Association between the Thr715Pro P-selectin gene polymorphism and soluble P-selectin levels in a multiethnic population in South London

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Introduction

Ethnic differences in cardiovascular disease (1, 2) and the apparent protection from coronary heart disease (CHD) seen in blacks cannot be entirely attributed to ethnic differences in traditional risk factors (3-5). Moreover, the importance of inflammatory processes in the development of CHD and atherosclerosis has recently been recognised (6).

The development of an atheromatous lesion requires the activation of the adhesion molecule pathway including expression of P-selectin by the endothelium and its release from platelets (7). Leukocytes are then attracted to and ‘roll’ along the endothelium, before becoming firmly attached and then migrating into the sub-intimal spaces where they take up lipids, become foam cells and form fatty plaques (8).

The Thr715Pro gene is located on chromosome 1q21 to 1q24 and it is highly polymorphic (9). The Thr715Pro (A/C) polymorphism is located in the consensus repeat (CR9) domain of exon 13 and substitutes a polar amino acid for a non-polar one at position 715 (9). The CR9 domain of the P-selectin protein is

Summary

The aim was to investigate whether the Thr715Pro P-selectin polymorphism is associated with soluble P-selectin (sP-selectin) levels in individuals from different ethnic groups. Plasma sP-selectin and Thr715Pro (A/C) P-selectin gene polymorphism were measured in 237 white (106 females), 177 black African origin (92 females) and 201 South Asian (94 females) individuals living in England. All were free from coronary heart disease (CHD), stroke and other cardiovascular disease, diabetes, drug therapy for hypertension or high lipids, hormone replacement therapy or oral contraceptive pill. The Thr715Pro C allele was rare in blacks (0.8%) and intermediate in South Asians (3.0%) compared to whites (11.2%; p < 0.001). sP-selectin levels were significantly lower in the individuals with the AC or CC compared to the AA genotype in both whites (-25% (95% C.I. -33.3 to -16.9); p < 0.001) and South Asians (-25.2% (-40.5 to -6.1); p < 0.012). There was insufficient power for this analysis in blacks. In conclusion, in whites and South Asians the C allele of the Thr715Pro P-selectin polymorphism is associated with lower sP-selectin levels. Lower levels of sP-selectin were not accounted for by this polymorphism in blacks, in whom the C allele was very rare.

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adjacent to the transmembrane domain (7, 10). A lower frequency of the Pro715 (C) allele has been found in patients with myocardial infarction (MI), suggesting that this polymorphism may be protective for MI (9). Although P-selectin is expressed as a functional membrane glycoprotein a shorter soluble isoform (sP-selectin) has also been reported (11). Studies which use soluble adhesion molecule levels as an indication of adhesion molecule expression have shown that circulating levels are associated with both overt cardiovascular disease and, to a lesser extent, atherosclerosis (7). Furthermore, the circulating levels of sP-selectin (12), C-reactive protein (CRP) (13), as well as plasminogen activator inhibitor (14), which has shown to be associated with thrombogenesis, are lower in individuals of African origin. It is therefore possible that variations in the adhesion molecule pathway may contribute to ethnic variations in CHD risk.

The purpose of this study was to investigate whether the Pro715 (C) allele is associated with lower levels of sP-selectin in individuals from different ethnic groups.

Materials and methods

Subjects and methods

705 essentially healthy untreated individuals were selected from the Wandsworth Heart & Stroke Study (WHSS) (3, 4) as previously described (12). In brief, individuals were selected if they did not have diabetes, were not on hypertension or lipid-lowering medication and were not taking the oral contraceptive pill or hormone replacement therapy. Subjects were selected if they did not have any previous history of ischaemic heart disease or stroke. Of these, 615 individuals had samples suitable for genetic analysis and did not differ significantly from the 90 who did not. Of the individuals studied, 237 were white (106 females), 177 were of African origin (92 females) and 201 were of South Asian origin (94 females). People of African origin were all first generation immigrants with both parents born in the country of origin (3). The Local Ethics Committee approved the study. All participants gave their informed consent to participate. Administration of questionnaire, determination of blood pressures, demographic and clinical and biochemical parameters have been described in detail previously (3, 4). Soluble plasma P-selectin (sP-selectin) levels were determined using commercially available ELISA kits (R & D systems Europe Ltd, Abingdon, U.K.) as previously described (12).

