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Polymorphisms in the ACE and PAI-1 genes are associated with recurrent spontaneous miscarriages

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BACKGROUND: Successful pregnancies require fine tuning of fibrinolytic activities in order to secure fibrin polymerization and stabilization of the placental basal plate as well as to prevent excess fibrin deposition in placental vessels and intervillous spaces. Fibrinolysis is tightly regulated by plasminogen activator inhibitor-1 (PAI-1). Endothelial PAI-1 synthesis is induced by angiotensin II, which is generated by angiotensin I-converting enzyme (ACE). METHODS: We studied the ACE deletion (D)/insertion (I) polymorphism and the PAI-1 4G/5G polymorphism in women with recurrent spontaneous miscarriages (RM). Both polymorphisms have been shown to be associated with ACE and PAI-1 expression levels respectively. A study group of 184 patients with a history of two or more consecutive unexplained spontaneous miscarriages was compared with a control group of 127 patients with uneventful term deliveries and no history of miscarriages. RESULTS: Our findings show: (i) homozygosity for the D allele of the ACE gene, which results in elevated PAI-1 concentrations and hypofibrinolysis, is associated with an elevated risk of RM; (ii) the combination of the D/D genotype with two 4G alleles of the PAI-1 promoter, which further increases PAI-1 plasma levels, is significantly more frequent in RM patients compared with controls. CONCLUSIONS: Based on these results, we recommend the incorporation of these two polymorphisms into the spectrum of thrombophilic mutations which should be analysed in individuals with recurrent spontaneous miscarriages. Patients homozygous for both the ACE D and PAI-1 4G alleles may benefit from the application of low molecular weight heparin as early as possible in the pregnancy in order to prevent uteroplacental microthromboses.

Key words: angiotensin I-converting enzyme/fibrinolysis/plasminogen activator inhibitor-1/pregnancy loss/thrombophilic gene polymorphism

Introduction

Recurrent spontaneous miscarriages (RM) affect up to 5% of fertile couples and, for many of them, the causes remain unexplained (Coulam *et al*., 1997). Haemorheologic disposition plays a role and states of hypercoagulability due to mutations of procoagulatory factors such as coagulation factor V (FV) or prothrombin (FII) have been extensively investigated and associated with RM or severe pregnancy complications (Blumenfeld and Brenner, 1999; Kutteh *et al*., 1999; Kupferminc *et al*., 1999; Younis *et al*., 2000; Pihusch *et al*., 2001; Rai *et al*., 2002; Sarig *et al*., 2002). Furthermore, hyperhomocysteinaemia seems to increase the risk of miscarriages. The most thoroughly investigated inherited risk factor for elevated homocysteine serum levels is homozygosity for the C677T mutation of the methylenetetrahydrofolate reductase (MTHFR) gene (Girling and de Swiet, 1998; Nelen *et al*., 2000; Nelen, 2001). However, the role played by the mutant MTHFR enzyme in pregnancy outcome is still not clear, and its effect might only be additive to other inherited or acquired thrombophilic factors (Sarig *et al*., 2002).

Pregnancies appear to require an even balance of coagulation and fibrinolysis in order to avoid excess fibrin accumulation in placental vessels and intervillous spaces as well as to secure fibrin polymerization and stabilization of the placental basal plate (Preston *et al*., 1996; Buchholz and Thaler, 2003). A successful implantation, on the other hand, needs endometrial vascular remodelling due to angiogenesis and vascular breakdown (Hickey and Fraser, 2000). Adequate fine tuning of fibrinolysis is mandatory in order to prevent haemorrhage. For most tissues involved, fibrinolytic activity has been demonstrated (Lockwood, 2001; Smith *et al*., 2001; Chakraborty *et al*., 2002). A variety of factors influencing fibrinolysis has been studied. For instance, the fibrin-stabilizing factor (FXIII) has been shown to play an important role in placentation during the first trimester of pregnancy (Kappelmayer *et al*., 1994). Deficiency of FXIII with the resulting lack of fibrin stability

