Thomas Jefferson University
Jefferson Digital Commons

Department of Medicine Faculty Papers | Department of Medicine

June 2008

Beta3 integrin haplotype influences gene regulation and plasma von Willebrand factor activity

Katie E. Payne
University of Leeds

Paul F. Bray
Thomas Jefferson University, paul.bray@jefferson.edu

Peter J. Grant
University of Leeds

Angela M. Carter
University of Leeds

Let us know how access to this document benefits you

Follow this and additional works at: http://jdc.jefferson.edu/medfp

Part of the Medical Genetics Commons

Recommended Citation

Payne, Katie E.; Bray, Paul F.; Grant, Peter J.; and Carter, Angela M., "Beta3 integrin haplotype influences gene regulation and plasma von Willebrand factor activity" (2008). *Department of Medicine Faculty Papers.* Paper 33.
http://jdc.jefferson.edu/medfp/33

This Article is brought to you for free and open access by the Jefferson Digital Commons. The Jefferson Digital Commons is a service of Thomas Jefferson University's Academic & Instructional Support & Resources Department (AISR). The Commons is a showcase for Jefferson books and journals, peer-reviewed scholarly publications, unique historical collections from the University archives, and teaching tools. The Jefferson Digital Commons allows researchers and interested readers anywhere in the world to learn about and keep up to date with Jefferson scholarship. This article has been accepted for inclusion in Department of Medicine Faculty Papers by an authorized administrator of the Jefferson Digital Commons. For more information, please contact: JeffersonDigitalCommons@jefferson.edu.

β_3 integrin haplotype influences gene regulation and plasma von Willebrand factor activity

Katie E. Payne [a], Paul F. Bray [b], Peter J. Grant [a], Angela M. Carter [a]

[a] *Academic Unit of Molecular Vascular Medicine, Leeds Institute for Genetics, Health and Therapeutics, University of Leeds, UK*

[b] *Jefferson Medical College and the Cardeza Foundation for Hematologic Research, Philadelphia, USA*

Abstract

The Leu33Pro polymorphism of the gene encoding β_3 integrin (*ITGB3*) is associated with acute coronary syndromes and influences platelet aggregation. Three common promoter polymorphisms have also been identified. The aims of this study were to (1) investigate the influence of the *ITGB*3 −400C/A, −425A/C and −468G/A promoter polymorphisms on reporter gene expression and nuclear protein binding and (2) determine genotype and haplotype associations with platelet $\alpha_{IIb}\beta_3$ receptor density. Promoter haplotypes were introduced into an *ITGB*3 promoter-pGL3 construct by site directed mutagenesis and luciferase reporter gene expression analyzed in HEL and HMEC-1 cells. Binding of nuclear proteins was assessed by electrophoretic mobility shift assay. The association of *ITGB*3 haplotype with platelet $\alpha_{IIb}\beta_3$ receptor density was determined in 223 subjects. Species conserved motifs were identified in the *ITGB*3 promoter in the vicinity of the three polymorphisms. The GAA, GCC, AAC, AAA and ACC constructs induced ~ 50% increased luciferase expression relative to the GAC construct in both cell types. Haplotype analysis including Leu33Pro indicated five common haplotypes; no associations between *ITGB*3 haplotypes and receptor density were found. However, the GCC-Pro33 haplotype was associated with significantly higher vWF activity (128.6 [112.1–145.1] %) compared with all other haplotypes (107.1 [101.2–113.0] %, p = 0.02). In conclusion, the GCC-Pro33 haplotype was associated with increased vWF activity but not with platelet $\alpha_{IIb}\beta_3$ receptor density, which may indicate *ITGB*3 haplotype influences endothelial function.

1. Introduction

Receptor-mediated interactions between leukocytes, endothelial cells and platelets and coagulation factors and extracellular matrix proteins at the sites of vascular injury are central to the pathogenesis of atherosclerosis and thrombosis [1]. β_3 integrin is a component of the platelet-specific $\alpha_{IIb}\beta_3$ receptor and the more widely distributed α_v β_3 receptor. Platelets play a key role in arterial thrombosis and the $\alpha_{IIb}\beta_3$ receptor is central to these processes as the mediator of platelet aggregation at the site of vascular injury. The α_v β_3 receptor is variably expressed by a number of cell types including endothelial cells and vascular smooth muscle cells [2]. Increased endothelial cell and vascular smooth muscle cell expression of α_v β_3 in coronary artery atherosclerotic plaques have been reported [3]. Consequently variants of the gene encoding β_3 integrin (*ITGB3*) may influence the development of cardiovascular disease through modification of the function of $\alpha_{IIb}\beta_3$ and α_v β_3.

Several polymorphisms in the *ITGB*3 gene have been identified including the Leu33Pro (Pl^A) variant and the −400C/A, −425A/C and −468G/A promoter polymorphisms [4]. Studies have demonstrated associations between the Pro33 allele (Pl^{A2}) and premature myocardial infarction (MI), ischemic stroke and restensosis following coronary angioplasty [5, 6]. Pro33 is associated with a lower threshold for $\alpha_{IIb}\beta_3$ activation in response to ADP, and enhanced α-granule release, clot retraction and adhesion to fibrinogen [7, 8]. Limited information is available regarding the functional effects of the −400C/A, −425A/C and −468G/A promoter polymorphisms. The aims of this study were to (I) investigate the influence of the −400C/A, −425A/C and −468G/A promoter gene polymorphisms on reporter expression and nuclear protein binding and (ii) determine genotype and haplotype associations with platelet $\alpha_{IIb}\beta_3$ receptor density.

