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## A NOVEL G/A AND THE 4G/5G POLYMORPHISM WITHIN THE PROMOTER OF THE PLASMINOGEN ACTIVATOR INHIBITOR-1 GENE IN PATIENTS WITH DEEP VEIN THROMBOSIS

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**Abstract** Plasma plasminogen activator inhibitor-1 (PAI-1) level was observed to be associated with sequence variations at the PAI-1 locus. Therefore, PAI-1 gene promoter was screened for possibly new polymorphisms and to investigate the contribution of these sequence variations to PAI-1 levels in patients with deep vein thrombosis (DVT). DNA was isolated from blood of 83 consecutive unrelated patients (42±11 years old) and from 50 apparently healthy subjects of similar age and gender distribution. Six fragments covering DNA sequence -1523 base pairs (bp) upstream from the start of PAI-1 gene transcription to +90 bp in the first exon, were amplified by polymerase chain reaction and analyzed by single-strand conformation polymorphisms. Two polymorphisms were found: a previously described 4G/5G deletion/insertion polymorphism -675 bp upstream from the start of transcription and a novel G/A single base substitution polymorphism further upstream at -844 bp. The two polymorphisms were in strong linkage disequilibrium. Significant differences between patients and controls were observed neither for the frequencies of the 4G/5G alleles (0.60/0.40 and 0.59/0.41, respectively) nor for the frequencies of the G/A alleles (0.33/0.67 and 0.41/0.59, respectively). The distribution of both polymorphisms was similar in idiopathic and secondary DVT as well as in first and recurrent DVT. In patients association between the 4G/5G genotypes and PAI activity was observed, with the highest values in the 4G/4G genotype (13.3 U/mL), median values in the 4G/5G genotype (9.8 U/mL) and the lowest values in the 5G/5G genotype (2.0 U/mL). Despite the lack of association between the G/A genotypes and plasma PAI-1 levels, electrophoretic mobility shift assay showed specific binding of a nuclear protein from human vascular endothelial cells extracts to both the G and the A variant, suggesting functional importance of this novel G/A polymorphism in regulating the expression of PAI-1 gene.

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**Key words:** PAI-1 gene, polymorphism, plasma PAI-1, deep vein thrombosis

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Considerable evidence supports the view that elevated plasma plasminogen activator inhibitor-1 (PAI-1), the principal rapid inhibitor of tissue-type plasminogen activator, is mainly responsible for the reduced fibrinolytic activity observed in patients with myocardial infarction (1-3) and in patients with deep vein thrombosis (DVT) (4, 5). In young patients with myocardial infarction cause-and-effect relation has been established between PAI-1 and risk of reinfarction (6). Increased levels of PAI-1 correlate also with the development of recurrent DVT (7), indicating that impaired fibrinolysis due to increased PAI-1 might have a role in the pathogenesis of vascular disease possibly by contributing to a pro-thrombotic state.

The cause of high PAI-1 in vascular disease is not clear. Increased plasma levels of PAI-1 are related to several anthropometric and metabolic factors of which body constitution, plasma levels of triglycerides and insulin seem to be the most important (8-10). In addition to these environmental features there is data suggesting that PAI-1 levels may be influenced by genetic polymorphism. Levels of PAI-1 have been found to relate to sequence variations at the PAI-1 locus. Multiallelic CA dinucleotide repeat polymorphism in the third intron, Hind III restriction fragment length polymorphism at the 3' end of the PAI-1 gene and a single guanosine deletion/insertion (4G/5G polymorphism) -675 base pairs (bp) upstream from the transcription start of the PAI-1 gene were associated with PAI-1 levels in myocardial infarction (11-14). It seems that sequence variations in the promoter of PAI-1 gene are of importance not only in regulating expression of the PAI-1 gene but influence also the relationship between metabolic factors such as triglyceride and PAI-1 levels (15, 16).

The aim of the present study was to screen PAI-1 gene promoter sequences for possibly new polymorphisms and to evaluate the contribution of these sequence variations to PAI-1 levels in patients with DVT.

## SUBJECTS AND LABORATORY METHODS

### *Subjects*

All subjects investigated were Caucasian, originated from Central Europe and were unrelated. They were asked to participate after they had given their full informed consent. The study was approved by the Slovene Ethical Committee.