and tendency towards hemorrhage as well as thrombosis is regarded as a risk factor for miscarriages (Anwar *et al*., 1999). Deficiency of FXII, in contrast, impairs fibrinolysis by decreasing plasminogen activation, and this has repeatedly been associated with RM (Braulke *et al*., 1993; Ogasawara *et al*., 2001). Minimal alterations in the fibrinolysis cascade leading to either hypo- or hyperfibrinolysis are therefore suspected to interfere with placentation and early pregnancy. Several factors influencing fibrinolysis, particularly the plasminogen activator inhibitors-1 and -2 (PAI-1 and PAI-2), have been shown to be up-regulated during implantation. They modify migration and invasive behaviour of extravillous trophoblast cells, and they also prevent additional haemorrhage during placentation (Feng *et al*., 2000; Lockwood, 2001).

PAI-1 is a key regulating element in the fibrinolysis cascade. Recently, associations between polymorphisms in the PAI-1 and ACE genes and PAI-1 plasma levels have been established. Endothelial PAI-1 expression is modulated by a 4G/5G polymorphism in the PAI-1 promotor, 675 bp upstream from the start site of transcription. PAI-1 expression is also influenced by angiotensin II plasma levels. Angiotensin II, a very potent vasoconstrictor, is generated by the angiotensin I-converting enzyme (ACE), which is well known for its role in blood pressure regulation. ACE expression is associated with a deletion (D)/insertion (I) polymorphism in intron 16 of the ACE gene. Comparable to the PAI-1 4G allele, the ACE D allele leads to an increased PAI-1 expression, resulting in reduced fibrinolysis (Kim *et al*., 1997).

These two genetic variations have been related to various vascular diseases such as myocardial infarction and deep vein thrombosis (Sartori *et al*., 1998, 2000; Iacoviello *et al*., 1998; Seino *et al*., 1998; Gardemann *et al*., 1999; Lane and Grant, 2000) as well as to pregnancy-related disorders such as severe pre-eclampsia, pregnancy-induced hypertension, or serious pregnancy complications such as growth retardation and stillbirth (Zhu *et al*., 1998; Fatini *et al*., 2000; Glueck *et al*., 2000; Yamada *et al*., 2000).

In order to evaluate whether ACE and PAI-1 expression levels affect the course of early pregnancies, we determined the prevalence of the ACE D/I and PAI-1 4G/5G polymorphisms in patients with RM.

Materials and methods

A total of 184 women who had experienced at least two unexplained consecutive spontaneous miscarriages before 25 weeks of gestation were studied after obtaining their informed consent. All patients were investigated ⩾2 months after the last pregnancy to exclude the following established causes of RM: uterine anomalies (gynaecological examination, vaginal ultrasound, hysteroscopy), hypo- or hyperthyroidism (TSH), polycystic ovary syndrome [LH and FSH at cycle day 7, testosterone, dehydroepiandrosterone sulphate (DHEAS), vaginal ultrasound], antiphospholipid syndrome [IgG and IgM anti-cardiolipin antibodies, IgG and IgM anti-β2-glycoprotein antibodies, anti-nuclear antibodies, Lupus anticoagulant, activated partial thromboplastin time (aPTT)]. None of the patients had deficiencies of antithrombin, protein C or protein S as determined by functional assays (Coumans *et al*., 1999). Karyotyping was performed on both partners in order to exclude patients with chromosomal aberrations known to cause RM. The material from previous miscarriages was also karyotyped whenever possible. Miscarriages due to aneuploidy or other chromosomal imbalances were not counted as unexplained miscarriages (Quenby *et al*., 2002). However, patients were evaluated for this study if they had at least two unexplained consecutive miscarriages. A total of 127 patients with one or more normal term deliveries after uneventful pregnancies and no history of miscarriages served as controls. All patients and controls were Caucasians.

Table I. Prevalence of the FVL and FII mutations and of the MTHFR genotypes in recurrent spontaneous miscarriage (RM) patients and controls

	FV		FII		MTHFR		
	–/–	+/–	–/–	+/–	CC	CT	TT
RM (n = 184)[a]	171	13	175	9	74	87	22
%	92.9	7.1	95.1	4.9	40.4	47.5	12.0
Controls (n = 127)	116	11	126	1	55	61	11
%	91.3	8.7	99.2	0.8	43.3	48.0	8.7
P (χ^2)	0.60		0.04		0.59	0.90	0.35
P (trend test)					0.41		

[a]n = 183 for MTHFR genotyping.
–/– = wild type; +/– = heterozygous mutation carrier.

