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Residual vein thrombosis and onset of post-thrombotic syndrome: Influence of the 4G/5G polymorphism of plasminogen activator inhibitor-1 gene

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ABSTRACT

Background: Plasminogen activator inhibitor-1 (PAI-1) is the most important inhibitor of plasminogen activator. The functional 4G/5G polymorphism of the gene coding for PAI-1 may affect PAI-1 plasmatic activity, influencing the imbalance between coagulation and fibrinolysis cascades.
In this prospective cohort analytic study, we investigated the role of this single nucleotide polymorphism in the persistence of thrombotic lesion and the occurrence of post-thrombotic syndrome.
Patients/Methods: In a group of 168 patients with post-surgical deep vein thrombosis of the legs, we analyzed the 4G/5G polymorphism in the promoter of PAI-1 gene and plasmatic PAI-1 activity.
Enrolled patients were divided in two groups: patients with 4G/5G polymorphism and increased PAI-1 activity (n = 85) and patients without 4G/5G polymorphism and normal PAI-1 activity (n = 83). All patients were treated according to current protocols and re-examined after 3, 12 and 36 months in order to evaluate the persistence of thrombotic lesion and the occurrence of post-thrombotic syndrome.
Results: We found a significantly increased PAI activity in carrier of the 4G allele, who experienced much more frequently a persistence of thrombosis after 3, 12 and 36 months and/or the development of post-thrombosis syndrome, in spite of the anticoagulant treatment.
Conclusions: These data not only confirm the role played by PAI-1 activity and by the 4G/5G SNP of the PAI-1 gene, but also suggest that current therapeutic protocols, recommending the administration of low weight molecular heparin and oral anticoagulant for the treatment of deep vein thrombosis, could be non sufficient for patients genetically predisposed to a less efficient clot lysis.

Introduction

Plasminogen activator inhibitor-1 (PAI-1) is an acute phase protein that is usually only expressed in adipocytes and endothelial cells, but it can be highly expressed by most cells in response to stress [1]. It reacts with single and two-chain t-PA and with two-chain u-PA inhibiting target proteinase by formation of a 1:1 stechiometric reversible complex; the rapid inhibition of both t-PA and u-PA involves a reversible high-affinity between three negatively charged amino acids of PAI-1 and highly positively charged regions in t-PA or in u-PA, binding to fibrin and inhibiting t-PA-mediated fibrin dissolution. [2].

A functional polymorphism has been identified in the promoter region of the gene coding for PAI-1 (the 4G/5G polymorphism) at the -675 position. This variant affects plasma concentrations of PAI-1, influencing the imbalance between coagulation and fibrinolysis cascades. The 4G allele is in fact associated with an increased transcription of the gene and, subsequently, with increased levels of PAI-1 [3,4]. The presence of the 4G allele, both in the heterozygotic and, especially, in the homozygotic state, can induce a reduced fibrinolytic activity, associated with thrombotic disorders in experimental models [5]. Discordant studies have been recently published regarding the relationship between this polymorphism and the risk of arterial and/or venous thromboembolism in human, despite the results obtained in experimental models [6–19].

Previously we have studied the extension of thrombotic lesions from calf veins to popliteal and femoral veins in a group of patients with vein thrombosis and found that carriers of the 4G allele experienced a more marked extension than non carriers. In the group of patients with more extensive thrombosis we also observed a reduced fibrinolytic plasma activity [20]. In this research we evaluated whether the presence of

Abbreviations: CEAP, clinical severity, etiology, anatomy, patophysiology; DVT, deep vein thrombosis; INR, international normalized ratio; OR, odds ratio; PAI-1, plasminogen activator inhibitor-1; PTS, post-thrombotic syndrome; SD, standard deviation; t-PA, tissue-type plasminogen activator; u-PA, urokinase-type plasminogen activator.

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Table 1
Baseline characteristics of carriers of the 4G allele by the advanced CEAP Classification.

Patients (n)				
12	C3s	Ep	Ad	Po11,14
12	C3s	Ep	Ad	Po 13,14
10	C3s	Ep	Ad	Po11,13,14
10	C0s	Ep	Ad	Po13,14
11	C0s	Ep	Ad	Po11,14
12	C0s	Ep	Ad	Po12,14
8	C1s	Ep	Ad	Po11,14
10	C1s	Ep	Ad	Po13,14

Table 3
Characteristics of carriers of the 4G allele by the advanced CEAP Classification at the end of the study.