2. Materials and methods

2.1. Bioinformatics

Bioinformatics analyses were carried out to compare the 5′ region of the human (accession number AY706100), chimp (AY413572), mouse (AY413573) and chicken (X75348) *ITGB*3 genes to determine regions of conservation. The first 500 bp 5′ to the ATG of each species were analyzed by FootPrinter against the default phylogenetic tree to indicate potential regulatory elements [9]. The software produced short regions of sequences (up to 10 bp in length) that were highly conserved (maximum of 2 bp difference) between species.

2.2. *Generation of variant ITGB*3 *reporter constructs*

The *ITGB*3 promoter region was cut from a previously reported *ITGB3*-pGL2 vector [10] and ligated into pGL3 by digestion with five units of KpnI and NheI, and ligation with T4 ligase. The wild type *ITGB*3 5′ region construct will be referred to as *ITGB3*-pGL3-GAC in relation to the more common alleles located at positions −468, −425 and −400. Haplotypes were introduced into the *ITGB3*pGL3-GAC construct by site directed mutagenesis using the GeneEditor™ in vitro Site Directed Mutagenesis system (Promega). Mutagenic oligonucleotides were designed with the relevant mismatch in the middle:

−468A: 5′-GGCATTCA**A**CAGATGTTTG-3′;
−425C: 5′-GTGTGAATGAATGA**C**ACTCGAGGTAGT
GG-3′;
−400A: 5′-GTGAATGTGT**A**CCAAGAATC-3′.

The −400A and −468A variants were introduced using the manufacturer's standard protocol. A modified protocol was utilized to generate the −425C variant; the mutagenic oligonucleotide was added at the alkaline denaturation stage, and the initial temperature for oligonucleotide annealing was increased to 83°C. Twenty colonies were picked for each mutagenesis reaction and successful mutagenesis confirmed by genotyping and sequencing. The sequence of the *ITGB*3 promoter (from the ATG codon to −2070) has been submitted to GenBank, accession number AY706100.

2.3. *Cell culture and transient transfections*

Human erythroleukemic (HEL 92.1.7) cells ($\alpha_{IIb}\beta_3$ expressing, representative of megakaryocytic cells) were grown in RPMI 1640 medium (Sigma), supplemented with 10% foetal calf serum (FCS) (First Link Ltd.), 2 mM glutamine and antibiotic mix (10,000 U penicillin, 10 mg streptomycin and 25 μg amphotericin B per ml). Cells were transiently co-transfected by electroporation with the *ITGB3*pGL3 constructs and Renilla Luciferase-CMV (pRL-CMV) vector (Promega), as the internal control, at a ratio of 50:1. For transfection, 1×10^7 cells per sample were pelleted and resuspended in 0.8 ml PBS, 10 μg of DNA was added and cells were electroporated at 500 μF, 0.4 kV (BioRad Gene Pulser). The electroporated cells were seeded in 25 ml of pre-warmed media and incubated at 37 °C and 5% CO2 for 48 h prior to reporter gene analysis.

Human Microvascular Endothelial cells (HMEC-1, αVβ3 expressing, representative of endothelial cells) were grown in MCDB 131 media (Invitrogen), supplemented with 10% FCS, antibiotic mix, 2 mM glutamine, 0.001% epidermal growth factor and 1 μg/ml hydrocortisone. HMEC-1 cells were transiently transfected with Lipofectin (Invitrogen): 5×10^5 cells were plated on 60 mm plates 24 h prior to transfection with 3 μg DNA (100:1 ratio of *ITGB3*-pGL3 constructs to pRL-CMV) and 20 ml Lipofectin, according to manufacturer's instructions, followed by incubation at 37°C and 5% CO_2 for 24 h prior to reporter gene analysis.

2.4. Luciferase reporter gene expression analysis

Luciferase reporter gene analysis was carried out using the Dual-Luciferase Reporter Assay system (Promega). Transfected HEL cells were pelleted by centrifugation, washed, and 1×10^7 cells lysed with 250 μl passive lysis buffer (PLB). Transfected HMEC-1 cells were washed, and lysed with 1 ml PLB. Renilla and firefly luciferase activities were determined in 20 ml of centrifuged cell lysate according to manufacturer's instructions using a PHL luminometer (Mediators). Firefly luciferase values were normalized to Renilla luciferase activity. Eight independent sets of transfections were carried out, and results are expressed as the activity of each construct relative to the *ITGB3*-pGL3-GAC construct.

