Determinants of Plasma Levels of Plasminogen Activator Inhibitor-1
A Study of Normotensive Twins

Maurizio Cesari, Maria Teresa Sartori, Giovanni M. Patrassi, Silvia Vettore, Gian Paolo Rossi

Abstract—We investigated whether plasma levels of the plasminogen activator inhibitor type 1 antigen (PAI-1:Ag) are genetically determined in monozygotic (MZ) and dizygotic (DZ) twins. Twenty-five pairs of healthy twins underwent measurements of PAI-1:Ag and other variables, including body mass index, mean blood pressure, plasma renin activity, insulin, and glucose. To ascertain the zygosity of twins, highly discriminating micro- and minisatellite systems with variable numbers of tandem repeats were analyzed by PCR amplification followed by polyacrylamide gel electrophoresis. Subjects were also genotyped for the 4G/5G polymorphism by PCR. Estimates of genetic variance and heritability were obtained for PAI-1:Ag, and for body mass index, mean blood pressure, plasma renin activity, glucose, and insulin by jointly examining data in a path analysis with TWINAN90. Results showed that 12 pairs of twins were MZ and 13 were DZ. All tests of genetic variance [within pair (WP): F = 6.24, P = 0.002; among component (AC): F = 2.62, P = 0.04; average absolute difference t test = 3.00, P = 0.004] showed significant genetic variance of PAI-1:Ag, but not of the other variables. Three tests of heritability (WP = 0.837, P = 0.002; AC = 1.791, P < 0.05; intraclass correlation: 1.180, P = 0.001) consistently showed significant PAI-1:Ag heritability. Additive genetic influences (A), dominance genetic effect (D), and random environmental influences (E) accounted for 0.714, 0.154, and 0.132 of PAI-1:Ag variance, respectively. No effect of different 4G/5G genotypes was found. Thus, these results show significant genetic variance and heritability of PAI-1:Ag and suggest that A is more important than both D and E in determining PAI-1:Ag variance. (Arterioscler Thromb Vasc Biol. 1999;19:316-320.)

Key Words: plasminogen activator inhibitor type 1 ■ fibrinolysis ■ gene polymorphism ■ twins ■ humans

Plasminogen activator inhibitor type 1 (PAI-1) is the key regulator of the activity of the fibrinolytic system, an important protective mechanism against thrombosis. Reduced fibrinolytic activity, mainly caused by increased plasma levels of PAI-1, is a common finding in many cross-sectional studies of patients with coronary artery disease (CAD) and recurrent myocardial infarction (MI), deep vein thrombosis, and stroke. Although extensive investigations have led to identification of the environmental factors regulating PAI-1:Ag and PAI-1 activity (PAI-1:act) levels in plasma (see for review), only limited information exists on their genetic determinants. To our knowledge the following 8 polymorphisms of the PAI-1 gene have been identified: two (CA)ₙ repeat polymorphisms, a HindIII restriction fragment length polymorphism, an insertion (5G)/deletion (4G) polymorphism on the promoter, ie, −675 bp upstream from the start of transcription of the PAI-1 gene, and 4 polymorphisms in the PAI-1 gene by use of the single-strand conformational polymorphism (SSCP) method. A major effect of the 4G/5G polymorphism on PAI-1:act was reported, but conflicting results are also available. In fact, a relationship between both PAI-1 antigen and activity levels and the 4G/5G polymorphism (the 4G homozygous having the highest mean plasma PAI-1 levels) has been shown in patients with CAD, non-insulin-dependent diabetes mellitus (NIDDM), and healthy control subjects. However, in contrast with these findings no significant association was found between both PAI-1 antigen and activity levels and the 4G/5G polymorphism or the HindIII RFLP in 189 patients with NIDDM, and between PAI-1:act levels and the 4G/5G polymorphism and four polymorphisms identified in the PAI-1 gene by use of the SSCP method, in healthy men aged 50 to 59 years. Thus, the issue of whether plasma PAI-1:act and/or plasma PAI-1:Ag are under the effect of a major gene is still under debate.

