Platelet adhesion to decorin but not collagen I correlates with the integrin α2 dimorphism E534K, the basis of the human platelet alloantigen (HPA)-5 system

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ABSTRACT

A single nucleotide polymorphism in the integrin α2 gene *ITGA2* (rs1801106; G1600A) creates the non-conservative amino acid substitution E534K, the basis of the human platelet alloantigen system HPA-5. Yet HPA-5 alleles do not influence binding of α2β1 to its primary ligand collagen I, and the effect of HPA-5 on platelet function has not been determined. We used a direct platelet adhesion assay to evaluate whether differential inheritance of HPA-5 alleles influences platelet adhesion to collagen I or an alternative ligand, decorin. Platelets from donors bearing one or more minor allele HPA-5b showed attenuated adhesion to purified decorin but not collagen I. Adhesion to decorin was significantly inhibited by human alloantibodies specific for HPA-5a but not by the collagen I sequence GFOGER or α2-specific inhibitory monoclonal antibodies. The minor allele 534K attenuates platelet adhesion to decorin but not collagen I, providing the first evidence of a functional effect of HPA-5 alleles.

Key words: platelet adhesion, decorin, collagen I, HPA-5 allele.

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Introduction

The rs1801106 alleles of the human α2 gene *ITGA2* produce a non-conservative amino acid substitution E534K, the basis of the clinically relevant HPA-5 alloantigen system,[1] but these alleles have never been found to affect α2β1 binding to collagen I, III or V.[1] Nonetheless, gene association studies suggest that the minor allele of rs1801106 (1600A) is associated with recurrence of stroke[2] and increased risk for breast cancer in women.[3]

In this report, we compare the adhesion of platelets from donors who differ in rs1801106 alleles to decorin and collagen I, and provide the first evidence that platelets bearing even one minor allele 534K show attenuated adhesion to decorin but not to collagen I.

Design and Methods

Approval from the CHOC Children's Hospital and The Scripps Research Institute institutional IRB was obtained for the drawing of blood samples from volunteers who had given prior written informed consent.

Murine monoclonal antibodies 6F1, 8C12 and HY101 were provided by Drs. B. Coller (Rockefeller University, New York, USA), Mark Ginsburg (University of California-San Diego, USA) and Mark Kahn (University of Pennsylvania, USA), respectively. Collagen I was purified from human placentae as described.[4] Anti-HPA-5a IgG was purified from sera gifted by Drs. Sentot Santoso (Giessen, Germany) and Diana Beardsley (New Haven, CT), and the specificities of IgG alloantibodies were confirmed by monoclonal antibody-specific immobilization of platelet antigen (MAIPA) assay.[5] Decorin was purified from cultured human fibroblast matrices without denaturing and/or precipitation as described.[6] The presence of decorin in a 90-150 kDa protein complex and the core protein decoron at roughly 46 kDa, and the absence of collagen I or III were established by immunoblot assay.

The SNPs analyzed in this study and the minor allele frequency (MAF) of each were: for integrin α2β1, *ITGA2* rs1126643 (0.38),[7] ITGA2 rs28095 (0.36),[8,9] and *ITGA2* rs1801106 (0.08);[1] for integrin αIIbβ3, *ITGA2B* rs5911 (0.41)[10] and *ITGB3* rs5918 (0.17);[11] for GP Ibα, *GP1BA* rs6065 (0.1);[12] for GPVI, *GP6* rs1613662 (0.16);[13] and for the purinergic receptor P2Y1, *P2RY1* rs1065776 (0.05).[14] Genotypes were determined using primer sequences in a primer extension based assay[15] or a customized Nanogen-based single nucleotide polymorphism (SNP) analysis (Nanogen Inc., San Diego, CA, USA).[16] SNPs were confirmed by direct Sanger sequencing.

Platelet receptor levels were measured in para-formaldehyde fixed whole blood by flow cytometry, as previously described[4,17] using murine monoclonal antibodies: 8C12 for α2β1, AP2 for αIIbβ3, AP1 for glycoprotein Ibα (GPIbα), and HY101 for GPVI. Bound monoclonal antibody was expressed as geometric mean fluorescence intensity

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(GMFI). Whole blood platelet count and mean platelet volume (MPV) were measured using a Coulter 9000 apparatus (Mallinckrodt Baker, Phillipsburg, NJ, USA).

The adhesion of platelets (1-30 x10^5 per well) to purified human decorin or collagen I adsorbed to microtiter plates under static conditions at 20°C after 60 min was measured by a colorimetric assay as described.[17] For inhibition assays, platelets were pre-incubated with peptide or antibody for 30 min at 20°C.[18]

Statistical calculations were performed using SigmaStat 3.01 (SPSS Inc., Chicago, IL, USA). Associations between discontinuous variables (e.g. SNPs) and continuous variables (platelet adhesion) were described by χ^2 analysis and *P* values were corrected for multiple testing. Pair-wise associations between continuous variables were analyzed by one-way ANOVA. All tests are two-sided and considered significant at $P<0.05$ after correction for multiple testing.