To analyse the D/I polymorphism in intron 16 of the ACE gene and the –675 4G/5G polymorphism in the promotor region of the PAI-1 gene, genomic DNA was extracted from leukocytes of patients and controls and amplified by PCR using gene-specific primers. Each 50 µl reaction contained 10 mmol/l Tris–HCl, pH 8.3, 50 mmol/l KCl, 1.5 mmol/l $MgCl_2$, 0.01% gelatin, 200 µmol/l of each dNTP, 20 µmol/l of each forward and reverse primer, ~200 ng of high molecular weight DNA, and 1.25 IU Taq DNA Polymerase (Sigma–Aldrich). After denaturation at 95°C for 3 min, DNA fragments were amplified for 35 cycles at 95°C for 30 s and at 72°C for 1 min (PAI-1), or for 40 cycles at 95°C for 20 s, at 56°C for 30 s, and at 72°C for 30 s (ACE). The PAI-1 4G/5G promoter genotype was determined according to Margaglione *et al*. (1994) by using a mutated oligonucleotide which contains a partial restriction site for the Bsl I enzyme (New England BioLabs), thus making it possible to identify the extra G base by restriction fragment length polymorphism analysis in 2% low melting point agarose gels (Gibco BRL Life Technologies). The ACE D/I genotype was characterized by the length of the PCR product, 190 bp in the case of the deletion and 490 bp in the presence of the insertion (Tiret *et al*., 1992). Analyses of the thrombophilic FV Arg_{506}→Gln (factor V Leiden; FVL) and prothrombin G20210A mutations as well as of the MTHFR C677T substitution were performed as described elsewhere (Pihusch *et al*., 2001).

For statistical analysis, results of the two groups were compared with the t-test or with the Pearson's χ^2-test for categorical variables. We performed a Kolmogorov–Smirnov test and the hypothesis that the data are normally distributed was not rejected. Association of RM with the ACE D/I and PAI-I 4G/5G polymorphisms and the MTHFR mutation was also tested with the Cochran–Armitage trend test. Stepwise logistic regression analysis was performed with the patients and the controls as the dependent variable and the mutations/polymorphisms in the factor V, prothrombin, MTHFR, PAI-1 and ACE genes as the explanatory variable. Statistical analyses were performed with the Statistical Package for Social Sciences (SPSS for Windows 9.0, SPSS Inc., USA) and the SAS statistical software (V8.1, SAS Institute Inc., USA).

Results

All 184 patients and the 127 women of the control group were investigated for the PAI-1 and ACE gene polymorphisms as well as for the FVL, FII and MTHFR (all patients but one) mutations. No homozygous FVL or FII G20210A carriers were identified. Heterozygotes for the FVL mutation and MTHFR genotypes were equally distributed among RM patients and control individuals (FVL: 7.1 versus 8.7%, $P = 0.60$; MTHFR-CC: 40.4 versus 43.3%, $P = 0.59$; MTHFR-CT: 47.5 versus 48.0%, $P = 0.90$; MTHFR-TT: 12.0 versus 8.7%, $P = 0.35$; trend test $P = 0.41$, all non-significant) (Table I). Heterozygosity for the FII mutation, by contrast, was significantly more prevalent among RM patients (4.9 versus 0.8%, $P = 0.04$), confirming our previously published data (Pihusch *et al.*, 2001).

Demographic data of the patients are shown in Table II in comparison with the 127 controls. RM patients were older, had fewer children, and (by definition) more miscarriages. The results concerning the ACE D/I polymorphism are given in Table III. The hypofibrinolytic D/D genotype was more prevalent in RM patients (32.1%) than in controls (23.6%; $P = 0.11$) although the difference was not significant. The data regarding the PAI-1 promoter polymorphism are shown in Table IV. There was a tendency towards more 4G/4G carriers among RM patients (39.1%) than in controls (32.3%), but the difference was not significant ($P = 0.22$).