Eighty-three consecutive patients (35 women and 48 men) aged 19 to 60 ( $42 \pm 11$ , mean  $\pm$  SD) years, were asked to participate at least three months, but on average  $18 \pm 10$  months (mean  $\pm$  SD) after acute DVT. At the time of the acute event clinical diagnosis of DVT was confirmed by at least one of the following methods: ultrasound vein imaging, impedance plethysmography, isotope or contrast venography. Location of DVT was in 75 (90%) cases proximal lower limb, in 3 (4%) distal lower limb and in 4 (5%) upper limb. One patient suffered thrombosis of vena cava. Sixty-five (78%) patients suffered a single event and in 18 (22%) DVT was recurrent. Perfusion/ventilation lung scanning was performed in patients in whom pulmonary embolism was suspected and a positive scan was established in 17 (20%) patients. In 34 (41%) patients no common factors predisposing to DVT could be established (idiopathic DVT). However, in 49 (59%) DVT was secondary to common predisposing factors such as surgery (N=17), trauma (N=15), bed-rest (N=10) and immobilization (N=10). In women the most frequent predisposing factors were oral contraceptives (N=12), hormonal replacement therapy (N=4), puerperium

(N=4) and pregnancy (N=3). At the time of blood sampling 25 (30%) patients were receiving oral anticoagulant treatment. None of the women was taking oral contraceptives or hormonal replacement therapy. When included in the study patients were screened for haemostatic defects predisposing to DVT.

Fifty apparently healthy subjects (23 women and 27 men) 21 to 60 ( $43 \pm 10$ , mean  $\pm$  SD) years old were asked to participate as controls. They were mainly students and members of the hospital staff with no DVT or other cardiovascular disorders in their history.

#### *Blood sampling and DNA isolation*

Blood samples were obtained from fasting subjects between 7 and 9 a.m. after a 20 min rest. Blood was sampled from an antecubital vein, in most cases without application of a tourniquet. For measurement of haemostatic factors blood flowed directly into precooled siliconized glass vacuum tubes with 0.13 mol/L trisodium citrate (1 volume of citrate to 9 volumes of blood), was placed in ice water and then centrifuged within one hour for 30 min at 2000 g and 4° C. Platelet poor plasma was transferred to small plastic vials, frozen in liquid nitrogen and stored at -70° C until analyzed. For biochemical assays blood was collected into vacuum tubes without an anticoagulant; after one hour serum was harvested and analyzed the same day.

For DNA analysis blood was sampled into K<sub>3</sub>-EDTA vacuum tubes (Becton Dickinson Vacutainer System Europe), thoroughly mixed with the anticoagulant by inverting the tube several times and stored at -70°. DNA was isolated using commercially available genomic DNA purification kit (Wizard™, Promega, Madison, WI, USA).

#### *Screening for PAI-1 gene promoter polymorphisms*

Six fragments covering the promoter between -1523 bp upstream from the start of the transcription site to +90 bp in the first PAI-1 gene exon (Fig. 1) were amplified by polymerase chain reaction. 200-500 ng of genomic DNA in a 25  $\mu$ L volume were used as a template for the following 6 pairs of primers (Vienna Biocenter, Austria): TAA GGG CCA CGG GGC CCG TAC TGG TCC ATA G and CAA TTC ACC ATC GTG TAG AAT GAC for fragment I, CTG ATT CTA CAC GAT GGT GAA TTG and CAC GAG CTG CCC GCT AGG ACT TGG GCC for fragment II, CCT GCC ATG AAT TGA CAC TC and GTG TCC AG ACT CTC TGT G for fragment III, CAC AGA GAG AGT CTG GAC AC and CTG CCA GGT TGA CTG TCT CG for fragment IV, CAG ACA GTC AAC CTG GCA GG and GTC TGA ACA GCC AGC GGG TC for fragment V, GAC CCG CTG GCT GTT CAG AC and GGC TGC TGA GCT GCA GGA ATT CAG CTG CTG for fragment VI. The primers were designed according to the published sequence of the PAI-1 gene (17). Conditions for polymerase chain reaction were: 94° C: 40 sec, 62° C: 40 sec, 72° C: 40 sec for 30 cycles.

Double stranded DNA from polymerase chain reaction was denatured to a single stranded DNA and analyzed for single-strand conformation polymorphisms (18) in a semi-automated programmable electrophoresis instrument (Phast system) using precast polyacrylamide gels (Phast gels 8-25 gradient). Electrophoresis was run under non-denaturing conditions (Phast gel native buffer strips, all Pharmacia, Sweden). Electrophoretic conditions were adjusted to obtain optimal separation of different DNA entities (pre electrophoresis: 400V, 5mA, 1W, 200Vh; loading: 400V, 0.5mA, 1W, 6Vh; separation: 400V, 5mA, 1W, 300Vh). Gels were silver stained (19).