Results and Discussion

To minimize any effects of the ITGA2 alleles C-52T and C807T, which are associated with the regulation of α2β1 expression, we selected 20 donors who differed at 1600 (rs1801106) but were all homozygous -52TT (rs1126643) and 807CC (rs28095).

Two groups of normal subjects were compared, one consisting of 10 donors who were homozygous 1600GG (designated GG), the second including one donor homozygous 1600AA and 9 donors heterozygous 1600GA (designated GA+AA). There were no other differences with respect to platelet count, mean platelet volume, surface expression of four selected platelet receptors, including α2β1, and allelic distribution of five other receptor genes (Table 1).

In the platelet adhesion assay, platelets from GG donors bound more strongly to decorin than those from GA+AA donors (Figure 1) (asterisks represent $P<0.01$), but the two groups bind equally well to collagen I ($P=0.73$).

The hexapeptide GFOGER (O=hydroxyproline) self-assembles into a collagen-like triple helix and is the collagen I sequence bound by the integrin α2 I domain.[18] Using platelets from 3 representative 1600GG donors (Figure 2): 1) GFOGER (200 μg/mL) poorly inhibits binding to decorin (mean inhibition of 10%), but strongly inhibits adhesion to collagen I (90-95% inhibition); 2) the control peptide GPP(10) (200 μg/mL) has no effect; 3) the collagen-inhibitory antibody 6F1 (10 μg/mL) blocks platelet adhesion to collagen I but not to decorin; and 4) purified IgG preparations (50 μg/mL) from 2 patients with anti-HPA-5a alloantibodies (Ab1, Ab2), but not normal control IgG, inhibit adhesion to decorin by 50-80%, but have no affect on adhesion to collagen I. Anti-HPA-1a IgG alloantibody also had no effect on adhesion to decorin (*data not shown*). Mean values statistically different from the mean obtained with non-inhibited platelets are indicated ($P<0.01$).

Our results provide the first evidence for an effect of the highly-immunogenic *ITGA2* SNP rs1801106 on integrin α2β1 function and define a new paradigm for alternative ligand binding. We propose that E534K alters the affinity of α2β1 for decorin and that this residue likely represents an important component of the decorin binding site.

E534K is located in the β-propeller domain of the integrin α2. The crystal structures of the integrin αV[19] and αIIb subunit[20] β-propeller domains have been determined, and one can align the homologous β strands of the α2 sequence using the secondary structure prediction Consensus Data Mining (CDM) algorithm.[21] Thus, E534K would be located within a loop sequence that is situated at the opposite face of the β-propeller and spatially distant from the loop sequence into which the I-domain is inserted. It remains to be determined whether simultaneous occupancy of both binding sites is possible or whether the

Table 1. Comparison of key subject parameters.

		GG	GA+AA	*P**
Males/females		7/3	6/4	1.0
Age (years)		35.4±7.6†	36.6±7.8	0.73
MPV‡ (fL)		6.8±1.5	6.1±0.8	0.21
Platelet count (×10^3/L)		211.3±49.7	197.5±42.9	0.52
GPIb (AP1)§		19.4±5.5	22.0±5.2	0.28
GPVI (HY101)§		21.4±6.5	23.6±5.1	0.41
Integrin 2 1 (8C12)§		7.2±2.2	7.9±1.4	0.39
Integrin IIb 3 (AP2)§		280.4±55.6	287.4±63.4	0.80
Candidate gene alleles				
GP1BA rs6065	major	19	17	0.61
	minor	1	3	
GP6 rs1613662	major	14	15	0.73
	minor	6	5	
ITGB3 rs5918	major	14	16	0.72
	minor	6	4	
ITGA2B rs5911	major	15	13	0.73
	minor	5	7	
P2YR1	major	18	19	1.0
	minor	2	1	

*P *value: GG* vs. *GA+AA;* †*Mean ± 1 standard deviation (SD);* ‡*Mean platelet volume;* §*Receptor (monoclonal antibody): geometric mean fluorescence intensity.*

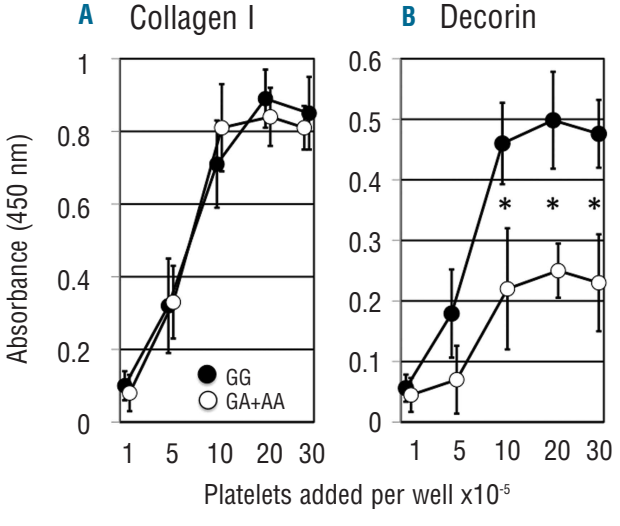

Figure 1. Platelet adhesion to (A) collagen I or (B) decorin. Each data point represents the mean±1 SD. GG (black) represents 10 donors homozygous for *ITGA2* 1600G; GA+AA (white) are one donor homozygous for 1600A and 9 heterozygous donors 1600GA. *$P<0.01$. Absorbance recorded for wells coated with BSA was subtracted as background from absorbance recorded for wells coated with collagen I or decorin.

