Genetics of fibrin clot structure: a twin study

Emma J. Dunn, Robert A. Ariëns, Marlies de Lange, Harold Snieder, John H. Turney, Timothy D. Spector, and Peter J. Grant

Coronary artery thrombosis following plaque rupture is an important feature of myocardial infarction, and studies have highlighted the role of coagulation in this condition. Although genetic and environmental influences on the variance in coagulation protein concentrations have been reported, there are no data on the heritability of structure/function of the final phenotype of the coagulation cascade, the fibrin clot. To assess genetic and environmental contributions to fibrin structure, permeation and turbidity studies were performed in 137 twin pairs (66 monozygotic, 71 dizygotic). The environmental influence ($\sigma^2$) on pore size ($K_s$) ($\sigma^2 = 0.61$ [95% confidence interval (CI), 0.45-0.80]) and fiber size ($\sigma^2 = 0.54$ [95% CI, 0.39-0.73]) was greater than the heritability ($h^2 = 0.39$ [95% CI, 0.20-0.55]) and 0.46 (95% CI, 0.27-0.62), respectively. After correction for fibrinogen levels, the environmental effect persisted for $K_s$ ($\sigma^2 = 0.61$), but genetic influence assumed a greater importance in determining fiber size ($h^2 = 0.73$). Multivariate analysis revealed an overlap in the influence of genetic and environmental factors on fibrinogen levels, $K_s$, and fiber size. Factor XIII B subunit showed environmental and genetic correlation with fibrinogen and fiber size and a genetic correlation with $K_s$. The results indicate that genetic and environmental influences are important in determining fibrin clot structure/function. (Blood. 2004;103:1735-1740)

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Introduction

Studies from the Scandinavian twin registries report an increased concordance of risk for death from coronary heart disease (CHD) in monozygotic (MZ) compared with dizygotic (DZ) twins.1 This supports an important role for shared genetic factors in determining mortality from CHD that is not explained by the recognized familial trends seen in conventional cardiovascular risk factors, such as diabetes mellitus, hypertension, smoking, and obesity.3

Coagulation/fibrinolytic proteins have been studied in several prospective studies of CHD risk, and elevated plasma concentrations of fibrinogen, factor VII (FVII), von Willebrand factor, and plasminogen activator inhibitor 1 (PAI-1) have been reported to relate to vascular risk and cardiovascular outcome.2-3 Variation in the genes coding for components of the coagulation and fibrinolytic system provides a potential mechanism to explain the heritable nature of interindividual susceptibility to atherothrombotic disease. However, although some genetic factors have been shown to have a major effect on the variance in plasma levels of a number of hemostatic cardiovascular risk factors,6-9 the relationship between individual polymorphisms in coding genes and risk of CHD has been inconsistent.10

Although the structure of the fibrin clot does not have a specific coding gene, because it is generated by the fluid phase of an activated coagulation cascade, the sum total of genetic and environmental influences on the component proteins will be integrated into those that affect fibrin structure/function. The structure of the fibrin clot has been studied in vitro using a variety of techniques,11 and it has been shown to be influenced by several factors including fibrinogen concentration12 and common polymorphisms in genes encoding Ao-fibrinogen (Thr312Ala)13 and the factor XIII (FXIII) A subunit (Val34Leu).14

Although genetic factors have been shown to influence plasma concentrations of hemostatic proteins6-9 and individual polymorphisms have been found to influence fibrin clot structure, no studies have assessed the overall influence of genetic factors on the fibrin clot. In this study, we used a sample of female twin pairs to apply quantitative genetic model fitting to estimate and contrast genetic and environmental effects on the structure of ex vivo fibrin clots. As both fibrinogen and FXIII are intimately involved in the production of the fibrin clot, we further investigated potential associations between fibrinogen, FXIII, and measures of fibrin structure/function and examined whether these were attributed to shared genes, environment, or both.

Patients, materials, and methods

Subjects

One hundred thirty-seven female, white twin pairs (274 subjects), ranging in age from 18 to 79 years, from the St Thomas’ UK Adult Twin Registry participated in the study. Twins from the registry were recruited from the general population through national media campaigns in the United Kingdom. St Thomas’ Hospital research ethics committee approved the study, and informed consent was obtained from participating twins. Zygosity was established in all subjects by standardized questionnaire and was confirmed by DNA fingerprinting. Subjects had no history of CHD or diabetes mellitus and were not taking oral anticoagulants or aspirin.

From the Academic Unit of Molecular Vascular Medicine, General Infirmary, Leeds; the Twin Research and Genetic Epidemiology Unit, St Thomas’ Hospital, London, United Kingdom; and the Georgia Prevention Institute, Department of Pediatrics, Medical College of Georgia, Augusta.