2.5. Electrophoretic mobility shift assay (EMSA)

Nuclear proteins were extracted from 5×10^6 HEL and HMEC-1 cells according to the method described by Andrews and Faller [11]. Fluorescein-labelled sense oligonucleotides were synthesized with the polymorphic variant in the centre, as follows:

-468G/A: forward 5′-AGAGAAGGCATTCA$^{G}/_{A}$CAGATGTTTGCCAG-3′
reverse 5′-CTGGCAAACATCTG$^{C}/_{T}$TGAATGCCTTCTCT-3′
-425A/C: forward 5′-GTGTGAATGAATGA$^{A}/_{C}$ACTCGAGGTAGTGG-3′
reverse 5′-CCACTACCTCGAGT$^{T}/_{G}$TCATTCATTCACAC-3′
-400C/A: forward 5′-GTGGGTGAATGTGT$^{C}/_{A}$CAAGAATCCAGCG-3′
reverse 5′-CGCTGGATTCTTG$^{G}/_{T}$ACACATTCACCCAC-3′

Sense and antisense oligonucleotides were annealed and gel purified, and 1.5 pmol

used in each binding reaction. EMSA reactions were performed in binding buffer (20% glycerol, 20 mM Tris, pH 7.5, 100 mM KCl, 1 mM DTT) with 0.1 μg/μl poly dI/dC and 35 μg of nuclear protein extract. For competition assays, 1-, 10-and 50-fold molar excess of unlabelled specific or non-specific competitor (SP1: forward 5′-ATTCGATCGGGGCGGGGCGAGC-3′ and reverse 5′-GCTCGCCCCGCCCCGATCGAA-3′) were added. Reaction mixes were incubated at RT for 30 min and then electrophoresed on an 8% polyacrylamide gel with 5% stacking gel in 0.25× TBE (22.5 mM Tris-borate and 0.5 mM EDTA, pH 8.3) at 200V for 6h at 15°C. The gel was visualized using a FX Molecular Imager (BioRad).

2.6. Genotyping of ITGB3 polymorphisms

The −468G/A polymorphism were genotyped using an Assays-by-Design^{SM} SNP genotyping assay (Applied Biosystems):

forward primer 5′ -GGCAAGAAAAAAATTAGTGAATAATAAAGGACTGA-3′

reverse primer 5′-ACTCGAGGTAGTGGGTGAATGT-3′

probes 5′-AAGGCATTCA$^{G}/_{A}$CAGATG-3′

Probes were labeled with VIC™ for detecting the −468G allele and 6-FAM™ for detecting the −468A allele, and following PCR amplification, fluorescence detection was carried out using an ABI PRISM 7700 SDS (Applied Biosystems). The −425A/C polymorphism was genotyped by PCR-RFLP with primers: forward 5′-CGAGGCTCTTCATGGACCTA3′ and reverse 5′-CACATTCACCCACTACCTCGA<u>A</u>T-3′ and digestion with ApoI (the underlined base is a mismatch to introduce an ApoI site in the presence of the −425A allele). The −400C/A polymorphism were genotyped by PCR–RFLP with primers: forward 5′-AGTGAATAATAAAGGACTGAACCG-3′ and reverse 5′-GCGCTCGCATCTCGTC-3′ and digestion with RsaI. The Leu33Pro polymorphism was genotyped as previously described [12].

2.7. Subject recruitment and analysis of αIIbβ$_3$ receptor expression

Subjects (n = 233) were recruited by the Baylor College of Medicine Thrombosis Research Group for analysis of the relationship between *ITGB*3 polymorphisms and *α*IIb*β*$_3$ receptor expression. The study population comprised healthy subjects, recruited through local advertising, who were free from a history of cardiovascular disease and were not taking aspirin (n = 138), as previously described [13] and subjects who had previously (>3 months) undergone coronary artery bypass surgery (CABG, n = 85) who were taking aspirin. All subjects gave informed consent according to a protocol approved by the Baylor College of Medicine institutional review board. Platelet *α*IIb*β*$_3$ receptor expression was measured by flow cytometry using phycoerythrin-conjugated monoclonal antibody HIP8 (anti-CD41a; BD-Pharmingen) and expressed as mean fluorescent intensity, as previously described [14]. Fibrinogen concentration was determined by the Clauss method and vWF activity was determined using a ristocetin cofactor assay, as previously described [14].

2.8. Statistical analysis

Non-normally distributed variables were log-transformed to enable parametric analyses. Comparisons between two groups of continuous variables were carried out using Student's independent *t*-test and more than two groups by ANOVA. Data are presented as mean (geometric mean) with either S.D. (antilog S.D.) or 95% confidence intervals (CI). Age did not conform to normal distribution and is presented as median with 25th and 75th percentiles in descriptive statistics. Differences between categorical variables were ascertained using Chi-square analysis. All statistical analyses were performed using SPSS for Windows version 12 (SPSS Inc).

3. Results

3.1. Bioinformatics analysis of the ITGB3 promoter

FootPrinter analysis (Fig. 1) indicated conserved motifs in the *ITGB*3 5′ region located in the region up to 500 bp from the ATG codon. The −400C/A polymorphism is within a motif conserved between human and chimp (TGTGTCCCAA) and the −425A/C and −468G/A polymorphisms are between conserved motifs. The evolutionary conservation of regions within the 5′ gene regulatory region indicated that these sequences might be functionally relevant. To determine if the promoter polymorphisms were functional, the luciferase reporter gene system was utilized together with analysis of nuclear protein binding.