Genetic analysis

Genomic DNA was extracted from whole blood as previously described using Nucleon BACC DNA extraction kit (15). Primers were designed by Primer3, and used to amplify the A-715C polymorphism. The sequence of the sense oligonucleotide primer was 5’-AAATTTGACCTTGGCAGGTT-3’ and that of the antisense primer was 5’-AGCTGTGAAATGCTCAGA-AC-3’. PCR (polymerase chain reaction) was performed in a total volume of 25 µl containing 100 ng of DNA, 12.5 pmol of each primer, 200 µM dNTPs, 1.5 mM MgCl₂, and 0.5U Redhot DNA polymerase (Abgene Epson, U.K.). After an initial denaturation at 94°C for 5 min, amplification was carried out by 35 cycles of 94°C for 30 sec, 51°C for 60 sec and 72°C for 60 sec, and a final extension at 72°C for 10 min. The PCR product (198bp) was then digested using Eco91II (BstEII) (Helena Biosciences, Sunderland, U.K.), and the digested products run on a 3% agarose gel and visualised under UV light by ethidium bromide staining. Genotype was confirmed by direct sequence analysis of both strands on an ABI 377 automated sequencer. Samples with A-715A, A-715C and C-715C genotype confirmed by sequencing were used for internal controls for the verification of the digestion assay. To prevent observer bias, the investigator was unaware of the sample origin and all gels were cross-checked by a separate individual.

Statistical analysis

Plasma levels of sP-selectin were positively skewed; therefore analyses were performed on log transformed data and results are presented as geometric means and 95% confidence intervals (C.I.). Differences between ethnic groups (adjusted for age and sex) were tested using analysis of co-variance. Differences between ethnic groups in smoking were adjusted using age standardisation with the direct method (3). Associations between plasma levels and genotype were done using analysis of variance and co-variance. Differences in genotype and allelic frequency between ethnic groups were evaluated with Fisher’s exact test or chi square tests as appropriate. A 2-sided p value < 0.05 was considered statistically significant.

Results

There were marked ethnic differences in the baseline characteristics and cardiovascular risk factors (Table 1). sP-selectin levels were lower in people of African origin (geometric mean 57 ng/ml (95% C.I. 54 to 60) than white (72 ng/ml (69 to 75)) and South Asian (72 ng/ml (68 to 75)); p < 0.001). The frequency of the Thr715Pro P-selectin genotype varied significantly between ethnic groups (Whites: 81.0% (AA), 15.6% (AC) and 3.4% (CC); South Asian: 95.0% (AA) 4.0% (AC) and 1.0% (CC); African origin: 98.3% (AA), 1.7% (AC); p < 0.001 by Fisher’s exact test). Likewise, there were significant differences between whites and South Asian (Fisher’s exact test p < 0.001) and white and people of African origin (Fisher’s exact test p < 0.001).

The allele frequencies were as follows: White 88.8% (A), 11.2% (C); South Asian 97.0% (A), 3.0% (C); people of African origin 99.2% (A), 0.8% (C). There was a significant difference in the total group due to ethnicity ($\chi^2 = 48.83$ p < 0.001; 2df). Likewise, there was a significant difference between white and
South Asian ($\chi^2 = 21.27$, $p < 0.001$; 1df) and white and people of African origin ($\chi^2 = 34.32$, $p < 0.001$; 1df).

The C allele was rare in people of African origin (only 3 heterozygotes and no homozygote), so further analyses by genotype were carried out in white and South Asian only. The frequency of the Thr715Pro P-selectin gene polymorphism did not vary by smoking status in white ($\chi^2 = 0.75$; $p = 0.94$) or in South Asian ($\chi^2 = 2.86$; $p = 0.58$). Likewise, the frequency of this polymorphism was not affected by gender in white ($\chi^2 = 0.42$; $p = 0.81$) or South Asian ($\chi^2 = 2.35$; $p = 0.31$).