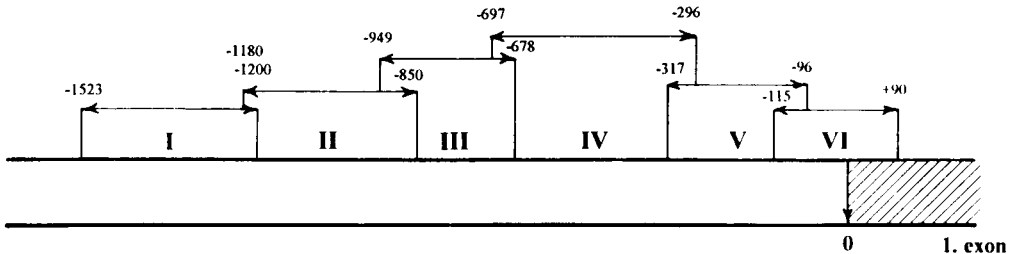


FIG. 1

Scheme of the PAI-1 gene promoter. Six fragments covering the promoter from -1523 bp from the start of the transcription to +90 bp in the first exon, amplified by polymerase chain reaction are shown.

Single-strand conformation polymorphisms found in polymerase chain reaction fragments III and IV were confirmed by direct sequencing in two homozygous subjects. Polymerase chain reaction products were first purified on purification columns (Qiaquick, Qiagen, USA) and then precipitated by ethanol. They were sequenced directly using the dideoxy method with DNA polymerase (AmpliTaq, Perkin Elmer, USA) and capillary electrophoresis equipment (Abi Prism 310, Perkin Elmer, USA). Primers purified by high performance liquid chromatography were the same as those utilized for the polymerase chain reactions.

#### *Electrophoretic mobility shift assays*

A 28-mer oligonucleotide was designed for each type of the polymorphic G/A sequence: TTA GCG GGC AGC TCG AGG AAG TGA AAC T for the G variant and TTA GCG GGC AGC TCG AAG AAG TGA AAC T for the A variant. Complementary oligonucleotides for each variant were synthesized by an Applied Biosystems Oligonucleotide Synthesizer (USA). Equimolar amounts of the complementary oligonucleotides were annealed, and radioactivity labeled by filling in the overhangs with Klenow enzyme in the presence of [ $\alpha$ - $^{32}$ P] dATP. Labeled probes were purified on 7% polyacrylamide gel, eluted and precipitated with ethanol. Nuclear extracts were prepared from human vascular endothelial cells (HUVECs) according to Dignam and coworkers (20) with exception of a modified buffer C, which was used during nuclear extraction (20 mM HEPES-KOH, pH 7.9, 420 mM NaCl, 400 mM  $(\text{NH}_4)_2\text{SO}_4$ , 1.5 mM  $\text{MgCl}_2$ , 0.2 mM EDTA, 0.5 mM dithiothreitol, 25% glycerol). Cells were either unstimulated or treated with lipopolysaccharide. In electrophoretic mobility shift assays, 1-5 $\mu$ g of nuclear protein were incubated with 0.3-1.0  $\mu$ L of radioactivity labeled probe ( $10^5$  cpm/ng) in binding buffer (100 mM HEPES-KOH, pH 7.9, 5 mM EDTA, 25 mM  $\text{MgCl}_2$ , 250 mM KCl, 5mM dithiothreitol, 50 % glycerol). Protein-DNA complexes were loaded on 5% polyacrylamide gel and run 2-3 hours at 150 V. The gel was dried on Whatman 3MM paper and autoradiographed overnight.

*Haemostasis and biochemical assays*

PAI-1 antigen was determined by an enzyme-linked immunosorbent assay and PAI activity by a chromogenic substrate assay utilizing commercially available kits (Imulyse™ PAI-1 and Spectrolyse®/fibrin, Biopool, Sweden, respectively). Resistance to activated protein C was estimated by a modified activated partial thromboplastin time (Coatest®, Chromogenix, Sweden). Antithrombin III, protein C and plasminogen were determined by amidolytic assays (all Berichrom, Behring, Germany) according to the instructions of the manufacturer. Glucose, triglyceride, total-cholesterol, high-density lipoprotein (HDL) -cholesterol and low-density lipoprotein (LDL) -cholesterol were determined by routine biochemical methods. Body mass index was expressed as: (body weight in kg)/(body height in m)<sup>2</sup>.

*Statistical methods*

The skewness of PAI-1 antigen, triglyceride and glucose distributions was normalized by log-transformation, while PAI activity was square-rooted, in order to permit use of parametric methods. In the tables the transformed values are presented as anti-log means with 95% confidence intervals (PAI-1 antigen, triglyceride) or squared means with 95% confidence intervals (PAI activity). The t-test and analysis of variance were used to compare values between the two groups of subjects and between different genotypes. Alternatively, non-parametric Kruskal-Wallis analysis of variance was utilized in order to establish differences between non-transformed PAI-1 antigen and PAI activity levels in various genotypes. Pearson's correlation coefficient was used to evaluate associations between PAI-1 and other variables. To compare PAI-1 antigen and PAI activity levels in different genotypes, these values were adjusted for the triglyceride levels and body mass index with the use of analysis of covariance.