Studies of twins are increasingly popular in molecular genetics, because the comparison of monozygotic (MZ) and dizygotic (DZ) twins offers a unique and powerful method of partitioning genetic and environmental sources of covariance of quantitative traits which may be relevant for late-onset diseases, such as atherosclerosis, hypertension, and related cardiovascular disease. They can also provide estimates of the additive (A) genetic effects, which combine independently over different alleles at the same genetic locus and

Received April 27, 1998; accepted July 1, 1998.
From the Department of Clinical and Experimental Medicine, Clinica Medica 4 (M.C., G.P.R.) and Institute of Medical Semiotics, II Chair of Internal Medicine, University of Padua Medical School (M.T.S., G.M.P., S.V.), Italy.
Correspondence to G.P. Rossi, MD, FACC, Clinica Medica 4, Department of Clinical and Experimental Medicine, University Hospital, Via Giustiniani, 2, 35126 Padova, Italy. E-mail gprossi@ux1.unipd.it
© 1999 American Heart Association, Inc.
Arterioscler Thromb Vasc Biol. is available at http://www.atvbaha.org
over different loci, dominant (D) effect, ie, allelic interactions at the same locus, the environmental effects common to cotwins (C, ie, shared family environment), and the environmental (E) variance unique to individuals on the variance of quantitative traits such as the components of the clotting cascade. Therefore, this study was undertaken to investigate the genetic and environmental components of the plasma levels of PAI-1 in identical and fraternal normotensive twins.

Subjects and Methods

Twins

The twins investigated in this study were enrolled from an Italian association of twins based in Padua, Italy. To ascertain whether twins were MZ or DZ, some highly discriminating micro- and minisatellite systems with a variable number of tandem repeats were analyzed. Three amplified fragment-length polymorphisms (ApoB, D1S80, and YNZ22) and 4 short tandem repeats (UMACTPB2, HUMTH01, HUMFES/FP5, and HUMMVWA31) were assessed by separate PCR amplifications of genomic DNA followed by polyacrylamide gel electrophoresis and silver staining. No subject was smoking or taking any drugs or medications known to affect the fibrinolytic tests for at least 15 days before the study. None participated in any competitive sports training or reported daily intake of alcohol. All subjects were healthy and showed normal fasting levels of serum glucose and insulin. Blood pressure (BP) was measured with a mercury sphygmomanometer using Korotkoff phase V for diastolic and according to the WHO guidelines. All gave an informed written consent, and the Institutional Human Subjects Review Committees of the University of Padua Medical School approved the study protocol.

Blood Sampling and Biochemical Measurements

Blood was collected after an overnight fast and 15-minute supine rest without stasis from an antecubital vein. Five milliliters of whole blood with 100 μL Na2EDTA 6% was immediately put on ice. After centrifugation at 3000 g (at 4°C for 10 minutes), separate aliquots of plasma and buffy coat were stored at −40°C and tested within 1 month. Plasma renin activity was measured by a commercially specific PCR following the procedure described by Falk et al.19 with minor modifications. In particular, allele-specific oligonucleotides (PAI 2d: 5′-TGCAGCCAGCCACGTGATTGTCTAG-3′, PAI 4G) coupled with a common upstream primer (PAI 1u: 5′-ACGTGGGG-3′) were used. A common upstream primer (PAI 1u: 5′-ACGTGGGG-3′) was used to generate a positive control in each PCR. The reaction was performed in a total volume of 25 μL with 3 μL of extracted DNA. The mixture contained 50 mmol/L KCl, 10 mmol/L Tris-HCl (pH 9), 2.5 mmol/L MgCl₂, 95 mmol/L dNTPs, 3 pmol primer PAI 1u, 25 pmol PAI 2d, and 50 pmol PAI 4G or PAI 5G. For each PCR, 1.5 U Taq DNA polymerase (Pharmacia Biotech, Uppsala, Sweden) was used after a hot start. The mixture was subjected to the following cycling steps: 94°C, 5 minutes for 1 cycle; 94°C, 1 minute; 58°C, 2 minutes for 5 cycles; 94°C, 1 minute, 65°C, 45 seconds, 72°C, 1 minute 15 seconds for 25 cycles; 65°C, 5 minutes for 1 cycle. The PCR products were run in TBE 0.5× buffer in a 4% agarose gel stained with ethidium bromide.