3.2. Influence of ITGB3 −468/−425/−400 haplotypes on luciferase reporter gene expression and nuclear protein binding profile

Luciferase reporter gene analyses indicated significant differences in reporter gene expression according to *ITGB*3 promoter haplotype in both HEL ($p = 0.035$) and HMEC-1 cells ($p = 0.048$), as shown in Fig. 2. Similar luciferase expression patterns were obtained from the HEL and HMEC-1 cells. The GAA, GCC, AAC, AAA and ACC constructs induced ~ 50% increased luciferase expression relative to the GAC construct whereas the GCA and ACA constructs showed similar expression to GAC in both cell types. These results indicated that the three polymorphisms in the *ITGB*3 promoter may be functional and lead to a difference in nuclear protein binding. Each polymorphic variant was, therefore, analyzed for differences in nuclear protein binding with HEL and HMEC-1 nuclear protein extracts. No difference in the nuclear protein binding profile was observed for the −400C/A, −425A/C and −468G/A allelic variants (data not shown).

3.3. Association of ITGB3 polymorphisms and haplotype with platelet $\alpha_{IIb}\beta_3$ receptor density

The relationship between *ITGB*3 polymorphisms and $\alpha_{IIb}\beta_3$ receptor density was investigated in 223 subjects. The characteristics of the study population, including ethnicity, are presented in Table 1. The mean receptor density (expressed as mean fluorescence intensity, MFI) was 136 (range: 81–192). There was no significant difference in mean receptor density between the different ethnic groups studied (Caucasian: 137 [S.D. 18]; African American: 134 [S.D. 19]; Mexican American: 135 [S.D. 16]; Asian: 143 [S.D. 27]; other: 131 [S.D. 22], $p = 0.59$). $\alpha_{IIb}\beta_3$ receptor density was significantly inversely correlated with BMI ($r = -0.264$, $p < 0.001$) and fibrinogen ($r = -0.155$, $p = 0.02$) but there was no correlation between $\alpha_{IIb}\beta_3$ receptor density and vWF activity ($r = -0.019$, $p = 0.78$). There was no significant correlation between receptor density and age ($r = -0.096$, $p = 0.16$) and no significant difference in $\alpha_{IIb}\beta_3$ receptor density between men and women, or between smokers and non-smokers (data not shown). There was no significant difference in receptor density between CABG patients taking aspirin and healthy subjects not taking aspirin, nor was there a significant difference in receptor density between those with and without a history of MI, or hypertension (data not shown).

The genotype distributions of the −400C/A, −425A/C, −468G/A and Leu33Pro polymorphisms conformed to Hardy Weinberg equilibrium. The −400C/A polymorphism was in significant linkage disequilibrium with the −425A/C ($D' = -1.0$, $p = 0.006$) and −468G/A polymorphisms ($D' = 0.93$, $p < 0.0001$) but not with the Leu33Pro polymorphism. The −425A/C polymorphism was in significant linkage disequilibrium with the −468G/A ($D' = -0.66$, $p = 0.04$) and the Leu33Pro ($D' = 0.67$, $p < 0.0001$) polymorphisms. There was no significant linkage disequilibrium between the −468G/A and Leu33Pro polymorphisms. Six promoter haplotypes were identified, as shown in Table 2 (denoted A–F), with the four most frequent haplotypes accounting for 98% of the 446 chromosomes analyzed. Inclusion of the Leu33Pro polymorphism in haplotype analysis gave rise to 11 different haplotypes, as shown in Table 2 (denoted 1–11), with the five most common haplotypes accounting for 94% of the 446 chromosomes analyzed.

Analysis of the relationship of individual SNPs with receptor density did not indicate any association of genotype with receptor density, as shown in Table 3. The relationship of receptor density with promoter haplotype and haplotype including Leu33Pro was also analysed; there was no significant difference in receptor density according to *ITGB*3 haplotypes (data not shown).

3.4. Association of ITGB3 polymorphisms and haplotype with plasma fibrinogen and vWF activity

There was no significant difference in fibrinogen according to *ITGB*3 genotype, as shown in Table 3, or haplotype (data not shown). There was no significant association of the −400C/A and −468G/A polymorphisms with vWF activity, however, there was a significant association of the −425A/C polymorphism and a borderline association of Leu33Pro with vWF activity, as shown in Table 3. Analysis of the relationship of promoter haplotypes and haplotypes including Leu33Pro with vWF activity did not indicate any significant associations (data not shown), however, the data was suggestive of potential differences in vWF activity, particularly in haplotype combinations including haplotype 3 (GCC-Pro33). To investigate this further we created a dummy variable including all subjects with haplotype combinations including haplotype 3 ($n = 43$) compared with subjects homozygous for haplotype 1 (GAC-Leu33) and compared vWF activity: vWF was significantly higher in subjects possessing haplotype 3 (128.6 [112.1, 145.1]%) compared to subjects homozygous for haplotype 1 (106.3 [98.1, 114.5] %, $p = 0.02$). vWF was also significantly higher in subjects possessing haplotype 3 when compared with all other subjects (107.1 [101.2, 113.0]%, $p = 0.02$).

vWF activity differed significantly by ethnicity, with African American subjects having significantly lower vWF (96.6 [S.D. 37.8] %) than Caucasian subjects (120.4 (S.D. 44.1) %, $p = 0.02$ after Sheffe post hoc analysis). Furthermore, although *ITGB*3 haplotype did not differ significantly by ethnicity, haplotype 3 was less prevalent in African Americans (10.4%) than Caucasians (21.4%, $p = 0.08$). Therefore we analysed the relationship of haplotype 3 with vWF activity accounting for ethnicity and haplotype 3 remained significantly higher in those possessing haplotype 3 ($p = 0.03$).