As both polymorphisms influence PAI-1 plasma levels and thereby plasmin and fibrin concentrations (Figure 1), we also analysed combinatorial effects (Table V). This revealed a significant difference in the prevalence of the hypofibrinolytic combination of the PAI-1 4G/4G and ACE D/D genotypes,

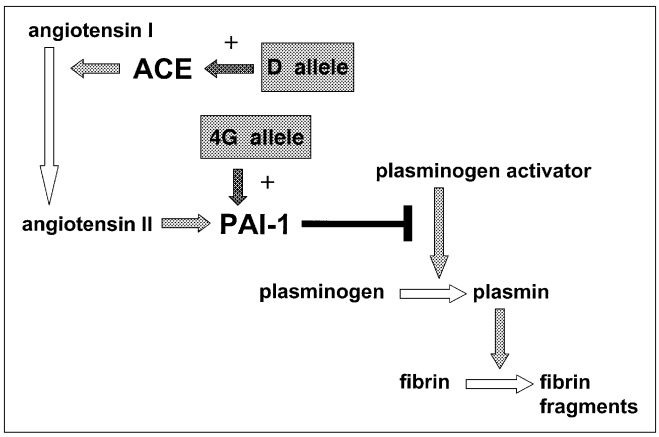

Figure 1. A scheme of the angotensin/fibrinolysis pathway, illustrating the key role of angiotensin I-converting enzyme and plasminogen activator inhibitor-1. PAI-1 expression is increased by the 4G allele of the PAI-1 gene and by the D-allele of the ACE gene via angiotensin II.

Table II. Demographic data of recurrent spontaneous miscarriage (RM) patients and controls (means and range)

	RM	Controls	*P*
n	184	127	
Age[a]	35.0 (21–49)	32.8 (19–45)	< 0.01
Pregnancies	4.2 (2–12)	1.5 (1–4)	< 0.01
Miscarriages	3.2 (2–11)	0 (0–0)	< 0.01
Deliveries	0.6 (0–4)	1.5 (1–4)	< 0.01

[a]At the time of consultation.

Table III. ACE D/I polymorphism in recurrent spontaneous miscarriage (RM) patients and controls

	I/I	D/I	D/D	I allele frequency	D allele frequency
RM ($n = 184$)	42	83	59	167	201
%	22.8	45.1	32.1	45.4	54.6
Controls ($n = 127$)	26	71	30	123	131
%	20.5	55.9	23.6	48.4	51.6
P (χ^2)	0.62	0.06	0.11	0.45	0.45

Table IV. PAI-1 4G/5G polymorphism in recurrent spontaneous miscarriage (RM) patients and controls

	5G/5G	4G/5G	4G/4G	5G allele frequency	4G allele frequency
RM ($n = 184$)	37	75	72	149	219
%	20.1	40.8	39.1	40.9	59.1
Control ($n = 127$)	28	58	41	114	140
%	22.0	45.7	32.3	45.2	54.8
P (χ^2)	0.68	0.39	0.22	0.28	

Table V. Combinations of the PAI-1 4G/5G and ACE D/I polymorphisms in recurrent spontaneous miscarriage (RM) patients ($n = 184$) and controls ($n = 127$)

ACE		PAI-1						*P* (trend test)
		5G/5G		4G/5G		4G/4G		
		n	%	*n*	%	*n*	%	
I/I	RM	10	5.4	19	10.3	13	7.0	0.40
	controls	6	4.7	8	6.3	12	9.5	
	P (χ^2)	0.78		0.22		0.45		
D/I	RM	17	9.2	32	17.4	34	18.5	0.67
	controls	12	9.5	36	28.3	23	18.1	
	P (χ^2)	0.95		0.02		0.93		
D/D	RM	10	5.4	24	13.0	25	13.6	0.02
	controls	10	7.9	14	11.0	6	4.7	
	P (χ^2)	0.39		0.59		0.01		
P (trend test)		0.44		0.76		0.02		

