>10</td>
<td>1</td>
<td>32</td>
<td>11/43 (25.6)</td>
</tr>
<tr>
<td>≥3</td>
<td>6</td>
<td>1</td>
<td>28</td>
<td>7/35 (20.0)</td>
</tr>
<tr>
<td>Total</td>
<td>18</td>
<td>7</td>
<td>100</td>
<td>25/125(20.0)</td>
</tr>
</tbody>
</table>

*aPregnancy loss: preclinical, first trimester (clinical) or second trimester.*
carrier. They concluded that first trimester repeated miscarriages are not associated with APCR.

Leiden mutation carriers and patients with no APC-resistance in our study group showed comparable live birth rates, 2071 (28.2%) and 104/319 (32.6%) respectively. This is in contrast to the report by Brenner et al. (1997) who found 19% live births in carriers of the Leiden mutation compared with 33% in patients with normal APC-resistance (P < 0.03). This may be due to the fact that their study group comprised 39 patients with at least three miscarriages whereas our study included patients with a various number of miscarriages.

Why do some pregnancies in patients with APC-resistance end as an early or late pregnancy loss whereas other pregnancies result in live births? A recent study (Dizon-Townson et al., 1997) is interesting in this regard and may shed some light on the answer to that question. They found a twofold increase in the carrier frequency for Leiden mutation in abortuses compared with unselected pregnant women and, even more remarkable, a 10-fold increase in the carrier state for the factor V mutation when the pathologically examined placentae of women at birth contained more than 10% infarctions compared with those with less than 10% infarctions. Therefore the existence of a carrier state in the conceptus may play a more important role regarding the fate of the pregnancy than the maternal carrier state since the inheritance is considered autosomal dominant (Zoller, 1994) and thus some fetuses may be affected while the others may not.

The 14.4% of Leiden mutation carriers in our study group was significantly higher than in the control group (5.6%). Since matching was done for ethnicity and age it is quite obvious that the group of patients with repeated miscarriages had a higher prevalence of hereditary thrombophilia than women with no history of miscarriage. Thus, we found an APCR rate of approximately 1/7 after one pregnancy loss occurring any time between the preclinical stage and the second trimester. After two or more pregnancy losses the APCR rate was close to 1/5.

APCR may be associated not only with an increased rate of pregnancy loss but also with a five to 10-fold increased risk of maternal thrombotic events in the heterozygous and 50 to 100-fold in homozygous patients (Dalback, 1996). We maintain therefore that searching for this thrombophilic factor after repeated spontaneous abortions for which no reason had been revealed after a thorough work-up is appropriate because it may be the first clue to the existence of thrombophilia. We do not suggest, however, screening of women who plan pregnancy for the existence of APC-resistance, in agreement with others (Rouse et al., 1997).

Consecutive miscarriages in a given couple show a non-random distribution with respect to chromosomal complements, thus the high percentage (44.3%) of chromosomal abnormalities found in our patients who had no APCR is expected. These correlate well with the 50% chromosomal aberrations associated with fetal loss between 8 and 15 weeks of gestation. The much lower percentage (22.7%) of chromosomal abnormalities revealed in Leiden mutation carriers may suggest that another factor was also involved in causing the miscarriages. However, statistical analysis seems irrelevant here since for obvious reasons only a part of the miscarriages in the two groups could have been analysed.

We have a preliminary experience (unpublished) with 14 patients who had 1–6 pregnancy losses before APCR was diagnosed (none had anticardiolipin antibodies) and were administered a low molecular weight heparin, clexane 40 mg/day (Rhône-Poulenc Rorer, Netanya, Israel) throughout their next conception. Only two of the 14 women aborted and the other 12 delivered healthy newborns. No complications known to be related to the anticoagulant occurred in those 14 patients. Although we think that these results are encouraging it is premature to conclude that enoxaparine is of any benefit in preventing recurrent miscarriages. Certainly, prospective randomized trials are warranted to answer the question of the efficacy of antithrombotic treatment in preventing pregnancy loss in these patients.

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References


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