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Reprints: P. J. Grant, Academic Unit of Molecular Vascular Medicine, G-Floor, Martin Wing, The General Infirmary at Leeds, Leeds LS13EX, United Kingdom; e-mail: p.j.grant@leeds.ac.uk.

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Blood sampling

Venous blood samples were taken between 8 and 10 AM after an overnight fast. Within 5 minutes, blood was taken from the co-twin into 0.13 trisodium citrate containers (Becton Dickinson, Oxford, United Kingdom) at room temperature. Within 1 hour of collection, samples were centrifuged at 2560 g for 20 minutes to obtain platelet-poor plasma, frozen in aliquots in liquid nitrogen, and stored at −40°C until analysis.

Biochemical assays

Fibrinogen levels were determined using the Clauss method, FXIII A and B subunits were analyzed with in-house sandwich enzyme-linked immunosorbent assay (ELISA), and FXIII activity was analyzed with a 5 (biotinamido) pentamidine incorporation assay.

Turbidity measurements of ex vivo fibrin clots

Turbidity measurements were performed as described previously. Lag phase was recorded (in seconds) as the time taken for the absorbency (at 350 nm) of the plasma samples to increase by 0.01 from baseline (ie, time zero reading) from addition of thrombin and calcium. Lag phase represents the length of time required for fibrin protofibrils to grow to sufficient length to allow lateral aggregation to occur, and it is sensitive to a variety of factors including fibrinogen concentration and rate of fibrinoprotein A cleavage. Clot turbidity is directly proportional to the average cross-sectional area of the fibers, and the maximum absorbency at 350 nm reflects the average size of the fibrin fibers and the number of protofibrils per fiber. Maximum absorbance was recorded as the absorbency value at 15 minutes minus the baseline (time zero) reading. All turbidity curves reached a plateau within 15 minutes of analysis, and 2 replicate measurements were performed for each sample. Only the results of maximum absorbance are reported here. Whereas the inter- and intra-assay coefficients of variation (CV) for measurement of maximum absorbance were acceptable (0.049 and 0.018, respectively), lag-time measurement had a normal intra-assay CV (0.051) but a large inter-assay CV (0.286), which introduced an erroneously large common environmental factor into the genetic model-fitting analysis; therefore, the results are not reported here.

Clot permeation measurements

Liquid permeation studies were performed as described previously, and measure flow rates through fibrin clots formed from plasma samples. Two replicate clots for each sample were analyzed. The technique enables calculation of the permeation coefficient (Ks), which represents the surface of the gel allowing flow through the network. This gives an indication of the pore size within the fibrin clot and can indirectly provide an estimation of the thickness of the fibrin fibers.

Statistical analysis

Raw data for maximum absorbance and the FXIII A subunit were normally distributed, but data on fibrinogen levels, Ks, FXIII activity, and FXIII B subunit needed log transformation to obtain normal distributions. All variables were adjusted for age and body mass index (BMI; weight/height 2 [kg/m²]) before genetic analyses, except for lag time and factor FXIII activity, for which no influence of age or BMI could be detected.

Quantitative genetic model fitting of twin data

Twin methodology makes use of the fact that MZ twins share identical genotypes, whereas DZ twins share on average 50% of their genes. It is assumed that both types of twins share their common family environment to the same extent, so any greater similarity between MZ compared with DZ twins reflects genetic influences. A higher MZ than DZ intraclass correlation (r) provides a first impression of the magnitude of genetic influence as reflected by the classic formula to estimate heritability: \( h^2 = 2(r_{MZ} - r_{DZ}) \). Model-fitting analysis of twin data, however, has some major advantages over the classic twin methodology and is now standard in twin research. The technique is based on the comparison of the covariances (or correlations) in MZ and DZ twin pairs and allows a more extensive separation of the observed phenotypic variance into its genetic and environmental components: additive (A) or dominant (D) genetic components and common (C) or unique (E) environmental components. E also contains measurement error. Dividing each of these components by the total variance yields the different standardized components of variance. For example, heritability (\( h^2 \)) can be defined as the proportion of the total variance attributable to additive genetic variation.