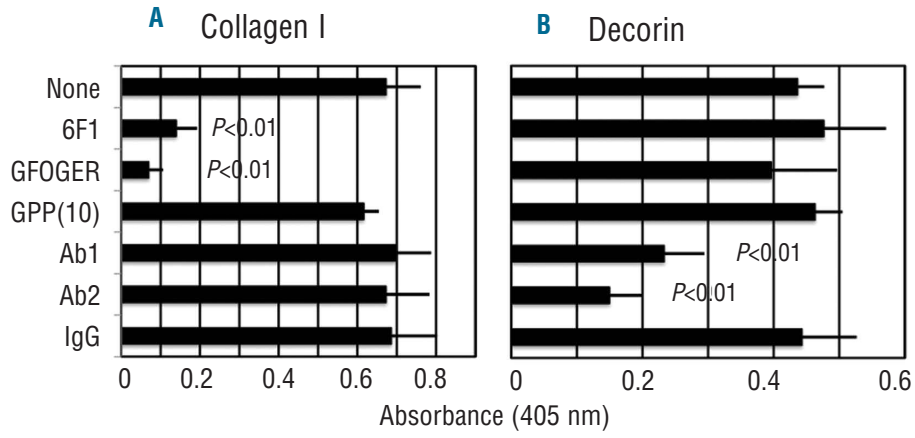

Figure 2. Inhibition of platelet adhesion to collagen I or decorin. Bars and horizontal lines represent the mean ±1 SD for results obtained for donors homozygous for *ITGA2* 1600G. Platelet adhesion to (A) collagen I or (B) decorin was measured in the absence of inhibitor (none) or after preincubation with 6F1 (10 μg/mL); GFOGER (200 μg/mL); control peptide GPP(10) (200 μg/mL); Ab1 or Ab2 (IgG containing anti-HPA-5a; 50 μg/mL)); or non-immune IgG (50 μg/mL).

binding of one ligand modulates the binding of the second, through direct or allosteric effects.

The lack of a significant difference in expression of GPIbα, GPVI or αIIbβ3 argues that these important receptors are not primary contributors to adhesion to decorin. The lack of a difference in α2β1 levels also eliminates a quantitative effect of this integrin. Regarding these other receptor alleles, we are aware that the two cohorts in this study are likely too small to make statistically valid conclusions regarding the lack of association of these gene variants with decorin binding activity. However, the need to compare donors with the relatively rare 1600 (rs1801106) A allele (MAF, 0.08) precludes the study of sample sizes sufficiently large to exclude any other gene association with unquestionable statistical power. Nonetheless, given this caveat, the differences in frequencies of other receptor alleles were not statistically significant.

These results expand our understanding of the decorin and collagen I interactions described in murine studies of cell adhesion and cancer biology, where decorin and collagen I often have opposite effects that are both mediated by α2β1. Decorin knockout (Dcn(-/-) murine embryonic fibroblasts exhibit greater adhesion to collagen and greater migration on collagen substrates,[22] while decorin attenuates the aggressiveness and metastasis of tumor cells with diverse histological backgrounds.[23,24]

Indirect evidence for an effect of the immunogenic *ITGA2* SNP rs1801106 on the pathobiology of α2β1 in humans has been obtained. In a case-control study of 500 female Caucasian breast cancer patients and 500 healthy control subjects,[3] the 1600AA genotype was significantly associated with breast cancer (odds ratio 3.12; 95% confidence interval 1.11-8.77), while carriers of the 1600G allele were less likely to have a histological grade 3 or 4 cancer (P=0.003). An association of this SNP with cerebrovascular disease has also been implied by the finding that the 1600AA genotype correlated with the extent of neurological symptoms and the recurrence of stroke.

Our results represent the first evidence of a physiological effect of HPA-5 alleles and provide important clues regarding the nature of the decorin binding site on α2β1, thus creating a novel paradigm for alternative ligand binding sites (i.e. those not inherent in the I-domain) in this and other integrins. A more comprehensive study of the α2β1 binding site for decorin and other proteglycans and its relationship to hemostasis and thrombosis is clearly warranted.

Authorship and Disclosures

The information provided by the authors about contributions from persons listed as authors and in acknowledgments is available with the full text of this paper at www.haematologica.org.

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