Patients (n)				
11	C4aS	Es	Ad	Po11
11	C4aS	Es	Ad	Po13
9	C4aS	Es	Ad	Po11,14
10	C4aS	Es	Ad	Po13,14
10	C4bS	Es	Ad	Po11
10	C4bS	Es	Ad	Po12,14
11	C1a	Es	Ad	Pr11
13	C1a	En	An	Pn13,14

the 4G/5G polymorphism would influence the persistence of thrombotic lesion and the occurrence of post-thrombotic syndrome in a group of patients with post-surgical deep vein thrombosis in a prospective 36 months follow-up study.

Subjects and methods

From a total number of 1867 patients referred to the Department of Angiology of the University Hospital of Palermo for the suspicion of a deep vein thrombosis (DVT), we selected and enrolled, according to the inclusion and exclusion criteria of the protocol, 168 consecutive patients with post-surgical deep vein thrombosis (DVT) of the legs documented by echo-Doppler.

The inclusion criteria were: presence of post-surgical thrombosis of femoral and/or popliteal veins and age between 45 and 70 years.

The exclusion criteria were: presence of a cancer, malignant haematological diseases, hyperhomocysteinemia, hyperviscosity syndromes or the presence of antiphospholipid antibodies, the presence of factor V Leiden or prothrombin 20210 G-A mutation and a clinical history of previous episodes of deep-venous thrombosis or of pulmonary embolism. The presence of cancer was ruled-out by tumour markers dosage. Hyperhomocysteinemia was diagnosed by the Medical Systems Immulite and Immulite 1000 analyzers DPC – Diagnostic Products Corporation USA. The Brookfield viscosimeter at high and low shear rates was used to exclude haemorheological disorders such as blood hyperscosity. Using the Elisa test kit that provides a quantitative in vitro assay for human autoantibodies of the IgM or IgG class against cardiolipin in serum, we ruled out patients with antiphospholipid antibodies: the upper limit of the normal range (cut-off) was 12 Phospholipid-IgG or IgM-units/ml. Lastly, the presence of the Factor V Leiden and the G20210A mutation for the protrombin gene was excluded by means of the same method used for 4G/5G polymorphism.

In the enrolled patients we analyzed the PAI-1 promoter polymorphism 4G/5G using Nuclear Laser Medicine methods [21,22], which included three steps: DNA isolation from fresh or frozen blood with ethylenediaminetetracetate or citrate anticoagulant; PCR amplification using an Amplification Mix containing biotinylated primers added to Taq DNA Polymerase (1U) and DNA template (25-200 ng); hybridization of amplification products with a test strip containing allele-specific oligonucleotide probes immobilized as an array of parallel lines. The tests strips were inserted and incubated for 30 min at 45 °C in a water-bath with shaking platform and subsequently two stringent washes at 45 °C were performed. For colour development 1 ml of conjugate solution containing streptavidin-alkaline phosphatase was added to the strip test and incubated in an orbital shaker at room temperature for 15 min. Each strip was added to 1 ml of colour developer. After an incubation period of 15 min. at room temperature in the dark, the genotype of the sample was determined.

We also studied plasmatic PAI-1 activity in all patients using the Berichrom PAI method – Dade Behring Inc. Newark U.S.A. The principle of this method is based on the deactivation of urokinase by PAI-1 contained in plasma. The remaining urokinase activity is determined by the conversion of plasminogen into plasmin. The resulting plasmin is measured by the segregation of a chromogen substrate at 405 nm (concentration through plasminogenolysis). The destructive alfa2-antiplasmin is deactivated through oxidation with chloramine T. In healthy adults PAI-1 activity lay between 0.3 and 3.5 U/ml.

Patients with DVT were then divided in two different groups:

1. patients with 4G/5G polymorphism and increased PAI-1 activity ($n = 85$, 48 males and 37 females), aged between 49 and 65 years (mean age 56.96 ± 4.70 years);
2. patients without 4G/5G polymorphism and normal PAI-1 activity ($n = 83$, 46 males and 34 females), aged between 47 and 68 years (mean age 57.10 ± 5.54 years).

All the enrolled patients were treated daily with nadroparin calcium anti-Xa at a dosage of 90 IU/kg body weight, given in two administrations daily [23–25]. At the same time, sodium warfarin was initiated and nadroparin administrated until the INR had stabilized (between 2 and 3). Heparin treatment was stopped in all patients after 5 or 6 days. The sodium warfarin treatment was prolonged for six months.

The adopted procedures were in agreement with the Helsinki Declaration of 1975 as revised in 1983 and were approved by the Ethic Council of the Department of Internal Medicine of the University of Palermo. All subjects gave their informed consent to participate to the study.