Extension from univariate to multivariate models also allows exploration of the question whether the origin of the covariance between the different variables is genetically and/or environmentally determined. Thus, we used multivariate analysis to determine to what extent shared genes, environment, or both can explain the relationship between fibrin structure measurements (traits). These analyses are based on the comparison of cross-trait/cross-twin correlations for MZ and DZ twins. If the correlation between fibrinogen of one twin and Ks level in the co-twin is larger in MZ than in DZ twins, it indicates that the genes influencing fibrinogen partly overlap with the genes that influence Ks. The extent of the overlap is reflected by the magnitude of the genetic correlation. When cross-trait/cross-twin correlations are absent for MZ and DZ twins, it suggests that environmental factors specific to the individual (E) contribute to the observed phenotypic correlation between fibrinogen and Ks. Thus, the genetic contribution \( r_{g} \) between 2 traits gives an indication of the amount of overlap between (sets of) genes influencing those traits; \( r_{g} \) is calculated as the (additive) genetic covariance \( \text{COV}_{A} \) between 2 traits divided by the square root of the product of the total genetic variance components of each of the traits. The genetic correlation between, for example, fibrinogen and Ks therefore equals: \( \text{COV}_{A} / \sqrt{(\text{V}_{A_{Fib}} \times \text{V}_{A_{Ks}})} \). Shared and unique environmental correlations are calculated in a similar fashion. All quantitative genetic modeling was performed with Mx, a computer program specifically designed for the analysis of twin and family data.

Model-fitting procedure

The significance of variance components A, C, and D was assessed by testing the deterioration in model fit after each component was dropped from the full model (ACE or ADE), leading to a parsimonious model in which the pattern of variances and covariances is explained by as few parameters as possible. Standard hierarchic \( \chi^2 \) tests were used to select the best-fitting model. Estimates of the genetic and environmental variance components and the appropriate 95% confidence interval (CI) were obtained from the best-fitting model.

Results

Table 1 summarizes the general characteristics and mean (untransformed) results of the hemostatic parameters in the MZ and DZ twin pairs. There was no significant difference for any of the parameters between the MZ and DZ twins, nor was there any effect of smoking, oral contraceptive, or hormone replacement therapy use on any of the hemostatic measurements. Age and body mass index (BMI) influenced some of the parameters measured (Table 2), and their effects were regressed out before genetic analysis.

The intraclass correlations of the MZ and DZ twins are shown in Table 3. The MZ twin correlations are higher than those for DZ twins, which suggests that genetic factors influence fibrin clot structure and hemostatic factor concentrations.

Results of quantitative model fitting, based on comparisons of observed and expected variance-covariance matrices using the normal theory of maximum likelihood, are summarized in Table 4, which contains the standardized variance components and 95% CI for the best-fitting univariate models for each measured parameter. For all the listed parameters, the data were best explained using a model with an additive genetic and a unique environmental component (AE models). The genetic component explained moderate to large proportions of the total variance in the parameters.
measured, ranging from 39% for Ks values to 89% for FXIII activity levels. The unique environmental component was found to explain substantial proportions of the total variance in fibrin clot structure; Ks values (61%), which represents pore size, and maximum absorbance, which is an indicator of fibrin fiber diameter (54%). When Ks and maximum absorbance were adjusted for maximum absorbance, which is an indicator of fibrin clot structure measurements, the environmental effect persisted for Ks (54%). When Ks and maximum absorbance were adjusted for maximum absorbance, the environmental effect persisted for Ks (54%).

In this study we quantified the impact of genetic and environmental factors on the structure of the ex vivo fibrin clot, and we have shown that environmental factors have a considerable influence on fibrin structure.
fibrin clot structure, mediated in part by an influence on fibrinogen levels. We have also shown that plasma levels of fibrinogen and FXIII subunits correlate with fibrin clot pore size (Ks) and fiber size (maximum absorbance) and that an overlap in genetic and environmental influences between these parameters contributes to these correlations. FXIII was included in the analysis because levels have been shown to influence fibrin clot structure in vitro.13,14,23,24

Although studies using family data to estimate genetic influence are confounded to some extent by cohort, age, and shared environmental factors, twin studies are able to discriminate between genetic and shared environmental sources of familial resemblance, and they have the advantage of matching for both age and unknown cohort effects.25 Although this study was limited to women because most twins in the St Thomas’ UK Adult Twin Registry are women for historical reasons,26 there is no reason to expect major differences to exist between men and women for the traits assessed; hence, the results should be of relevance to both sexes. However, strictly speaking, results of our study cannot be generalized to men. Future twin studies, aimed at systematically investigating sex differences in genetic and environmental influences on fibrin structure, should include opposite-sex twin pairs in addition to same-sex male twins.27 There was no effect of smoking, oral contraceptive use, or hormone replacement therapy on any of the hemostatic measurements. However, we cannot completely rule out that unmeasured environmental variables, such as dietary intake and physical activity, might have had an effect. If so, any increased sharing of such environmental variables between MZ and DZ twins might have caused bias in our genetic and environmental variance components.

