Original articles

The association of combined α and β fibrinogen genotype on plasma fibrinogen levels in smokers and non-smokers

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Abstract

Objective – To determine in healthy men: (1) whether an extended genotype of the fibrinogen gene cluster using the G/A^{-455} and the $BcII$ polymorphism of the β fibrinogen gene and $TaqI$ of the α fibrinogen gene explains a significantly larger proportion of variance in plasma fibrinogen levels in either smokers or non-smokers than a single polymorphism (G/A^{-455}); (2) whether there is any evidence for genotype-smoking interaction in the determination of fibrinogen levels.

Design – A cross sectional study of healthy, white men recruited at the screening for entry into the Thrombosis Prevention Trial.

Setting – The subjects were drawn from four general practices in the United Kingdom.

Results – The frequency of the rare alleles in the sample was 0.19 for the G/A^{-455} polymorphism (A^{-455}), 0.15 for $BcII$ ($B+$), and 0.27 for $TaqI$ ($T+$) alleles. BMI and age made significant contributions to the variance in plasma fibrinogen levels only in non-smokers of 5.4% and 2.3% respectively and, in the group as a whole, smoking accounted for 6.6% of the variance. In the non-smokers, of the individual polymorphisms only the G/A^{-455} showed a significant association with plasma fibrinogen levels ($p=0.03$). The mean fibrinogen in non-smokers homozygous for the G^{-455} allele was 2.54 g/l v 2.85 g/l in those homozygous for the A^{-455} allele, with the polymorphism explaining 3.6% of the variance in plasma fibrinogen levels in this group. On investigation of the association of fibrinogen levels with combined genotypes, the most significant effect was seen with the combination of the G/A^{-455} and $TaqI$ polymorphisms, with those with no “fibrinogen raising alleles” having a mean fibrinogen of 2.57 g/l v 3.10 g/l for those with four “fibrinogen raising alleles” ($p=0.0036$), and this combination explained 8.9% of the variance in plasma fibrinogen levels ($p<0.005$). Although the contribution to variance was greater with the $G/A^{-455}/TaqI$ combination than the G/A^{-455}

polymorphism alone (8.9% v 3.6%), this did not reach significance ($p=0.09$). By contrast, in the smoking group, the only significant contribution to the difference in plasma fibrinogen levels was the G/A^{-455} genotype alone which, after adjustment for BMI and age, contributed 3.8% to the variance ($p<0.05$). No interaction was shown between smoking and genotype.

Conclusion – These data suggest that in non-smokers an extended genotype using the G/A^{-455} β fibrinogen gene polymorphism and the $TaqI$ α fibrinogen gene polymorphism explains a larger proportion of the variance in plasma fibrinogen levels than any one polymorphism alone, but that smoking has an overriding effect so that other variables such as age and BMI make little additional contribution.

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Several prospective epidemiological studies have shown that plasma fibrinogen level is an important predictor of ischaemic heart disease and stroke.¹⁻⁶ The risk of developing peripheral vascular disease is also increased with increasing plasma fibrinogen levels.⁷⁻⁹ Fibrinogen is an acute phase protein whose levels rise in response to infection, inflammation, and trauma.¹⁰ Other factors which influence levels include age, obesity, hormonal changes, and diabetes.¹¹⁻¹⁵ Fibrinogen can be raised as a result of a variety of environmental factors, most particularly smoking.^{12 10-12 16} It is likely that a substantial part of the relationship between smoking and ischaemic heart disease is mediated through its effect on plasma fibrinogen levels.

Fibrinogen consists of two each of three separate chains, $A\alpha$, $B\beta$, and γ ,¹⁷ each encoded by a separate gene.¹⁸⁻²⁰ The fibrinogen gene loci lie in a cluster spanning approximately 50 kb on the long arm of chromosome 4.²⁰⁻²² The synthesis of the $B\beta$ chain is the rate limiting step for fibrinogen production²³⁻²⁵ and this has prompted investigation of genetic variation at these loci, especially the β fibrinogen promoter region, for association between such variation

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and differences in plasma fibrinogen levels. Several reports have shown such an association,²⁶⁻²⁹ but this has not been seen in all studies.^{30,31} More recently there has been a report of a gene-environment interaction between smoking and fibrinogen genotype²⁸ but other studies have not shown this finding.^{26,29}

In this report, the role of genetic variation at the fibrinogen gene locus has been studied in more detail. Recently, detection of five different polymorphisms involving both the α and the β fibrinogen gene loci using PCR has been described.³² It is therefore relatively quick and simple when compared to Southern blotting to elucidate the extended genotype of a person. To what extent further information is gained by using the different polymorphisms or an extended genotype has been investigated. Possible gene-environment interaction has also been sought.