Statistical Analysis

Results are expressed as mean±SD, or SEM, as appropriate.20 Comparison of subjects of the different genotypes was carried out with Kruskall–Wallis test for plasma renin activity, or with a 1-way ANOVA followed by Bonferroni’s multiple comparison test for the variables that showed a normal distribution. Because subjects in each twin pair could not be regarded as independent unrelated individuals, either twin 1 or twin 2 of each pair was considered for statistical comparison. Analysis was carried out with the SPSS for Windows™ statistical package (version 7.5, SPSS Inc, Chicago, Ill). A P value <0.05 was considered statistically significant. Analysis of twin data was carried out jointly with TWINAN90 (a generous gift of Prof. C.J. Williams), a program specifically developed for conducting analyses of twin data.21 To this end the consistency of the variable of interest with the normal distribution assumption and with the hypothesis of equal variance between zygosity was verified beforehand. Estimates of genetic variance, including the within-pair (WP) and among components (AC), were attained thereafter, and a test for genetic variance based on the average absolute difference between twins,22 which is standardized to yield an approximate t test for the null hypothesis of no genetic variance, was performed. Three estimates of heritability were also calculated; the first two were derived from the WP and AC estimates of genetic variance, respectively, according to the following equations:

\[
WP = \frac{2 \times (S_{MZ} - S_{DZ})/(S_{MZ} + S_{DZ})/4}{ac = (2AC)/(S_{MZ} + S_{DZ})/4}.
\]

The third estimate of heritability (h²) is based on intrapair correlation coefficients calculated from the MZ and DZ twins as follows: h² = 2(rMZ−rDZ)/(1−rMZ).

This approach allows the determination of the proportion of variance of PAI-1:Ag accounted for by an additive genetic influence (A) which combines independently over different alleles at the same genetic locus and over different loci, nonadditive genetic influences (D) (ie, allelic interactions at the same locus), environmental influences shared by cotwins within a family (C) and unique to individuals (E).21 This ANOVA-based method was chosen because it was more accurate than path-maximum-likelihood methods to estimate and test the significance of A because it does not require the assumption that C = 0.22 Finally, the results of the likelihood-based analyses were determined for models with A, D, and E (ADE); A, C, and E (ACE); A and E (AE); C and E (CE), and E.

This type of analysis was carried out also for body mass index (BMI), mean blood pressure (MBP), glycemia, and plasma fasting insulin.

Results

After ascertainment of zygosity, 12 twins were determined to be MZ and 13 DZ. The observed 4G/5G PAI-1 genotype distribution was in agreement with the Hardy–Weinberg proportion (4/4 = 0.14, 4/5 = 0.54, 5/5 = 0.32) in the whole population, as well as in the MZ and DZ subgroups (all P = NS by χ² test). Their demographic and biochemical characteristics, including PAI-1:Ag levels, are shown in Table 1. No significant difference between MZ and DZ twins and among different 4G/5G PAI-1 genotypes in all variables of interest was found (Table 1).

Joint analysis of the MZ and DZ twins data was carried out for the variable PAI-1:Ag with TWINAN90.21 A Kolmogorov–Smirnov goodness-of-fit test applied separately to the grouped pairs for each of the two zygosities confirmed that PAI-1:Ag levels followed a normal distribution (P > 0.15 for both zygosities), and therefore no transformation of the data was needed. The null hypothesis that the mean of the MZ and DZ twins absolute difference did not differ from zero was thereafter accepted (t = 1.62, with 18.7 degree of freedom, NS) and the equal variance hypothesis was not rejected (F = 0.61, NS). Estimates of...
genetic variance and intraclass correlation coefficients for both MZ and DZ twins were obtained, and a test for genetic variance was performed (Table 2). Both the preferred tests of genetic variance WP and average absolute difference tests\textsuperscript{21} and also the AC estimate resulted in being statistically significant. The three estimates of heritability were also calculated (Table 2), and the results were significant, thereby leading to acceptance of the hypothesis that PAI-1:Ag levels are heritable.\textsuperscript{21}

The relationship between PAI-1:Ag in each pair of identical and fraternal twins is shown as a scatter plot (Figure 1). A higher correlation coefficient was observed in MZ (\(r = 0.935, P < 0.0001\)) than in DZ (\(r = 0.345, \text{NS}\)) twins. The twin pair difference in PAI-1:Ag in MZ, and in DZ discordant and discordant for the 4G/5G genotype was also examined (Figure 2). To further investigate the components of PAI-1:Ag variance, the results of the likelihood-based analyses for different models (ADE, ACE, and the AE, CE, and E) were determined. Since the correlation coefficient of DZ twins was less than half of that of MZ twins the ADE was the preferred model (Table 2). The latter indicated that the larger proportion of PAI-1:Ag variance was caused by A, whereas smaller proportions were accounted for by D and E.