In whites, age- and sex-adjusted sP-selectin levels were significantly lower in the 45 individuals with the AC or CC genotype (57 ng/ml (52 to 63)) compared with the 192 individuals with the AA genotype (76 ng/ml (73 to 80)) (Difference 25% (-33.3 to -16.9); $p < 0.001$; (Fig. 1A). This difference was also maintained when females and males were analysed separately (85 females with AA genotype (71 ng/ml (66 to 76) compared with 21 with AC or CC genotype 56 ng/ml (49 to 64); Difference -21.3% (-32.4 to -8.3) $p = 0.003$); (107 males with AA genotype (81 ng/ml (76 to 86) compared with 24 with AC or CC genotype 58 ng/ml (51 to 66) (Difference -28.0% (-37.4 to -17.2); $p < 0.001$)).

Likewise, in the South Asians the 10 individuals with either the AC or CC genotype had significantly lower levels (53 ng/ml (42 to 67)) than the 191 individuals with the AA genotype (72 ng/ml (69 to 74)) (Difference -25.2% (-40.5 to -6.1); $p < 0.012$; (Fig. 1A).

In whites, the variation in age- and sex-adjusted soluble sP-selectin levels was graded across genotype ($p < 0.001$) (Fig. 1B) indicating an additive (dose-dependent) model of expression.

Table 1: Age and sex adjusted characteristics of 615 men and women aged 40-59 years of different ethnic groups living in Wandsworth, South London, 1994-96. Results are means or percentages (95% C.I.).

<table>
<thead>
<tr>
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<tr>
<td>Group</td>
<td>AA</td>
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<tr>
<td>Total group (n=237)</td>
<td>76 (73 to 80) (n=192)</td>
</tr>
<tr>
<td>Males (n=131)</td>
<td>81 (76 to 86) (n=107)</td>
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<tr>
<td>Females (n=106)</td>
<td>71 (66 to 76) (n=85)</td>
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<tr>
<td>Younger§ (n=123)</td>
<td>77 (72 to 82) (n=97)</td>
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<tr>
<td>Older§ (n=114)</td>
<td>76 (72 to 81) (n=95)</td>
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<tr>
<td>Current smokers (n=75)</td>
<td>79 (73 to 85) (n=63)</td>
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<tr>
<td>Ex-smokers (n=86)</td>
<td>79 (72 to 85) (n=69)</td>
</tr>
<tr>
<td>Never smoked (n=75)</td>
<td>68 (63 to 74) (n=59)</td>
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§ $\leq$ or $>$ median age of 48 years
This dose-dependent effect of the C allele on sP-selectin levels in whites was maintained in men and women separately, in younger and older participants as well as in smokers and non-smokers (Table 2).

In both whites and South Asians, sP-selectin levels were lower in individuals with the AC+CC genotype compared with the AA genotype even after multivariate analyses. Adjustments were made for increasing numbers and combinations of potential confounding risk factors including smoking, lipids, insulin, homocysteine, social class and blood pressure (Fig. 2 and Table 3). For example, in whites sP-selectin levels were lower by 25% (17.8 to 32.8%) (p < 0.001) in individuals with the AC and CC genotype compared with the AA genotype following adjustment for age, sex, smoking, BMI, triglycerides, systolic blood pressure and insulin levels. Similarly in South Asians, the values were also reduced (25.8% (6.5 to 41.2%); p = 0.012).

**Discussion**

sP-selectin levels vary by ethnic group independently of main confounders (12). This study shows for the first time that (a) the frequency of the Thr715Pro polymorphism varies substantially by ethnic group; (b) the C allele is less common in South Asians than whites and rare in people of African origin; (c) the presence of the C allele is associated with lower levels of sP-selectin in both whites and South Asians; (d) at least in whites, the C allele is associated with lower levels according to an additive (dose-dependent) model.

Soluble adhesion molecules are associated with CHD, atherosclerosis and CHD risk factors but the factors which govern the level of these circulating soluble adhesion molecule levels, including cell surface shedding and clearance have not been fully investigated (7). This study is the first to show that the frequency of the Thr715Pro polymorphism varies in different ethnic groups and that in South Asians, as shown previously in whites (9, 16, 17), the levels of circulating sP-selectin vary according to genotype. In whites, the C allele reduced sP-selectin levels dose-dependently suggesting an additive model of expression. However, despite this association and the reduced frequency of the ‘protective’ C allele in South Asians, there was no difference in sP-selectin levels between whites and South Asians.
Asians. More surprisingly, given that sP-selectin levels are significantly lower in African individuals, we did not find the expected excess of the C allele in these individuals. Hence, although this polymorphism contributes to sP-selectin levels in whites and South Asians it is unlikely to be responsible for the observed ethnic differences in sP-selectin levels. Whilst the exact role of the consensus tandem repeat domains are unknown it is possible that this polymorphism may alter intracellular transportation or secretion of P-selectin or that the 715Pro allele may be associated with a reduction in the membrane bound form of P-selectin (17). Studies investigating the CR4 and CR9 domains have also suggested that they may play a role in modulating P-selectin-leukocyte interactions (18, 10).