The finding that levels of FXIII B subunit correlated with fibrin clot structure to a greater extent than FXIII activity and A subunit level (which contains the catalytic activity that cross-links fibrin) was unexpected. There are 2 possible explanations for this finding. First, the B subunit may interact with other proteins that influence fibrin structure and function. Previous studies have found that levels of FXIII B subunit associate with other CHD risk factors, such as elevated fibrinogen levels and insulin resistance in persons with diabetes.28 Correlations between B subunit and fibrin structure measures could, therefore, reflect the impact of other cardiovascular risk factors, which associate with insulin resistance, on the structure of the fibrin clot. A second explanation for our findings may be that there is a direct effect of the B subunit itself on fibrin structure and function. The B subunit is composed of 10 modular domains, called Sushi domains,29 that are involved in protein–protein interactions. It has been reported that a splice variant of the γ-chain of fibrinogen, γ’, which occurs in 8% to 15% of fibrinogen, binds FXIII through its B subunit.20 Direct interaction between the B subunit and γ’ may modulate the reported influence of fibrinogen γ’ on fibrin clot structure, providing an alternative explanation for the observed effects. Each of these hypotheses, however, needs further investigation.

Results of the turbidity experiments indicated that although the measurement of maximum absorbance was a reliable test, the measurement of lag time was not. The lack of interassay reliability in its measurement resulted in the generation of bias in the (unreported) estimates of the components of variation on statistical analysis. This assay appears to be highly sensitive to environmental or experimental procedure-related influences, which are so great that they obscure the ability of the assay to detect true differences in lag time that would be induced by differences in the samples analyzed. In our study, the large inter-assay variability of the test translated into the presence of an erroneously large common environmental influence on variation in lag time, which explained nearly half the variation in measurements and was, therefore, not reported.

The structure of the fibrin clot is relevant in CHD because the thrombus in a coronary artery is composed of a platelet-rich fibrin mesh, and clinical studies have shown that differing structures relate to CHD risk.16,32,34 Fatah et al34 report that patients who experience first myocardial infarction (MI) before the age of 45 years form clots with tighter and less permeable fibrin networks than control subjects. This type of clot structure is more resistant to fibrinolysis,35 possibly because the small pores within the clot restrict the access of fibrinolytic enzymes.35,37

Atherosclerotic cardiovascular disease is the leading cause of morbidity and mortality in much of the industrialized world. The coagulation cascade plays a key role in this disease. The formation of a thrombus over a ruptured atheromatous plaque in a coronary

**Table 5. Phenotypic correlations among fibrinogen, FXIII activity and subunit levels, and fibrin structure/function parameters**

<table>
<thead>
<tr>
<th></th>
<th>Fibrinogen</th>
<th>Ks</th>
<th>Maximum absorbance</th>
<th>FXIII activity</th>
<th>FXIII A subunit</th>
<th>FXIII B subunit</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fibrinogen</td>
<td>1.00</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Ks</td>
<td>—0.37*</td>
<td>—0.16/—0.21</td>
<td>—0.17 (NS)</td>
<td>1.00</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Maximum absorbance</td>
<td>0.66* (0.24/0.42)</td>
<td>—0.29* (all rE)</td>
<td>1.00</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>FXIII activity</td>
<td>0.12 (NS)</td>
<td>—0.17 (NS)</td>
<td>0.01 (NS)</td>
<td>1.00</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>FXIII A subunit</td>
<td>0.22* (all rE)</td>
<td>—0.10 (NS)</td>
<td>0.19* (all rE)</td>
<td>0.29* (0.21/0.08)</td>
<td>1.00</td>
<td>—</td>
</tr>
<tr>
<td>FXIII B subunit</td>
<td>0.40* (0.23/0.17)</td>
<td>—0.22* (all rG)</td>
<td>0.34* (0.19/0.15)</td>
<td>0.24* (all rG)</td>
<td>0.66* (0.51/0.15)</td>
<td>1.00</td>
</tr>
</tbody>
</table>

Multivariate analysis and path tracing rules were used to quantify the extent to which genetic (G) and environmental (E) factors contribute to the phenotypic correlations (G/E).

*Significant at P < .05.