We performed a 36 months follow up, in all patients to evaluate the persistence of thrombotic lesion (after 3 and 12 months) and the occurrence of post-thrombotic syndrome (after 36 months). The persistence of thrombotic lesion and the occurrence of post thrombotic syndrome was evaluated by B-mode real-time echo-color-doppler with a machine ATL HDI 1500, Madison Co Ltd, Korea at three and twelve months and at the end of the follow-up. Ultrasound examination was performed by

Table 2
Baseline characteristics of non carriers of the 4G allele by the advanced CEAP Classification.

Patients (n)				
12	C3s	Ep	Ad	Po11,14
11	C3s	Ep	Ad	Po13,14
10	C3s	Ep	Ad	Po11,13,14
11	C0s	Ep	Ad	Po13,14
10	C0s	Ep	Ad	Po11,14
10	C0s	Ep	Ad	Po12,14
10	C1s	Ep	Ad	Po11,14
9	C1s	Ep	Ad	Po13,14

Table 4
Characteristics of non carriers of the 4G allel by the advanced CEAP Classification at the end of the study.

Patients (n)				
4	C4aS	Es	Ad	Po11,14
6	C4aS	Es	Ad	Po13
2	C4bS	Es	Ad	Po13,14
2	C4bS	Es	Ad	Po13
12	C1a	Ep	Ad	Pr11
13	C1a	Ep	Ad	Pr12
11	C1a	En	An	Pn11,14
33	C1a	En	An	Pn13,14

Table 5
Characteristics of the study population subdivided according to the genotype and persistence of thrombosis during the follow-up.

	4G/4G (n 4)	4G/5G (n 81)	5G/5G (n 83)	p
Age (years)	61,2 ± 2,9	56,7 ± 4,7	57,1 ± 5,5	ns
Men/Women (n)	1/3	47/34	46/37	ns
Persistence after 3 months (%)	100	75,3	47	< 0,001
Persistence after 12 months (%)	100	71,6	21,7	<0,001
Persistence and development of PTS (%) after 36 months (%)	100	70,4	16,9	<0,001

one investigator in a room at 22-25 ° C, with the patient in the supine position and at a 45° angle. For each examined vein a compression was performed by the probe, registering flow changes. In order to check the persistence of thrombotic lesion, the primary end-point was the absence of compressibility of the vein wall, while the secondary end-points were flow velocity and colour distribution variations. We also measured venous pressure in orthostatic position. During the follow-up, patients were considered positive for persistence of thrombosis when they presented complete or partial thrombotic occlusion in femoral or popliteal veins after 3 and 12 months. In order to evaluate residual vein thrombosis, we measured and recorded major and minor diameters of the venous segments before and after compression. Ultra-sonography findings were arbitrarily scored as normalized when residual thrombus occupied, after compression, less than 40% of the vein area calculated in the absence of compression, utilizing the same methodology used in previously published studies [26,27].

Moreover patients were considered positive for post-thrombotic syndrome when they presented persistence of thrombosis and a vein pressure value, in the supine position, > 90 mm Hg determined by echocolordoppler at the level of posterior tibial vein associated with pigmentation, varicose eczema or lipodermatosclerosis and atrophie blanche. We determined the vein pressure by a tensiometric cuff applied around the leg; we inflated the cuff until the 110 mm Hg, then we deflated the cuff: the vein pressure value were relieved when we listened to a sound as “a wind breath”.

Statistical analysis

Statistical analyses were performed using the SYSTAT DATA software package, version 12 (Systat, San Jose, CA, USA). Continuous variables normally distribuited were reported as means ± SD. Comparison between means were done using Student's t-test for unpaired data, whereas comparison between frequencies were done using Chi-square test. To investigate the association between the 4G/5G mutation and persistence of thrombosis and/or development of post-thrombotic syndrome logistic regression analysis was performed by calculating odd ratios (OR) and 95% confidence limits. The null hypothesis was rejected at a two-tailed <0.05.