The finding that people of African origin, who have the lowest CHD risk, have the lowest frequency of the protective allele is consistent with the results from the ECTIM (Etude cas-témoin de l’infarctus myocarde) study, in which the Pro715 allele was more frequent in individuals with a higher risk of MI in Belfast than those at lower risk from France (9). They suggested it might be an example of a protective allele that is found with greater frequency in high-risk than low-risk populations. The incidence of CHD is lower in Africans than Caribbeans and all black individuals with the C allele were of Caribbean origin (Fisher’s exact test p = 0.038). Our C allele frequency in whites without CHD (11.2%) was comparable to the estimates from the ECTIM study for non-MI patients (Belfast (17.4%), France (10.7%)) (9).

Previous studies have demonstrated an age- (16) and sex-dependence of sP-selectin levels (17). In both this and our previous study (12) we have found that sP-selectin levels vary with gender. Unlike Barbaux et al. (16) we have not found a relationship with age but the age range of our individuals is narrow. In this study we have clearly demonstrated that in whites the C allele was associated with sP-selectin levels in a dose-dependent manner, irrespective of gender, age or smoking status (Table 2). Furthermore, in both white and South Asian individuals, the difference in sP-selectin levels between those individuals with the AA genotype and those with either the AC or CC genotype was maintained following multiple regression. Increasing numbers and differing combinations of cardiovascular risk factors were adjusted for as seen in figure 2 and in Table 3. In a study of patients with coronary artery disease, Barbaux et al. reported an effect of smoking on sP-selectin levels which was confined to individuals with the Pro715 allele (16). Carter et al. demonstrated that the Pro715 allele was associated with lower levels of sP-selectin but only in non-smokers (17). In our population-based study of essentially healthy individuals we have shown that smoking is associated with an increase in sP-selectin levels (12), that the frequency of the polymorphism does not vary by smoking status and that the lower levels of sP-selectin in the presence of the C allele is independent of smoking status (Table 2).

The strengths and limitations of our population-based study design have been discussed previously (12). The study examined first-generation immigrants of ethnic minority groups with both parents born in the country of origin and belonging to the same ethnic background, thus markedly reducing the possible impact arising from an unknown degree of admixture. Significant linkage disequilibrium within the P-selectin gene has been demonstrated (9). We only studied one polymorphism and we cannot exclude the fact that our results may be explained by a combination of genetic variants or specific haplotypes that include the Pro715 allele. A recent study confirmed that the Pro715 allele is protective for MI and showed that two asparagine codons (S290N and N562D) are associated with a higher risk of MI in individuals from France and Ireland but only when carried on the same haplotype (19). In addition, we have previously demonstrated (12) that not only are sP-selectin levels significantly lower in individuals of African origin compared to whites but that levels are significantly different in Caribbean (62 (59 to 67) ng/ml; n = 111) compared to West African individuals (51 (47 to 55) ng/ml; n = 77). In this study we were
unable to investigate genotype frequency or the effect of genotype and ethnicity on sP-selectin levels in individuals of different African origin as the C allele was too rare. Further studies are therefore needed.

The idea that adhesion molecules may be predictors of atherosclerosis and of CHD risk is attractive, though still controversial (7, 20). Although the Thr715Pro polymorphism may protect individuals from MI (9), our results indicate that whilst the Pro715 allele is associated with reduced sP-selectin levels in whites and South Asians, the ‘so-called’ protective allele is paradoxically rare in people of African origin. This suggests that even if this polymorphism is causally related to sP-selectin expression in whites and South Asians, it does not play a role in determining ethnic differences in sP-selectin levels. Prospective studies are required to further investigate the effect of this and associated polymorphisms / haplotypes of the P-selectin gene on CHD risk in different ethnic populations.

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