4. Discussion

Regions of a gene that are conserved between species are likely to be functionally significant and bioinformatics analysis of the region of *ITGB*3 promoter containing the −400C/A, −425A/C and −468G/A polymorphisms indicated a number of regions conserved between species, suggesting they may be important in regulation of *ITGB*3 expression. Luciferase reporter gene analyses in cells representative of megakaryocyte and endothelial cells indicated modest (~50%) increases in reporter gene expression for the GAA, GCC, AAC, AAA and ACC constructs relative to the GAC construct, supporting a potential role for the polymorphisms in modulating gene expression. In contrast, EMSA analyses did not indicate any clear difference in nuclear protein binding pattern between the alleles of each polymorphism. However, luciferase reporter gene and EMSA analyses do not take into account the native chromatin structure of the human *ITGB*3 gene and therefore the inconsistency of results between the two methods does not in itself negate an effect of the polymorphisms on *ITGB*3 expression *in vivo*. Therefore, to determine whether the differences in reporter gene expression translated into differences in $\alpha_{IIb}\beta_3$ expression in platelets, genotype and haplotype associations with receptor expression were determined.

The extent of linkage disequilibrium between the promoter polymorphisms and the Leu33Pro polymorphism were similar to those reported by O'Halloran et al. [15]. Six of the eight potential promoter haplotypes were identified in the present study and inclusion of the Leu33Pro polymorphism resulted in the detection of 11 haploytpes, in keeping with those of O'Halloran et al. [15]. The prevalence of the three most common haplotypes (GAC-Leu33, AAA-Leu33, GCC-Pro33) were approximately equivalent in the two study groups, with the majority of Pro33 alleles associated with the GCC promoter haplotype and the Leu33 allele associated predominantly with the GAC promoter haplotype. There was some discrepancy in the occurrence of the less frequent haplotypes, which likely reflects ethnic heterogeneity in the present study.

We found a wide range in platelet $\alpha_{IIb}\beta_3$ expression which was not explained by *ITGB*3 promoter and Leu33Pro genotype or haplotype. Previous reports of the association of Leu33Pro with receptor density have been inconsistent [7]; however, the results of the present study lend further support for a lack of association of *ITGB*3 promoter polymorphisms and Leu33Pro with receptor density in unstimulated platelets. These results are inconsistent with the luciferase reporter gene results in which the common GCC and AAA promoter haplotypes were associated with a 50% increase in luciferase reporter gene expression compared with the GAC haplotype. The influence of the promoter polymorphisms on $\alpha_v\beta_3$ expression in human endothelial cells or smooth muscle cells cannot be inferred from the results of our study regarding $\alpha_{IIb}\beta_3$ receptor expression due to cell specific regulation of $\alpha_{IIb}\beta_3$ and $\alpha_v\beta_3$ receptor expression. Increased $\alpha_{IIb}\beta_3$ receptor expression is reported to arise from increased steady-state mRNA levels of both α_{IIb} and β_3 mRNA resulting from enhanced transcription [16]. In contrast, the expression of $\alpha_v\beta_3$ appears to be largely dependent upon regulation of β_3 expression, both at the transcriptional and post-transcriptional level [17, 18]. Therefore it cannot be excluded that the influence of the promoter polymorphisms on *ITGB*3 gene expression may have more of an influence on the ultimate expression of $\alpha_v\beta_3$ than on the expression of $\alpha_{IIb}\beta_3$.

We found an association of *ITGB*3 -425A/C and Leu33Pro polymorphisms and the GCC-Pro33 haplotype (hap3) with vWF activity. Since vWF is released from endothelial cell Weibel Palade bodies this may reflect an influence on endothelial cell function. Shear stress induces activation of endothelial cell $\alpha_v \beta_3$ [19] and it has been shown that shear stress also induces increased secretion of vWF from endothelial cell Weibel Palade bodies [20]. Therefore it is possible that shear stress elicits differential responses in endothelial cells expressing different *ITGB*3 haplotypes. Studies of the Leu33Pro polymorphism indicated differential effects on platelet function, with the Pro33 allele associated with enhanced outside-in signaling in platelets and increased αgranule secretion [21]. In addition, enhanced cell migration on vitronectin and osteopontin was observed in CHO cells expressing $\alpha_v \beta_3$ Pro33 [22], which may lend support for a functional effect of Leu33Pro in endothelial cells.

5. Conclusions

Bioinformatics and reporter gene analyses suggested that the *ITGB*3 −468G/A, −425A/C and −400C/A polymorphisms may be functional. However, promoter haplotypes and promoter haplotypes in combination with the Leu33Pro polymorphism were not significantly associated with $\alpha_{IIb}\beta_3$ receptor density, despite wide inter-individual differences in receptor density. The GCC-Pro33 haplotype was, however, associated with enhanced vWF activity, which may indicate *ITGB*3 haplotype influences endothelial function. Further studies are required to determine whether *ITGB*3 haplotype is associated with enhanced $\alpha_v \beta_3$ expression or activity.