Materials and methods

The 292 healthy men participating in this study were from four general practices being screened for entry into the Thrombosis Prevention Trial³³ and the selection procedures have previously been described.²⁶ Samples were collected and DNA extracted as described by Thomas *et al.*²⁶ Three of the polymorphisms described by Thomas *et al.*,³² G/A⁻⁴⁵⁵, C/T⁻¹⁴⁸, and T/G⁺¹⁶⁸⁹, are in complete linkage disequilibrium (except one of 596 chromosomes tested) and therefore just one of these, G/A⁻⁴⁵⁵, was used. The other two polymorphisms investigated were those detectable using the restriction enzymes *Bcl*I and *Taq*I, of the β fibrinogen and α fibrinogen genes respectively. The primers and conditions used for PCR amplification are given in Thomas *et al.*³²

The data were analysed using the statistical package SPSS.³⁴ Fibrinogen levels were log₁₀ transformed to normalise the data. Combined genotypes were devised by counting the number of alleles in any given genotype combination that were associated with higher fibrinogen levels. Although some associations did not reach significance in this study, the trend seen was similar to that in a previous study²⁷ and the designated "fibrinogen raising alleles" determined from this. These were the A⁻⁴⁵⁵, the *Bcl*I cutting, and *Taq*I non-cutting alleles. Thus,

A map of the α and β fibrinogen genes (not to scale) showing relative positions of the *Taq*I, *Bcl*I, and G/A⁻⁴⁵⁵ polymorphic sites and linkage disequilibrium coefficients. The transcription of the two genes is indicated by arrows above the text alpha and beta.

for an extended genotype involving two different polymorphisms there are five categories having 0, 1, 2, 3, or 4 "fibrinogen raising alleles". For the extended genotype involving all three polymorphisms there are seven possible categories. A quadratic term was calculated for each of the genotype groups and tested with the linear term in a multiple regression analysis. There was no evidence of non-linearity in any of the genotype groups except for the combined G/A⁻⁴⁵⁵/*Taq*I in non-smokers. The genotypes were therefore entered into any regression analysis as *n* categories except for the G/A⁻⁴⁵⁵/*Taq*I combination when dummy variables were used. In order to evaluate the relative contribution to the variance in plasma fibrinogen levels of the genotypes and their combinations, multiple regression analysis was used, first adjusting for BMI and age which are known to affect plasma fibrinogen levels and then entering the various genotypes or their combinations to see if any further significant effect was seen. The analysis was performed separately in smokers and non-smokers. Interaction terms were calculated between smoking and age, smoking and BMI, and smoking and genotype and entered into the multiple regression model.

Results

The locations within the fibrinogen gene cluster of the three variable sites used in the study are shown in the figure. The characteristics of the PCR fragments of each of the polymorphisms have been previously described.³² The overall frequencies of the polymorphic alleles in the subjects were 0.81 and 0.19 for the G/A⁻⁴⁵⁵ polymorphism, 0.85 and 0.15 for the *Bcl*I non-cutting (B-) and cutting (B+) allele respectively, and 0.73 and 0.27 for the *Taq*I non-cutting (T-) and cutting (T+) allele respectively. These frequencies are similar to those found in previous studies.^{27,30,31} The overall genotype frequencies were compared with those predicted from the Hardy-Weinberg equilibrium and no significant differences were detected.

The characteristics of the smokers and non-smokers are given in table 1 and, as expected, smokers have a significantly higher fibrinogen than non-smokers (2.93 g/l *v* 2.61 g/l, *p*<0.0001). Of the individual polymorphisms, only the G/A⁻⁴⁵⁵ in the non-smokers showed a significant association with unadjusted plasma fibrinogen levels, with those homozygous for the A⁻⁴⁵⁵ allele having 12.2% higher levels than those homozygous for the G⁻⁴⁵⁵ allele (*p*=0.03). In the smokers there was a trend, although not significant, towards higher fibrinogen levels in those possessing the A⁻⁴⁵⁵

Table 1 Fibrinogen, age, and BMI by smoking status

Smoking status (No)	Fibrinogen (g/l) (95% CI)	Age (y) (SD)	BMI (SD)
Non-smoker (172)	2.61 (2.53-2.69)	55.5 (5.8)	25.4 (2.8)
Smoker (120)	2.93* (2.81-3.05)	55.3 (5.8)	25.6 (2.9)

* *p*<0.0001.