The  $\chi^2$ -test was used to analyze deviation of the genotype distribution from the distribution that would be expected if the alleles were in the Hardy-Weinberg equilibrium and to test differences in frequencies of genotypes between patients and controls.

Calculations were performed using the GLM Procedure of the SAS/Stat program (SAS Institute Inc., Cary, NC, USA). Linkage disequilibrium between the 4G/5G and the G/A genotypes was calculated with the Genepop computer program (21). P value of <0.05 was taken as statistically significant.

## RESULTS

The patients and the controls had similar age and gender distribution. The two groups did not differ significantly in body mass index, fasting blood glucose, HDL-cholesterol and triglyceride levels. On the other hand, patients had higher levels of total- and LDL-cholesterol than controls (Table I).

In two of the PAI-1 promoter fragments single-strand conformation polymorphism was found. Fragment IV (Fig. 1) contained previously described 4G/5G deletion/insertion polymorphism at -675 bp from the transcription start resulting in alleles with either 4 or 5 guanines in a row. Fragment III (Fig. 1) contained a new hitherto undescribed single substitution of guanine to adenine (G/A polymorphism) further upstream at -844 bp (Fig. 2).

TABLE I

Anthropometric and metabolic data of DVT patients and healthy controls (means $\pm$ SD or means with 95% confidence intervals, NS: not significant).

	Patients (N=83)	Controls (N=50)	p
Women/Men (N)	35/48	23/27	NS
Age (years)	42.2 $\pm$ 11.4	43.9 $\pm$ 10.0	NS
Body mass index (kg/cm <sup>2</sup> )	26.2 $\pm$ 3.8	26.5 $\pm$ 3.5	NS
Glucose (mmol/L)	5.2 (5.1-5.4)	5.4 (5.2-5.6)	NS
Total Cholesterol (mmol/L)	6.2 $\pm$ 1.2	5.3 $\pm$ 1.0	<0.001
HDL-Cholesterol (mmol/L)	1.3 $\pm$ 0.4	1.3 $\pm$ 0.4	NS
LDL-Cholesterol (mmol/L)	4.1 $\pm$ 1.1	3.4 $\pm$ 0.9	<0.001
Triglyceride (mmol/L)	1.5 (1.3-1.7)	1.3 (1.2-1.6)	NS

Frequencies of the 4G and 5G alleles were 0.60/0.40 in patients and 0.59/0.41 in controls. Allele frequencies in the novel G/A polymorphism were 0.33/0.67 in patients and 0.41/0.59 in controls. The distribution of the 4G/5G and the G/A genotypes in both groups of subjects studied is shown in Tables II and III. There were no significant differences in the frequencies of different genotypes between patients and controls either for the 4G/5G or for the G/A polymorphism. In patients ( $\chi^2$ -test=5.46), but not in controls ( $\chi^2$ -test=1.97) the observed frequencies of the 4G/5G genotypes differed significantly from the distribution expected from the Hardy-Weinberg equilibrium. Distribution of the G/A genotypes differed both in patients ( $\chi^2$ -test=11.55) and in controls ( $\chi^2$ -test=3.95) from the expected equilibrium. The 4G/5G and the G/A polymorphisms were in strong linkage disequilibrium in both groups of subjects investigated (patients:  $p=0.001$ , controls:  $p=0.005$ ).

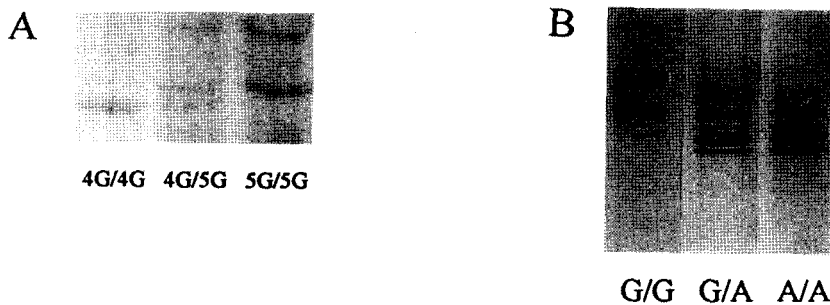


FIG. 2

Typical single-strand conformation polymorphism patterns of the 4G/5G polymorphism (A) and the G/A polymorphism (B).

TABLE II

Distribution of 4G/5G genotypes in DVT patients and in healthy controls.

Genotype N (%)	4G/4G	4G/5G	5G/5G
Patients (N=83)	25 (30.1)	50 (60.2)	8 ( 9.7)
Controls (N=50)	15 (30.0)	29 (58.0)	6 (12.0)

The distribution of the 4G/5G and the G/A genotypes did not change significantly if patients with resistance to activated protein C (N=9) and with deficiencies of antithrombin III (N=1), protein C (N=4) or plasminogen (N=1) were excluded. Likely, the distribution of the 4G/5G and the G/A genotypes was not significantly different between the subgroups of patients with idiopathic and secondary DVT or with single and recurrent DVT (data not shown).