The same statistical analysis applied to the same data set did not reveal any evidence of genetic variance and heritability for serum glucose, insulin, and mean blood pressure. Statistically significant correlation coefficients for mean blood pressure and serum glucose, both in MZ and DZ twins, and in either MZ or DZ twins (for body mass index and serum insulin, respectively) were found. However, all tests of genetic variance and heritability were not statistically significant for serum glucose, insulin, and mean blood pressure. Only BMI showed a statistically significant AC genetic variance and heritability (\(P = 0.011\)) and a borderline significant intraclass correlation coefficient (\(P = 0.042\)).

**Discussion**

In this study, to gain insight into the relative effects of genes and environment on PAI-1:Ag, we compared the plasma levels of PAI-1:Ag of fraternal (DZ) and identical (MZ) twins selected from a Caucasian population, who were young, healthy, and normotensive and had normal plasma levels of PAI-1:Ag. Their zygosity status was carefully established both at the phenotypic and at the molecular level by assessing some highly discriminating variable number of tandem repeats micro- and minisatellite systems. In addition utmost care was also taken to ensure an accurate measurement of PAI-1:Ag both during sample collection and at assay. Data were analyzed with a strategy selected because it takes into account:

| TABLE 2. Output of Path Analysis Carried Out With TWINAN90 Jointly on MZ and DZ Twins to Assess the Genetic Variance and Heritability of PAI-1:Ag |
|--------------------------|-----------------|-----------------|-----------------|-----------------|
| PAI-1:Ag                  | Estimates       | Variance        | F Ratio         | P Value         |
| Genetic variance          |                 |                 |                 |                 |
| WP                       | 63.50           | 783.6           | 6.24            | 0.002           |
| AC                       | 135.9           | 24 583.5        | 2.62            | 0.040           |
| Intrapair correlations    |                 |                 |                 |                 |
| MZ                       | 0.935           | <0.0001         |                 |                 |
| DZ                       | 0.345           | 0.106           |                 |                 |
| Average absolute difference | 3.00             | 17.3            | 0.004           |                 |
| t test                   |                 |                 |                 |                 |
| Heritability              |                 |                 |                 |                 |
| WP                       | 0.837           | 0.002           |                 |                 |
| AC                       | 1.791           | 0.040           |                 |                 |
| Intrapair correlations    |                 |                 |                 |                 |
| MZ                       | 1.180           | 0.001           |                 |                 |
| DZ                       | 0.657           | 0.132           |                 |                 |

*Figure 1.* Scatter plot of WP plasma levels of PAI-1:Ag with regression lines and 95% confidence intervals. MZ (closed circles) and DZ (open circles) twins are shown. The correlation was significant (\(P < 0.001\)) in MZ but not in DZ twins.
consideration the contribution of shared environment and therefore allows investigation of the proportion of variance of PAI-1:Ag accounted for by an additive genetic influence (A), nonadditive genetic influences (D) (ie, allelic interactions at the same locus), environmental influences shared by cotwins within a family (common, C) and unique to individuals (E). At variance with a recent study of Swedish twins investigating the genetic influences of PAI-1:act with a multivariate genetic technique (LISREL),23 we used TWINAN90, an ANOVA-based method specifically developed for conducting analyses of twin data by Williams et al,21 because this approach was found to be more accurate than path-maximum-likelihood methods for estimating and testing the significance of A.22

By using this strategy, we found strong evidence of genetic variance and heritability of PAI-1:Ag in these subjects (Table 2). When phenotypic covariance was partitioned into additive genetic effects (A), dominant genetic components (D), environmental effects common to cotwins (C), and error variance (E) components, the estimates indicated that more than 85% was accounted for by A and D (Table 2), thereby suggesting that most of PAI-1:Ag variance is genetically determined. This contention is also supported by the finding of high and statistically significant correlation coefficients only in MZ and not in DZ twins. These results concerning PAI-1:Ag are in sharp contrast with those concerning other demographic, biochemical, and hemodynamic variables, which are of potential interest for cardiovascular risk, such as fasting serum glucose, insulin, and mean blood pressure.