Table 2 Unadjusted plasma fibrinogen levels according to genotype and smoking status

RFLP	Smoking status	Fibrinogen (g/l) 95% CI (No)			p value
		GG	GA	AA	
G/A ⁻⁴⁵⁵	Smoker	2.84 2.70–2.98 (75)	3.07 2.84–3.33 (42)	3.28 1.60–6.76 (3)	0.13
		2.54 2.45–2.63 (115)	2.74 2.58–2.92 (49)	2.85 2.29–3.55 (8)	
	Non-smoker				0.03
BcII	Smoker	2.85 2.72–2.99 (83)	3.12 2.85–3.41 (35)	2.96 2.01–4.36 (2)	0.16
		2.55 2.46–2.64 (123)	2.77 2.57–2.98 (37)	2.61 1.92–3.57 (4)	
	Non-smoker				0.10
TaqI	Smoker	2.93 2.77–3.10 (62)	2.97 2.78–3.18 (48)	2.67 2.26–3.15 (7)	0.52
		2.66 2.54–2.78 (88)	2.55 2.41–2.69 (62)	2.49 2.24–2.76 (9)	
	Non-smoker				0.37

One way analysis of variance.

Table 3 Mean (geometric, unadjusted) plasma fibrinogen level by combined G/A⁻⁴⁵⁵ and TaqI genotype and smoking status

G/A ⁻⁴⁵⁵ /TaqI genotype (No of "fibrinogen raising alleles")	Fibrinogen (g/l) 95% CI (No)			
	Smoker		Non-smoker	
AA/T–T– (4)	3.28 2.17–4.98 (3)		3.10 2.20–4.36 (4)	
AA/T–T+ GA/T–T– (3)	3.13 2.78–3.53 (23)	3.15 2.83–3.50 (26)	2.93 2.68–3.20 (29)	2.95 2.72–3.20 (33)
GG/T–T– AA/T+T+ GA/T–T+ (2)	2.86 2.70–3.03 (55)		2.52 2.43–2.62 (74)	
GG/T–T+ GA/T+T+ (1)	2.95 2.70–3.22 (29)	2.87 2.75–3.00 (91)	2.52 2.35–2.70 (46)	2.52 2.44–2.61 (126)
GG/T+T+ (0)	2.67 2.62–3.15 (7)		2.57 2.20–3.00 (6)	
p value*	0.35	0.07	0.0036	0.0001

* From analysis of variance on log transformed, unadjusted fibrinogen values.

Table 4 Contribution to variance in plasma fibrinogen levels using a multiple regression model

Smoking status	Variable					
	BMI (No)	Age (No)	Polymorphism			
			BcII (No)	G/A ⁻⁴⁵⁵ (No)	TaqI (No)	G/A ⁻⁴⁵⁵ /TaqI (No)
Non-smoker	5.4%† (172)	2.3%† (172)	2.4%† (164)	3.6%‡ (172)	1.4%* (159)	8.9%§ (159)
Smoker	0.1%* (120)	1.5%* (120)	2.5%* (120)	3.8%† (120)	0.3%* (117)	4.4%* (117)

BMI and age were entered first into the regression model and then genotype entered and the additional contribution shown in the table.

* Not significant, † p<0.05, ‡ p<0.01, § p=0.005.

allele, with those homozygous for this allele having fibrinogen levels 15.5% higher than those homozygous for the G⁻⁴⁵⁵ allele (table 2). For the BcII polymorphism (β fibrinogen) highest plasma fibrinogen levels were observed in the heterozygotes and the lowest in those homozygous for the non-cutting allele for both smokers and non-smokers (table 2). The numbers were small in those homozygous for the cutting allele. This is similar to the trend seen in a previous study.²⁷ For the TaqI poly-

morphism (α fibrinogen), no significant association was shown with plasma fibrinogen levels in smokers or in non-smokers. However, in both groups a trend toward lower levels in those with one or two cutting alleles was observed, with those homozygous for T+ having 8.9% lower levels than those homozygous for T– in smokers and 6.4% lower levels in non-smokers (table 2).

The association between plasma fibrinogen levels and combined genotypes was investigated and the most significant effect was seen with the combination of the G/A⁻⁴⁵⁵ and TaqI genotypes (table 3). This reached significance in the non-smokers but not in the smokers. The number of categories of genotypes was reduced to investigate whether the presence of 3–4 "fibrinogen raising alleles" compared with the presence of 0–2 "fibrinogen raising alleles" showed a more significant difference. In the non-smokers, fibrinogen levels were 17% higher (2.95 g/l v 2.52 g/l, p=0.0001) and in smokers, levels were 10% higher (3.15 g/l v 2.87 g/l, p=0.07).