In subjects investigated PAI-1 antigen and PAI activity significantly correlated with triglyceride levels ( $r=0.43$  and  $r=0.37$ , respectively), body mass index ( $r=0.35$  and  $r=0.17$ , respectively) and total cholesterol ( $r=0.20$  and  $r=0.25$ , respectively). Analysis of covariance revealed significant influence of both triglyceride level and body mass index on PAI-1 antigen and PAI activity, therefore adjustments for these two independent variables were performed (Tables IV and V).

The patients and controls had similar levels of PAI-1 antigen (12.8, 10.6-15.4 ng/mL vs 13.3, 10.2-17.4 ng/mL) and PAI activity (11.4, 8.8-14.3 IU/mL vs 9.1, 6.3-12.5 IU/mL). For PAI-1 antigen analysis of variance indicated no significant interactions between groups of subjects and different 4G/5G genotypes. For PAI activity interactions were almost statistically significant using parametric analysis of variance ( $p=0.067$ ) and significant ( $p=0.034$ ) using non-parametric test. In patients the lowest PAI activity was observed in the 5G/5G genotype, intermediate in the 4G/5G genotype and the highest in the 4G/4G genotype (Table IV). For the G/A genotypes neither the patients nor the controls differed significantly in PAI-1 antigen and PAI activity levels (Table V).

TABLE III

Distribution of G/A genotypes in DVT patients and in healthy controls.

Genotype N (%)	G/G	G/A	A/A
Patients (N=83)	2 ( 2.4)	50 (60.2)	31 (37.4)
Controls (N=50)	5 (10.0)	31 (62.0)	14 (28.0)

TABLE IV

PAI-1 antigen (ng/mL) and PAI activity (U/mL) levels in DVT patients and healthy controls with different 4G/5G genotypes. Non-adjusted values and values adjusted for the triglyceride level and body mass index are shown (Means with 95% confidence intervals).

<i>Genotype</i>	N	<i>PAI-1 antigen</i>		<i>PAI activity</i>	
		Non-adjusted	Adjusted	Non-adjusted	Adjusted
<i>Patients</i>					
4G/4G	25	14.1 (10.3-19.3)	12.9 ( 9.3-17.9)	14.5 (10.3-19.4)	13.3 ( 8.7-18.9)
4G/5G	48	13.1 (10.1-16.9)	13.3 (10.2-17.3)	11.7 ( 8.4-15.6)	9.8 ( 6.6-13.7)
5G/5G	8	8.4 ( 5.6-12.5)	9.0 ( 4.5-17.9)	3.1 ( 0.2- 9.6)	2.0 ( 0.0- 8.5)
<i>Controls</i>					
4G/4G	15	10.7 ( 6.8-16.9)	14.1 ( 9.3-21.5)	7.0 ( 2.7-13.3)	10.0 ( 5.1-16.6)
4G/5G	29	14.1 ( 9.9-20.1)	13.7 (10.2-18.3)	9.7 ( 6.0-14.4)	9.6 ( 6.1-13.8)
5G/5G	5	18.5 ( 7.2-47.8)	14.8 ( 7.4-29.7)	12.7 ( 4.4-25.3)	10.9 ( 3.3-23.0)

TABLE V

PAI-1 antigen (ng/mL) and PAI activity (U/mL) levels in DVT patients and healthy controls with different G/A genotypes. Non-adjusted values and values adjusted for the triglyceride level and body mass index are shown (Means with 95% confidence intervals).

<i>Genotype</i>	N	<i>PAI-1 antigen</i>		<i>PAI activity</i>	
		Non-adjusted	Adjusted	Non-adjusted	Adjusted
<i>Patients</i>					
G/G	2	15.2 (12.2-18.8)	13.7 ( 2.9-65.0)	14.2 (13.6-14.8)	15.7 ( 0.3-54.0)
G/A	48	12.5 (10.0-15.8)	12.6 ( 9.7-16.4)	9.9 ( 6.8-13.6)	8.3 ( 5.4-11.9)
A/A	31	13.1 ( 9.4-18.3)	12.9 ( 9.5-17.4)	13.7 ( 9.5-18.6)	12.8 ( 8.4-18.0)
<i>Controls</i>					
G/G	4	13.4 ( 7.1-25.0)	16.7 ( 7.7-36.6)	9.7 ( 4.6-16.6)	11.1 ( 2.6-25.4)
G/A	27	13.1 ( 9.3-18.7)	13.7 (10.3-18.3)	8.4 ( 4.9-12.8)	9.0 ( 5.6-13.3)
A/A	13	13.7 ( 8.1-23.3)	13.7 ( 9.0-20.8)	10.7 ( 5.2-18.1)	11.4 ( 6.0-18.4)