Table VI. Logistic regression analysis of mutations and polymorphisms analysed

Gene	*P*
FV	0.55
FII	0.09
MTHFR	0.57
ACE	0.71
PAI-1	0.92
ACE and PAI-1	0.03

which was present in 13.6% of the RM patients, but only in 4.7% of the controls ($P = 0.01$). In contrast, individuals carrying the 4G/5G–D/I genotype (17.4% of the patients versus 28.3% of the controls; $P = 0.02$) appeared to have a lower risk of RM. The Cochran–Armitage trend test confirmed the relationship between a combination of the PAI 4G/4G and ACE D/D genotypes and RM ($P = 0.02$). Logistic regression analysis also demonstrated a significant influence of these two factors combined ($P = 0.03$), while the FVL mutation and the MTHFR C677T genotype were not significant variables. In addition, the impact of the FII mutation was verified ($P = 0.09$; Table VI). By stepwise logistic regression, the combination of the PAI-1 4G/4G and ACE D/D genotype was a significant positive explanatory variable for miscarriages ($P = 0.01$) and a significant independent risk factor for miscarriages with an odds ratio of 3.2.

Discussion

In our study, patients with unexplained RM had an increased prevalence of the ACE D/D genotype compared with the controls (32.1 versus 23.6%, $P = 0.11$), although this was not statistically significant. Fatini *et al*. (2000) found a similar association of the D/D genotype with first trimester miscarriages. Recently, they reported the ACE D/I polymorphism to be a stronger risk factor for RM than the two more well-established thrombophilic mutations FVL and FII G20210A as well as another ACE polymorphism. The association of D/D homozygosity with other pregnancy complications such as pregnancy-induced hypertension and pre-eclampsia, however, is controversial (Zhu *et al*., 1998; Morgan *et al*., 1999). Glueck *et al*. (2000) stated that homozygosity for the 4G allele of the PAI-1 gene represents a serious risk for pregnancies, predisposing to prematurity, intrauterine growth retardation, miscarriage, and stillbirth. An earlier study in polycystic ovary syndrome (PCOS) patients with or without miscarriages also performed by Glueck *et al*. (1999), in contrast, did not reveal a significant difference with regard to the 4G/5G polymorphism of the PAI-1 gene. However, PCOS patients with at least one pregnancy were more likely to be heterozygous or homozygous carriers of the 4G allele than the healthy controls. Our findings also confirm a tendency of homozygous 4G allele carriers to be bound for miscarriages.

None of these studies, however, investigated the cumulative risk associated with a combination of the two polymorphisms which are both known to influence PAI-1 levels (Figure 1). Both proteins affect the same downstream mechanism and combinatorial effects have been proposed to increase the incidence of diabetic macroangiopathy (Kimura *et al*., 1998). Our study demonstrated that the combination of homozygosity for the PAI-1 4G and ACE D alleles was significantly more prevalent among RM patients. Both the 4G allele and the D allele lead to an increased PAI-1 expression (Figure 1), which may result in hypofibrinolysis. Gris *et al*. (1997) postulated in a study of 500 patients that high PAI-1 levels may be a risk factor for RM. Excess fibrin accumulating in spiral arteries and within the intervillous space may well impede perfusion and prevent normal development of the pregnancy.

FVL and the MTHFR C677T mutation, in contrast, do not appear to significantly predispose to first or second trimester RM in our group of patients (Pihusch *et al*., 2001). Using logistic regression analysis, we were also unable to find an association between heterozygosity for FVL and homozygosity for the PAI-1 4G allele ($P = 0.25$). This is in conflict with data reported by Glueck *et al*. (2001), showing a higher incidence of this combination in patients with severe pregnancy complications.

In conclusion, our results demonstrate that homozygosity for the ACE D allele is a risk factor for RM. Homozygosity for the ACE D and PAI-1 4G alleles additionally amplifies the RM risk and this may very well be exerted by their common effect to increase PAI-1 expression. Analysis of these two polymorphisms should therefore be included in the routine work-up of patients with RM. The clinical implications of these data need to be addressed in a prospective study to answer the question whether or not double homozygous individuals should be treated with low molecular weight heparin.

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