A multiple regression analysis was performed for all combinations of genotypes having first entered BMI then age. In non-smokers, BMI made a contribution to variance of 5.4% and age a smaller contribution of 2.3% (table 4). BMI and age made no significant contribution in the smokers. In the non-smokers, genotype explained 2.4% (p<0.05) and 3.6% (p<0.01) of the variance in plasma fibrinogen levels for the BcII polymorphism and the G/A⁻⁴⁵⁵ respectively. The TaqI polymorphism made a smaller contribution of 1.4% which did not reach significance. However, the combination of the G/A⁻⁴⁵⁵ and TaqI polymorphisms together explained 8.9% of the variance (p<0.005) (table 4). The combination of the three genotypes together did not increase the contribution.

In the smokers, using multiple regression analysis, G/A⁻⁴⁵⁵ genotype explained 3.8% variance in plasma fibrinogen levels (p<0.05) but no other genotype or combination made a significant contribution. When the group was analysed as a whole, the effect of smoking contributed 6.6% of the variance in the plasma fibrinogen levels. No statistically significant interaction could be shown between genotypes or their combinations and smoking in determining plasma fibrinogen levels.

Discussion

Of the three polymorphisms, the G/A⁻⁴⁵⁵ was associated with the greatest effect on plasma fibrinogen level and accounted for 3.6% and 3.8% of the variance in non-smokers and smokers respectively. The combination of the G/A⁻⁴⁵⁵/TaqI polymorphisms in the non-smokers accounted for 8.9% of the variance in plasma fibrinogen levels but this did not differ significantly from the G/A⁻⁴⁵⁵ alone (p=0.09). The G/A⁻⁴⁵⁵/BcII combination did not contribute any further to the variance in plasma fibrinogen levels than the G/A⁻⁴⁵⁵ polymorphism alone. These results are not surprising since the G/A⁻⁴⁵⁵ and the BcII

polymorphisms are in strong linkage disequilibrium ($\Delta=0.85$, $p<0.001$), whereas the G/A^{-455} and the $TaqI$ polymorphisms are not ($\Delta=-0.09$, $p>0.1$).³² However, the $TaqI$ polymorphism on its own was not associated with a significant effect on plasma fibrinogen levels although a trend of lower fibrinogen level with the T+ genotype was seen in both smokers and non-smokers. A previous smaller study using the $TaqI$ and $BclI$ polymorphisms showed that the T+ allele was associated with a lowering effect on plasma fibrinogen level and the B+ allele a raising effect, with these polymorphisms explaining 4.2% and 9.0% of the phenotypic variance respectively.²⁷ The effect of the combination of these two genotypes on fibrinogen level was additive explaining 14.7% in total of the variance,²⁷ which is similar to the size of the effect seen here with the combination of the G/A^{-455} and $TaqI$ genotypes.

The studies by Connor *et al*³⁰ and Berg and Keirulf³¹ showed no significant effect of genotype on plasma fibrinogen levels but correction for BMI was not made in either study; in this study it made a significant contribution in the non-smokers. In the smokers BMI made no significant contribution and neither did age, possibly because the effect of smoking is so strong that it overrides lesser effects in studies of this size and with a relatively narrow age and BMI range. No statistically significant interaction was seen between genotype and smoking. In contrast, Green *et al*²⁸ found a significant interaction between smoking status and genotype in determining plasma fibrinogen levels in healthy Swedish controls, but not in the patient group, who consisted of young survivors of myocardial infarction.

In this study, those who had a history of coronary artery disease were excluded. Since a high fibrinogen level has consistently been shown to be a predictor of coronary events it seems likely that a disproportionate number of people with higher fibrinogen levels would have been excluded. This would decrease the chances of showing an association between genotype and plasma fibrinogen level and might explain why the fibrinogen level is lower than expected in the $BclI$ B+B+ group, which has previously been shown to be associated with higher fibrinogen levels.²⁷ There is also a significantly lower percentage of smokers in the oldest age group, 19% in those ≥ 65 years *v* 47% in the 55-65 year olds and 38% in the 45-55 year olds (likelihood ratio $\chi^2=5.86$, $p=0.04$) which would decrease the likelihood of showing a smoking-genotype interaction.

Detection of these fibrinogen genetic polymorphisms using PCR rather than Southern blotting is both technically simpler and more rapid, enabling large population samples to be genotyped easily and quickly. Using the extended genotype G/A^{-455} and $TaqI$ explains a greater proportion of the sample variance in plasma fibrinogen levels than G/A^{-455} alone, but extending the genotype to include the $BclI$ polymorphism does not contribute any further information. Further study in a younger population before the onset of ischaemic heart disease should help to clarify the relationship

between genetic variation and plasma fibrinogen levels. Gene-environment interaction needs further investigation, paying attention to different age groups and with a detailed history of smoking characteristics.

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