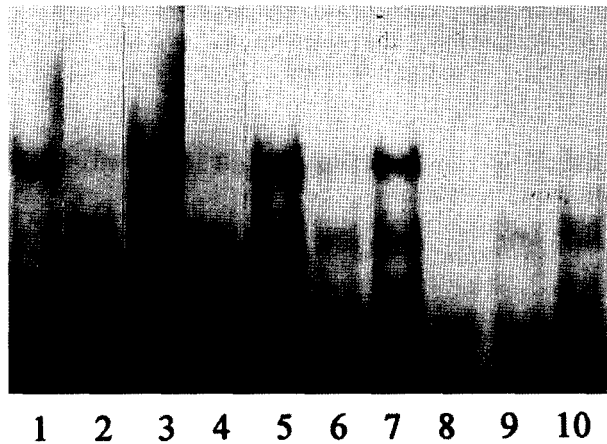


FIG. 3

Specific binding of human vascular endothelial cells (HUVECs) nuclear extracts to G/A polymorphism. G variant (lanes 1-4) and A variant oligonucleotide (lanes 5-8) incubated with non-induced HUVECs nuclear extracts without (lanes 1 and 5) and with 100-fold excess of unlabeled oligonucleotide (lanes 2 and 6), or incubated with lipopolysaccharide-induced HUVECs without (lane 3 and 7) and with 100-fold excess of unlabeled oligonucleotide (lane 4 and 8); A variant oligonucleotide competed with unlabeled G oligonucleotide variant, incubated with non-induced HUVECs nuclear extracts (lane 9); G variant oligonucleotide competed with unlabeled A oligonucleotide variant, incubated with non-induced HUVECs nuclear extracts (lane 10).

To investigate whether the G/A polymorphic site specifically binds to nuclear proteins electrophoretic mobility shift assays were performed with two double stranded oligonucleotides corresponding to the G and the A variant, using nuclear extracts from HUVECs. With both variants specific binding was observed. Binding activity was similar for non-induced and lipopolysaccharide-induced nuclear extracts and for the A and G variant. One major specific protein-DNA complex was formed. Competition reactions were performed with 100-fold excess of the unlabeled oligonucleotide for each type of the sequence. The strong reduction of the protein-DNA complexes in all competition reactions indicated specificity of binding. In addition, the formation of the complex with the A variant oligonucleotide could be competed with an excess of the G variant and vice versa, suggesting that similar proteins bind to both forms (Fig. 3).

## DISCUSSION

In this study a novel polymorphism in the promoter of the PAI-1 gene is described. This polymorphic site is located -844 bp upstream from the transcription start and represents a single guanine to adenine substitution (G/A polymorphism) according to the published sequence (17). The distribution of the G/A genotypes both in the patient and in the control group differed from

the distribution that would be expected from the Hardy-Weinberg equilibrium, with an over dominance of heterozygotes. The excess of heterozygotes might be due to the balancing selection which is one of the important factors for maintaining polymorphisms in natural populations. The novel G/A polymorphism was in strong linkage disequilibrium with the previously described 4G/5G polymorphism. Such allelic association was expected because of the vicinity of their chromosomal location. Presumably, the guanine to adenine substitution in the novel polymorphism happened in the distant past because all four possible haplotypes were present.

This study is to the best of our knowledge also the first report on the 4G/5G polymorphism in patients with DVT. It shows a relationship between the 4G/5G genotype and plasma PAI-1 activity levels and thus supports previous findings on such associations in patients after myocardial infarction (11-14) and in patients with non-insulin-dependent diabetes mellitus (15), with the highest PAI-1 levels in the 4G/4G genotypes and the lowest in the 5G/5G genotypes. In spite of a strong correlation between PAI activity and PAI-1 antigen levels in this study (data not shown) and approximately 40% higher PAI-1 antigen levels in patients with the 4G/4G genotype compared to the 5G/5G genotype (Table IV), relationship between the 4G/5G genotypes and PAI-1 antigen did not reach the level of statistical significance. This lack of significant association between the 4G/5G genotypes and PAI-1 antigen levels may be explained in part by different efficiencies with which various forms of PAI-1 (active, latent, complexed) are detected with the PAI-1 antigen assay and/or by contribution of latent platelet PAI-1 to PAI-1 antigen levels determined in plasma, possibly blurring the differences between the genotypes.