**Table 6. Genetic and environmental correlations from the best-fitting multivariate model**

<table>
<thead>
<tr>
<th></th>
<th>Fibrinogen</th>
<th>Ks</th>
<th>Maximum absorbance</th>
<th>FXIII activity</th>
<th>FXIII A subunit</th>
<th>FXIII B subunit</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fibrinogen</td>
<td>1.00</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Ks</td>
<td>—0.38*</td>
<td>1.00</td>
<td>—0.27*</td>
<td>—0.13</td>
<td>—0.18</td>
<td>0.02</td>
</tr>
<tr>
<td>Maximum absorbance</td>
<td>0.48*</td>
<td>—0.33</td>
<td>1.00</td>
<td>0.12</td>
<td>0.25*</td>
<td>0.36*</td>
</tr>
<tr>
<td>FXIII activity</td>
<td>0.12</td>
<td>—0.23</td>
<td>—0.03</td>
<td>1.00</td>
<td>0.53*</td>
<td>0.20</td>
</tr>
<tr>
<td>FXIII A subunit</td>
<td>0.21</td>
<td>—0.06</td>
<td>0.17</td>
<td>0.25*</td>
<td>1.00</td>
<td>0.50*</td>
</tr>
<tr>
<td>FXIII B subunit</td>
<td>0.39*</td>
<td>—0.47*</td>
<td>0.33*</td>
<td>0.27*</td>
<td>0.73*</td>
<td>1.00</td>
</tr>
</tbody>
</table>

Genetic correlations are shown in the lower left hand corner of the table, and environmental correlations are shown in the upper right hand corner of the table.

*Significant at P < .05.
artery is the precipitant of MI. Hemostatic and fibrinolytic protein concentrations are associated with CHD risk.\(^2\) As a consequence, there has been a great deal of interest in determining the influence of genetic factors on hemostatic proteins and on CHD risk. Associations have been found between some hemostatic gene polymorphisms and plasma levels of the individual proteins, but there is still no reproducible evidence for the role of any hemostatic polymorphism in the development of CHD or MI.\(^6\) We have demonstrated that polymorphisms in FXIII (Val34Leu), B/Thr312Ala affect fibrin structure/function in vitro,\(^13\) making these polymorphisms candidates for a component of the genetic contribution to variance in fibrin structure/function. However, a clinical study of the first-degree relatives of subjects with severe coronary artery disease demonstrated differences in fibrin structure/function in relatives compared with controls that were not attributable to classical risk factors or to possession of the FXIIIVal34Leu or the AThr312Ala polymorphisms.\(^6\)

The results of our current study make the lack of consistent associations between individual genetic polymorphisms and CHD less surprising. Polymorphisms in the PAI-1, FVII, and fibrinogen genes were found to have a minimal impact on the overall total genetic contribution to plasma levels, ranging from less than 0.01% (fibrinogen) to 11% (FVII) in a recent study of healthy families.\(^3\)

More intriguingly, the impact of genetic factors appears to be greatest in the precipitant of MI. Hemostatic and thrombotic factors that influence the phenotype of the fibrin clot, particularly environmental factors, which can be influenced via intervention, to reduce future atherothrombotic risk. Multiple genes influence the phenotype of fibrin clot structure/function, and our results show there is a modest genetic influence on its variance. This combination indicates that simple gene association studies of the thrombotic component of vascular disease should be approached with circumspection.

Atherosclerosis is a complex disorder with multiple risk factors influenced by environmental and genetic factors. Although genetic polymorphisms are likely to have been present within populations for thousands of years, atherosclerotic disease has only reached epidemic proportions within the last century, suggesting that this situation can only have arisen through the development of adverse genetic or environmental interactions. The Framingham Study\(^4\) demonstrated a decline in CHD in the cohorts studied in sequential decades from 1950 to 1970, reflecting changes in environmental factors that influenced CHD over this time period (decline in smoking, improvement in diet, and management of hypertension and dyslipidemia).\(^4\) Studies demonstrate that migrants acquire the risks associated with their country of destination, lending additional weight to the importance of environmental factors.\(^5\) It is feasible that known vascular risk factors, including glycation, oxidized lipoproteins, and meny contribute to the environmental component of variance in the fibrin clot. This is an area worthy of further study.

The development of CHD involves life-long interactions between genetic and environmental factors that influence a person’s risk for disease. The fibrin clot plays an important role in the development of the thrombotic complications of atherosclerotic disease, and our results indicate environmental factors have a significant influence on its structure. A key issue for future research will be identifying factors that determine the structure/function of the fibrin clot, particularly environmental factors, which can be influenced via intervention, to reduce future atherothrombotic risk. Multiple genes influence the phenotype of fibrin clot structure/function, and our results show there is a modest genetic influence on its variance. This combination indicates that simple gene association studies of the thrombotic component of vascular disease should be approached with circumspection.

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