Acknowledgements

This study was funded through grants from the British Heart Foundation (Ph.D. studentship FS/2001012, KEP) and the Heart, Lung and Blood Institute of the National Institutes of Health (HL65229, PB).

References

[1] Jang Y, Lincoff AM, Plow EF, Topol EJ. Cell adhesion molecules in coronary artery disease. J Am Coll Cardiol 1994;24:1591–601.
[2] Wilhide CC, Jin Y, Guo Q, et al. The human integrin β_3 gene is 63 kb and contains a 5′-UTR sequence regulating expression. Blood 1997; 90:3951–61.
[3] Hoshiga M, Alpers CE, Smith LL, Giachelli CM, Schwartz SM. $\alpha_v \beta_3$ integrin expression in normal and atherosclerotic artery. Circ Res 1995;77:1129–35.
[4] Negrier C, Grenier C, Attali O, Dechavanne M, Vinciguerra C. Identification of new and known polymorphisms in glycoprotein IIb and IIIa genes by denaturing gradient gel electrophoresis. Platelets 1998;9:374–80.
[5] Weiss EJ, Bray PF, Tayback M, et al. A polymorphism of a platelet glycoprotein receptor as an inherited risk factor for coronary thrombosis. N Engl J Med 1996;334:1090–4.

[6] Carter AM, Catto AJ, Bamford JM, Grant PJ. Platelet GPIIIa Pl^A and GPIb variable number tandem repeat (VNTR) polymorphisms and markers of platelet activation in acute stroke. Arterioscler Thromb Vasc Biol 1998;18:1124–31.
[7] Michelson AD, Furman MI, Goldschmidt-Clermont P, et al. Platelet GP IIIa Pl(A) polymorphisms display different sensitivities to agonists. Circulation 2000;101:1013–8.
[8] Vijayan KV, Liu Y, Dong JF, Bray PF. Enhanced activation of mitogen-activated protein kinase and myosin light chain kinase by the Pro33 polymorphism of integrin β_3. J Biol Chem 2003;278:3860–7.
[9] Blanchette M, Tompa M. FootPrinter: a program designed for phylogenetic footprinting. Nucleic Acids Res 2003;31:3840–2.
[10] Jin Y, Wilhide CC, Dang C, et al. Human integrin β_3 gene expression: evidence for a megakaryocytic cell-specific cis-acting element. Blood 1998;92:2777–90.
[11] Andrews NC, Faller DV. A rapid micropreparation technique for extraction of DNA-binding proteins from limiting numbers of mammalian cells. Nucleic Acids Res 1991;19:2499.
[12] Carter AM, Ossei-Gerning N, Wilson IJ, Grant PJ. Association of the platelet Pl(A) polymorphism of glycoprotein IIb/IIIa and the fibrinogen Bβ448 polymorphism with myocardial infarction and extent of coronary artery disease. Circulation 1997;96:1424–31.
[13] Yee DL, Sun CW, Bergeron AL, Dong JF, Bray PF. Aggregometry detects platelet hyperreactivity in healthy individuals. Blood 2005;106:2723–9.
[14] Yee DL, Bergeron AL, Sun CW, Dong JF, Bray PF. Platelet hyperreactivity generalizes to multiple forms of stimulation. J Thromb Haemost 2006;4:2043–50.
[15] O'Halloran AM, Curtin R, O'Connor F, et al. The impact of genetic variation in the region of the GPIIIa gene, on Pl^{A2} expression bias and GPIIb/IIIa receptor density in platelets. Br J Haematol 2006;132:494–502.
[16] Zutter MM, Fong AM, Krigman HR, Santoro SA. Differential regulation of the $\alpha_2\beta_1$ and $\alpha_{IIb}\beta_3$ integrin genes during megakaryocytic differentiation of pluripotential K562 cells. J Biol Chem 1992;267:20233–8.
[17] Witzenbichler B, Kureishi Y, Luo Z, et al. Regulation of smooth muscle cell migration and integrin expression by the Gax transcription factor. J Clin Invest 1999;104:1469–80.
[18] Feng X, Clark RA, Galanakis D, Tonnesen MG. Fibrin and collagen differentially regulate human dermal microvascular endothelial cell integrins: stabilization of α_v/β_3 mRNA by fibrin. J Invest Dermatol 1999;113:913–9.
[19] Tzima E, del Pozo MA, Shattil SJ, Chien S, Schwartz MA. Activation of integrins in endothelial cells by fluid shear stress mediates Rho-dependent cytoskeletal alignment. EMBO J 2001; 20:4639– 47.
[20] Galbusera M, Zoja C, Donadelli R, et al. Fluid shear stress modulates von Willebrand factor release from human vascular endothelium. Blood 1997;90:1558–64.
[21] Vijayan KV, Liu Y, Sun W, Ito M, Bray PF. The Pro33 isoform of integrin β_3 enhances outside-in signaling in human platelets by regulating the activation of serine/threonine phosphatases. J Biol Chem 2005;280:21756–62.
[22] Sajid M, Vijayan KV, Souza S, Bray PF. PlA polymorphism of integrin β_3 differentially modulates cellular migration on extracellular matrix proteins. Arterioscler Thromb Vasc Biol 2002;22: 1984–9.