The same studies showing the relationship between the 4G/5G genotype and PAI-1 in patients (11, 12) failed to show such a relationship in control subjects. Similarly, no such association was established in healthy controls investigated in this study. The discrepancy between DVT patients and healthy controls could probably be explained either by a relatively low number of healthy individuals investigated (only 6 healthy controls had the 5G/5G genotype) and/or by different interactions between determinants of PAI-1 in plasma in patients and controls. It has been observed that the influence of triglyceride on PAI-1 seems to be genotype dependent (15, 16). Since patients studied had increased total- and LDL-cholesterol indicating disturbed lipoprotein metabolism, it might be speculated that interactions between genotype and metabolic factors affected plasma levels of PAI-1 in the patient but not in the control group.

In young patients after myocardial infarction the prevalence of the unfavorable 4G allele was higher than in population-based controls (13). Association between the 4G/4G genotype and coronary artery disease was observed also in non-insulin-dependent diabetes mellitus (22). However, in the present study distribution of the 4G/5G genotypes and prevalence of the 4G allele in patients was not different from the distribution and prevalence observed in healthy controls. Different genotypes were also not associated with the severity of the disease, since the distribution was different neither between patients with single or recurrent DVT nor between patients with idiopathic or secondary DVT.

The regulation of PAI-1 expression *in vivo* is complex. PAI-1 behaves as an acute phase reactant, and increasing plasma level are observed in response to inflammation and trauma. The polymorphism(s) in the promoter region could be envisaged to alter either the response of PAI-1 to a specific physiological regulator or the basal level of PAI-1. For the 4G/5G polymorphism in

vitro assays of PAI-1 gene promoter activity demonstrated significantly higher activity of the 4G than the 5G allele under conditions of cytokine stimulation, such as are found in the acute phase (12). On the other hand Eriksson and coworkers (13) observed in accordance with the previous study (12) sequence-specific binding to the 4G and 5G alleles of two factors present in a nuclear extract derived from liver, smooth muscle and endothelial cells. The 5G allele bound an additional factor to an overlapping binding site, which acted as a transcriptional repressor. This sequence-specific binding of nuclear proteins was observed in unstimulated cells indicating that the 4G/5G polymorphism influences the basal level of transcription. Our data seem to support the latter possibility since DVT patients investigated were relatively long after the acute phase of the disease and yet, increased PAI-1 activity was observed with increasing number of the 4G alleles.

Although no association was observed between the novel G/A polymorphism and plasma PAI-1 levels neither in patients nor in healthy controls, this finding does not exclude a possible role of this novel polymorphism in regulating plasma PAI-1 levels. An evidence suggesting functional importance was obtained by electrophoretic mobility shift assays, demonstrating specific binding of a nuclear protein to the G and the A allele. Similar binding was observed with nuclear extracts from unstimulated and stimulated HUVECs. In spite of the latter observation importance of the G/A polymorphic site in up-regulation of PAI-1 levels during the acute phase can not be excluded. It was concluded that this novel G/A polymorphic site might be similarly to the 4G/5G polymorphic site involved in promoter activity of the PAI-1 gene with presumably pathogenetic importance in thrombosis.

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#### REFERENCES

1. JOHNSON, O., MELLBRING, G., NILSSON, T. Defective fibrinolysis in survivors of myocardial infarction. *Int J Cardiol* 6, 380-382, 1984.
2. PARAMO, J.A., COLUCCI, M., COLLEN, D., VAN DE WERF, F. Plasminogen activator inhibitor in the blood of patients with coronary artery disease. *Br Med J Clin Res Ed* 291, 573-574, 1985.
3. AZNAR, J., ESTELLES, A., TORMO, G., SAPENA, P., TORMO, V., BLANCH, S., ESPANA, F. Plasminogen activator inhibitor activity and other fibrinolytic variables in patients with coronary artery disease. *Br Heart J* 59, 535-541, 1988.