Results

Clinical characteristics of carriers and non carriers of the 4G allele at the baseline of the study are shown in Tables 1 and 2. In the selected population, composed 168 consecutive patients with post-surgical DVT, 4 subjects were homozygous for the 4G allele, 81 were carrier of the 4G/5G genotype and 83 of the 5G/5G genotype. After three months, all patients who were homozygous for the 4G allele (100%) and 61 of those who were eterozygous (75.3%) had a persistence of deep vein thrombosis while among carrier of the 5G/5G genotype the frequency of persistence was significantly lower (39 patients, 47%; p <0.001). This trend was confirmed even in long term follow-up: 100% (n = 4) of 4G/4G carriers and 71.6% (n = 58) of 4G/5G carries presented a persistence of thrombosis after 12 months as well as 100% (n = 4) and 70.4% (n = 57) presented a persistence of thrombosis and a post-thrombotic syndrome at the end of the study (after 36 months) compared with the 21.7% (n = 18) and 16.9% (n = 14) of homozygous for the wild type allele, respectively (p < 0.001 for both groups). The clinical and anatomic sign's changes between baseline and 36 months follow-up are shown in Tables 3 and 4, respectively for carriers and non carriers of the 4G allele.

Data about genotype distribution and persistence of thrombosis after 3, 12 and 36 months and the development of post-thrombotic syndrome are listed in Table 5.

Comparing PAI activity at baseline and after 3, 12 and 36 months follow-up, a significant difference was found between groups, being PAI activity much more enhanced in carries of the 4G than 5G allele (p < 0.001, Table 6).

Our results showed that 4G/5G polymorphism, causing an increased PAI-1 activity, could have a very strong and significant influence on the persistence of thrombosis after short-term analysis and is associated with an elevated incidence of post-thrombotic syndrome in the long term follow-up.

Discussion

A decreased fibrinolytic activity has been reported in several thrombotic diseases, including arterial and venous thrombosis [28–31]. This decrease has been related with increased levels of PAI activity and with the presence of the 4G allele in young subjects with myocardial infarction [3]. This polymorphism was thus considered a risk factor for thrombotic disorders, even if these data have not been confirmed in other studies [32].

Several surveys have suggested that increased in vivo levels of PAI-1 promote fibrin deposition. This deposition, associated with increased PAI-1 expression, was observed in the aging-process and may contribute to the development of thrombosis. Sartori [33] and Stegnar [4] demonstrated a PAI-1 induced hypofibrinolysis, related to the presence of the 4G allele among patients with deep vein thrombosis. To date, there are no studies demonstrating a correlation between DVT onset and persistence, 4G/5G polymorphism and a fibrinolytic activity decrease, in patients with increased levels of PAI-1.

Moreover, the role of the 4G/5G polymorphism in the pathogenesis of DVT is not universally recognized. In a previous study we hypothesized that the progression of thrombosis may be partially associated

Table 6
PAI-1 activity distribution in the different genotypes.

	4G/4G (n 4)	4G/5G (n 81)	5G/5G (n 83)	p
PAI-1 at baseline (U/ml)	5,22 ± 0,08	4,12 ± 0,89	1,29 ± 0,66	< 0,001
PAI-1 after 3 months (U/ml)	5,12 ± 0,14	4,17 ± 0,89	1,29 ± 0,65	<0,001
PAI-1 after 12 months (U/ml)	5,10 ± 0,13	4,19 ± 0,91	1,30 ± 0,65	<0,001
PAI-1 after 36 months (U/ml)	4,99 ± 0,11	4,17 ± 0,93	1,31 ± 0,65	<0,001

with a decreased fibrinolytic activity related with the 4G/5G polymorphism and we showed an overall incidence of thrombosis progression higher than other studies in which patients were not assessed for the genetic profile [20].

Taking into account that we excluded from this study patients with the presence of factor V Leiden and/or G20210A prothrombin mutation, we think that the persistence of thrombosis and the onset of post-thrombotic syndrome could also be related to the reduced fibrinolytic activity related to the presence of 4G/5G polymorphism and to the increase in PAI-1 activity. Moreover, in the presence of thrombotic vein occlusion, the flow velocity is very low near to the thrombus. We believe that this very low velocity might favour the persistence and progression of thrombotic lesions, especially in carriers of the 4G allele, because of a reduced fibrinolytic activity. However, our data cannot be considered exhaustive because of the limited number of cases involved. Nevertheless, if these results will be confirmed by further studies, they may have clinical practice implications for the management and treatment of venous thrombosis in carriers of the 4G polymorphism. It should be in fact underlined that the 4G/5G polymorphism is not rare in the general population, with a frequency of the heterozygous genotype approaching the normal genotype one, both in our survey and in several published researches [20,34,35]. The use of the oral anticoagulant seems to be inadequate in preventing the progression of thrombosis in this population. For these reasons further preventive and therapeutic strategies should be identified for the management of patients at high risk of developing thrombosis and post-thrombotic syndrome and a phamacogenetic approach could be proposed and should be validated by large-scale studies.

Conflict of Interest Statement

No conflict of interest to declare.

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