Figures and Tables

Fig.1.

FootPrinter analysis of conserved motifs in the 5′ regions of the human, chimp, mouse and chicken *ITGB*3 genes. Five hundred base pairs of the 5′ region of the human, chimp, mouse and chicken *ITGB*3 genes underwent foot printing analysis to determine if there were any regions of conservation between the different species. (Panel A) Identical motifs detected in each species' sequence have the same number. The phylogenetic tree on the right hand side shows the evolutionary relationship between the different species. (Panel B) Conserved motifs are underlined, several motifs between −90 and −140 overlapped. The positions of the −400C/A, −425A/C and −468G/A are in bold and underlined.

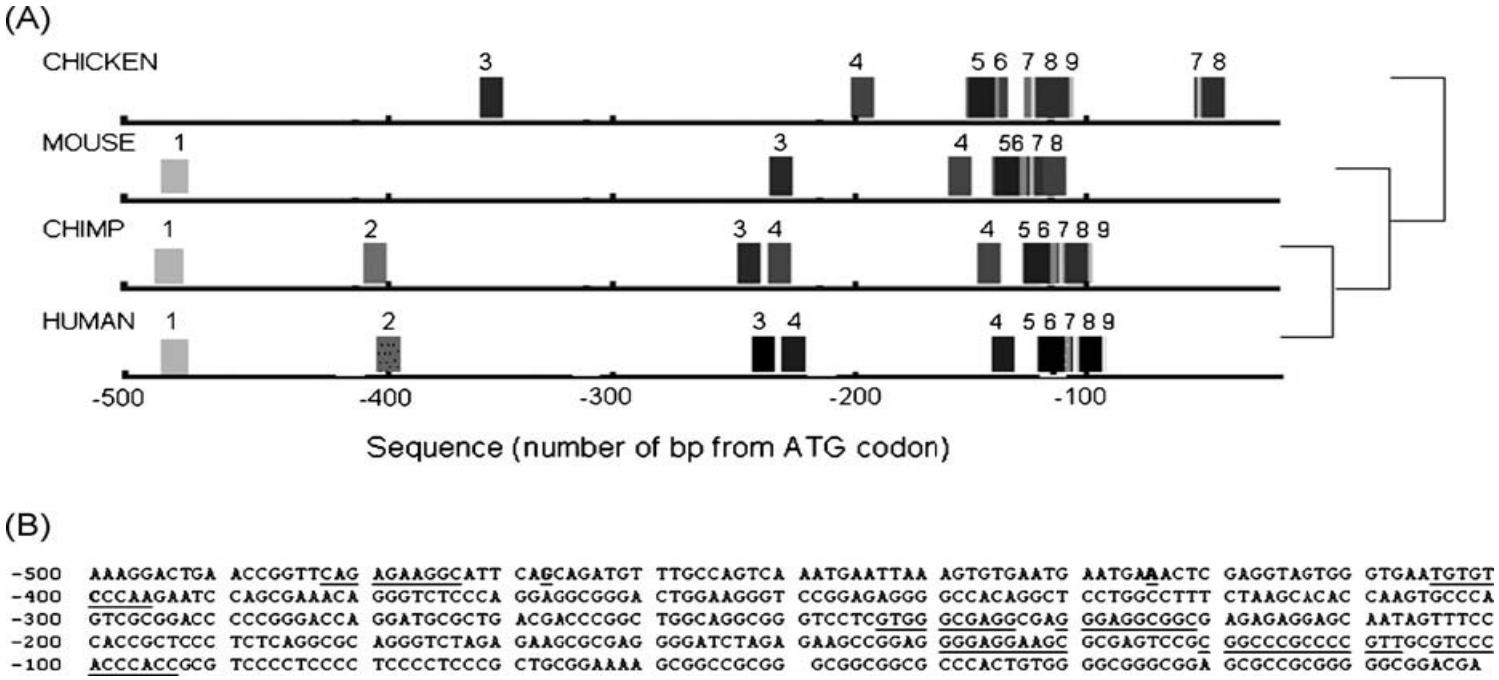

Fig. 2.

Influence of the −468G/A, −425A/C and −400C/A polymorphisms in the 5′ gene regulatory region of *ITGB*3 on firefly reporter gene expression in HEL and HMEC-1 cells. Firefly luciferase values were normalized to Renilla luciferase activity and expressed relative to the wild type GAC construct. (Panel A) firefly/renilla luciferase values for HEL cells transiently transfected with each construct. ANOVA analysis, p = 0.02. (Panel B) firefly/renilla luciferase values for HMEC-1 cells transiently transfected with each construct. ANOVA analysis, p = 0.048.