4. JORGENSEN, M., BONNEVIE-NIELSEN, V. Increased concentration of the fast-acting plasminogen activator inhibitor in plasma associated with familial venous thrombosis. *Br J Haematol* 65, 175-180, 1987.
5. JUHAN-VAGUE, I., VALADIER, J., ALESSI, M.C., AILLAUD, M.F., ANSALDI, J., PHILIP-JOET, C., HOLVOET, P., SERRADIMIGNI, A., COLLEN, D. Deficient t-PA release and elevated PA inhibitor levels in patients with spontaneous or recurrent deep venous thrombosis. *Thromb Haemostas* 57, 67-72, 1987.
6. HAMSTEN, A., DE FAIRE, U., WALLDIUS, G., DAHLEN, G., SZAMOSI, A., LANDOU, C., BLÖMBACK, M., WIMAN, B. Plasminogen activator inhibitor in plasma: Risk factor for recurrent myocardial infarction. *Lancet* 2, 3-9, 1987.
7. SCHULMAN, S., WIMAN, B. AND THE DURATION OF ANTICOAGULATION (DURAC) TRIAL STUDY GROUP. The significance of hypofibrinolysis for the risk of recurrence of venous thromboembolism. *Thrombos Haemostas* 75, 607-611, 1996.
8. VAGUE, P., JUHAN-VAGUE, I., AILLAUD, M.F., BADIER, C., VIARD, R., ALESSI, M.C., COLLEN, D. Correlation between blood fibrinolytic activity, plasminogen activator inhibitor level, plasma insulin level, and relative body weight in normal and obese subjects. *Metabolism* 35, 250-253, 1986.
9. JUHAN-VAGUE, I., VAGUE, P., ALESSI, M.C., BADIER, C., VALADIER, J., AILLAUD, M.F., ATLAN, C. Relationships between plasma insulin, triglyceride, body mass index, and plasminogen activator inhibitor 1. *Diabete Metab* 13, 331-336, 1987.
10. JUHAN-VAGUE, I., THOMPSON, S.G., JESPERSEN, J., ON THE BEHALF OF THE ECAT ANGINA PECTORIS STUDY GROUP. Involvement of the hemostatic system in the insulin resistance syndrome, a study of 1500 patients with angina pectoris. *Arterioscler Throm* 13, 1865-1873, 1993.
11. DAWSON, S., HAMSTEN, A., WIMAN, B., HENNEY, A., HUMPHRIES, S. Genetic variation at the plasminogen activator inhibitor-1 locus is associated with altered levels of plasma plasminogen activator inhibitor-1 activity. *Arterioscler Thrombos* 11, 183-190, 1991.
12. DAWSON, S., WIMAN, B., HAMSTEN, A., GREEN, F., HUMPHRIES, S., HENNEY, A.M. The two allele sequences of a common polymorphism in the promoter of the plasminogen activator inhibitor-1 (PAI-1) gene respond differently to interleukin-1 in HepG2 cells. *J Biol Chem* 268, 10739-10745, 1993.
13. ERIKSSON, P., KALLIN, B., VAN'T HOOFT, F.M., BÅVENHOLM, P., HAMSTEN, A. Allele-specific increase in basal transcription of the plasminogen-activator inhibitor 1 gene is associated with myocardial infarction. *Proc Natl Acad Sci USA* 92, 1851-1855, 1995.
14. YE, S., GREEN, F.R., SCARABIN, P.Y., NICAUD, V., BARA, L., DAWSON, S.J., HUMPHRIES, S.E., EVANS, A., LUC, G., CAMBOU, J.P., ARVEILER, D., HENNEY, A.M., CAMBIEN, F. The 4G/5G genetic polymorphism in the promoter of the plasminogen activator inhibitor-1 (PAI-1) gene is associated with differences in plasma PAI-1 but not with risk of myocardial infarction in the ECTIM study. *Thrombos Haemostas* 74, 837-841, 1995.

15. PANAHLOO, A., MOHAMED-ALI, V., LANE, A., GREEN, F., HUMPHRIES, S.E., YUDKIN, J.S. Determinants of plasminogen activator inhibitor 1 activity in treated NIDDM and its relation to a polymorphism in the plasminogen activator inhibitor 1 gene. *Diabetes* 44, 37-42, 1995.
16. MANSFIELD, M.W., STICKLAND, M.H., GRANT, P.J. Environmental and genetic factors in relation to elevated circulating levels of plasminogen activator inhibitor-1 in caucasian patients with non-insulin-dependent diabetes mellitus. *Thrombos Haemostas* 74, 842-847, 1995.
17. BOSMA, P.J., VAN DEN BERG, E., KOOISTRA, T. Human plasminogen activator inhibitor-1 gene. Promoter and structural gene nucleotide sequences. *J Biol Chem* 263, 9129-9141, 1988.
18. ORITA, M., IWAHANA, H., KANAZAWA, H., HAYASHI, K., SEKIYA, T. Detection of polymorphisms of human DNA by gel electrophoresis as single-strand conformation polymorphisms. *Proc Natl Acad Sci* 86, 2766-2770, 1989.
19. BASSAM, B.J., CAETANO-ANOLLES, G., GRESHOFF, P.M. Fast and sensitive silver staining of DNA in polyacrylamide gels. *Anal Biochem* 196: 80-83, 1991.
20. DIGNAM, J.D., LEBOVITZ, R.M., ROEDER, R.G. Accurate transcription initiation by RNA polymerase II in a soluble extract from isolated mammalian nuclei. *Nucleic Acids Res* 11, 1475-1489, 1983.
21. RAYMOND, M., ROUSSET, F. Genepop (Version 1.2): Population genetics software for exact tests and ecumenicism. *J Heredity* 86, 248-249, 1995.
22. MANSFIELD, M.W., STICKLAND, M.H., GRANT, P.J. Plasminogen activator inhibitor-1 (PAI-1) promoter polymorphism and coronary artery disease in non-insulin-dependent diabetes. *Thromb Haemostas* 74, 1032-1034, 1995.