In a recent study of white nuclear families from a healthy French population, variables related to the insulin resistance syndrome were found to explain a major part of PAI-1 variance (49% in fathers and 29% in mothers), whereas five PAI-1 gene polymorphisms accounted for a negligible proportion (3% in women and none in men) of PAI-1 variability.24 These findings differed from those of a Swedish study of twins in which the proportion of PAI-1:act levels accounted for by heritability and individual-specific environmental factors was 42% and 36%, respectively.23 Although these differences could be caused by several methodological factors, such as measurement of PAI-1:Ag versus PAI-1:act, study design, statistical technique used, in our view the most likely explanation relates to the different selection criteria of the study populations. It is in fact conceivable that our selection of young healthy normotensive twins with normal PAI-1 levels might have led to higher estimates of genetic variance, compared with those studies in which older subjects with higher BMI values and a much wider dispersion of PAI-1 levels were investigated.23,24

The 4G/5G PAI-1 polymorphism has been proposed as a determinant of the plasma levels of PAI-1:Ag. It has also been put forward as a risk factor for acute myocardial infarction in patients with angina pectoris and NIDDM.11,12,25 although the prognostic role of the 4G/5G polymorphism in the development of MI is still under debate.9,26,27 When classified by 4G/5G genotype, our twins were demographically similar and had superimposable blood pressure values (Table 1). The 4G or 5G allele frequency was consistent with the Hardy–Weinberg proportion, both in the whole twins population sample and when the twins were classified according to their zygosity. Although this finding makes a selection bias unlikely and suggests that the present results can be representative of the general population, it must be acknowledged that the observed 4G/5G genotype distribution in our series corresponds with a 4G allele frequency of 0.41, which is somewhat lower than the frequency of 0.54 to 0.56 reported in most studies in Caucasian populations (see for review9,26). Nevertheless, the present results do not support the contention that 4G/5G polymorphism plays a major role in determining PAI-1:Ag, because no significant difference between different 4G/5G genotypes was found.

Were the 4G/5G polymorphism exerting a major effect on PAI-1:Ag, the WP difference of 4G/5G concordant DZ twins would be expected to be close to that of MZ twins and lower than that of 4G/5G discordant DZ twins. In contrast, we found that the WP difference of 4G/5G concordant DZ twins differs significantly from that of MZ and was similar to that of DZ discordant twins (Figure 2). This finding suggests a role of additional, possibly still unidentified polymorphisms on the PAI-1 locus and/or of other quantitative trait loci contributing significantly to PAI-1:Ag, an issue which obviously deserves further specific research.

In conclusion, our results demonstrate that investigation of twins provides a useful model to assess the relative effects of environmental and genetic factors on quantitative traits such as PAI-1:Ag. Besides confirming that PAI-1:Ag levels are genetically determined, they indicate a major effect of A, ie, of an additive genetic influence which combines independently over different alleles at the same genetic locus and over different loci, and minor influences of both dominance genetic effect (ie, allelic interactions at the same locus) and environmental influence unique to individuals.

References


Figure 2. WP difference of plasma levels of PAI-1:Ag in MZ twins and in DZ twins concordant and discordant for the 4G/5G genotype. A significant difference between MZ twins and 4G/5G concordant DZ twins was found, whereas 4G/5G discordant twins had values similar to those of concordant DZ twins.


Determinants of Plasma Levels of Plasminogen Activator Inhibitor-1: A Study of Normotensive Twins
Maurizio Cesari, Maria Teresa Sartori, Giovanni M. Patrassi, Silvia Vettore and Gian Paolo Rossi

doi: 10.1161/01.ATV.19.2.316
Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 1999 American Heart Association, Inc. All rights reserved.
Print ISSN: 1079-5642. Online ISSN: 1524-4636

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://atvb.ahajournals.org/content/19/2/316

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Arteriosclerosis, Thrombosis, and Vascular Biology can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Arteriosclerosis, Thrombosis, and Vascular Biology is online at:
http://atvb.ahajournals.org/subscriptions/