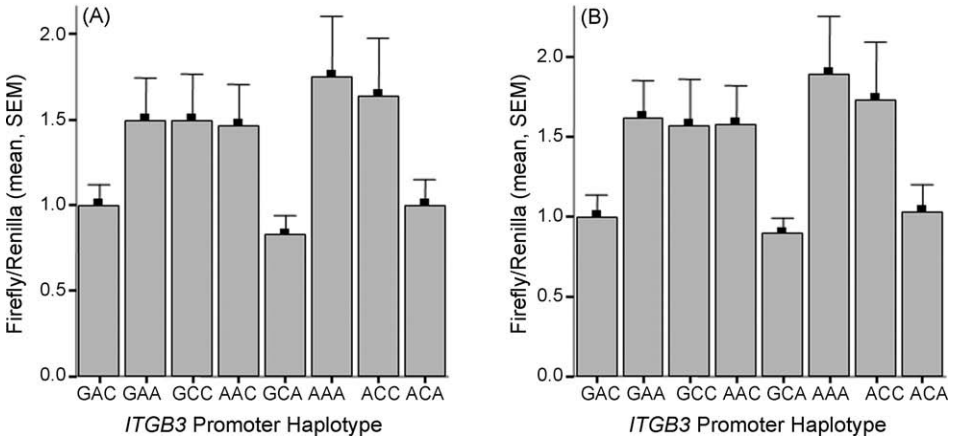

Table 1

Characteristics of study subjects

	Subjects (*n* = 223)
Age (years)	42.3 (40.1–44.5)
Male, *n*	112 (0.50)
BMI (kg/m^2)	26.6 (5.63)
Current smokers, *n*	23 (0.10)
Ethnicity	
Caucasian, *n*	131 (0.59)
African American, *n*	49 (0.22)
Mexican American, *n*	28 (0.13)
Asian, *n*	13 (0.06)
Other, *n*	2 (0.01)
Fibrinogen (gl^{-1})	3.39 (1.35)
vWF activity (%)	110.6 (42.9)

CABG, *n*	85 (0.38)
Previous MI, *n*	40 (0.18)
Hypertension, *n*	59 (0.27)
Diabetes, *n*	26 (0.12)
Aspirin[a], *n*	85 (0.38)

Age presented as median (25th and 75th percentiles). Other variables presented as mean or geometric mean (S.D. or antilog S.D.) or number (frequency). CABG: coronary artery bypass graft.
[a] all subjects taking aspirin were CABG patients.

Table 2

ITGB3 haplotype frequencies of study subjects

Promoter haplotye	Number of chromosomes (frequency)	Promoter and Leu33Pro haplotype	Number of chromosomes (frequency)
hapA: GAC	286 (0.64)	hap1: GAC-Leu33	279 (0.62)
		hap7: GAC-Pro33	7 (0.02)
hapB: AAA	69 (0.15)	hap2: AAA-Leu33	61 (0.13)
		hap6: AAA-Pro33	8 (0.2)
hapC: GCC	63 (0.14)	hap5: GCC-Leu33	20 (0.04)
		hap3: GCC-Pro33	43 (0.10)
hapD: AAC	22 (0.05)	hap4: AAC-Leu33	22 (0.05)
		AAC-Pro33	0
hapE: GAA	4 (0.01)	hap8: GAA-Leu33	3 (<0.01)
		hap9: GAA-Pro33	1 (<0.01)
hapF: ACC	2 (<0.01)	hap10: ACC-Leu33	1 (<0.01)
		hap11: ACC-Pro33	1 (<0.01)

Table 3

The relationship of *ITGB*3 polymorphisms with platelet $\alpha_{IIb}\beta_3$ receptor density, plasma fibrinogen and plasma vWF activity

	-468G/A		-425A/C		-400C/A		Leu33Pro	
	Mean (95% CI)	*p*	Mean (95% CI)	*p*	Mean (95% CI)	*p*	Mean (95% CI)	*p*
$\alpha_{IIb}\beta_3$ (MFI)	GG: 136 (133, 139)	0.12	AA: 136 (133, 139)	0.85	CC: 137 (134, 140)	0.62	LL: 137 (134, 140)	0.97
	GA: 139 (134, 143)		AC: 138 (133, 143)		CA: 137 (132, 142)		LP: 136 (131,141)	
	AA: 126 (119, 133)		CC: 138 (131, 144)		AA: 128 (120, 137)		PP: 138 (133, 143)	
Fibrinogen (gL^{-1})	GG: 3.36 (3.21,3.52)	0.62	AA: 3.36 (3.21,3.51)	0.33	CC: 3.40 (3.26,3.55)	0.92	LL: 3.35 (3.20, 3.50)	0.17
	GA: 3.51 (3.24,3.80)		AC: 3.60 (3.29,3.94)		CA: 3.43 (3.14,3.74)		LP: 3.68 (3.36, 4.02)	
	AA: 3.40 (3.06,3.78)		CC: 3.35 (2.60,4.31)		AA: 3.58 (3.30,3.90)		PP: 3.32 (2.67, 4.12)	
vWF activity(%)	GG: 112.7(105.1, 120.4)	0.47	AA: 105.4(99.6, 111.2)	0.01	CC: 113.1(106.0, 120.1)	0.42	LL: 106.8(100.5, 113.2)	0.06
	GA: 108.3(99.3, 117.3)		AC: 124.4(110.0, 138.8)		CA: 105.3(95.2, 115.3)		LP: 121.8(109.6, 134.0)	
	AA: 97.1(76.7, 117.5)		CC: 139.7(76.9, 202.5)		AA: 99.4(70.2, 128.7)		PP: 131.6(73.9, 189.3)	

Data presented as mean or geometric mean (95% confidence intervals).

MFI: mean fluorescence intensity.