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Platelet adhesion to decorin but not collagen I correlates with the integrin \( \alpha_2 \) 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 exhibited 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.
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

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

Murine monoclonal antibodies 6F1, 8C12 and HY101 were provided by Drs. B. Coller (Rockefeller University, New York, NY, 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 antigens (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 \( \alpha 2 \beta 1 \), \( ITGA2 \) rs1126643 (0.38) (7), \( ITGA2 \) rs28095 (0.36)(8, 9), and \( ITGA2 \) rs1801106 (0.08)(1); for integrin \( \alpha llb\beta 3 \), \( ITGA2B \) rs5911 (0.41) (10) and \( ITGB3 \) rs5918 (0.17) (11); for GP Ib\( \alpha \), \( GP1BA \) rs6065 (0.1)(12); for GPVI, \( GP6 \) rs1613662 (0.16) (13); and for the purinogenic 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 \( \alpha 2\beta 1 \), AP2 for \( \alpha llb\beta 3 \), AP1 for glycoprotein Ib\( \alpha \) (GPIb\( \alpha \)), and HY101 for GPVI. Bound monoclonal antibody was expressed as geometric mean fluorescence intensity (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\(^{\circ}\)C after 60 min was measured by a colorimetric assay, as described (17). For inhibition assays, platelets were preincubated with peptide or antibody for 30 min at 20\(^{\circ}\)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 Chi-Square 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 $\alpha_2\beta_1$ expression, we selected twenty 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 ten donors who were homozygous 1600GG (designated GG), the second including one donor homozygous 1600AA and nine 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 $\alpha_2\beta_1$, 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 $\alpha_2$ I domain.(18) Using platelets from three representative 1600GG donors (Figure 2): 1) GFOGER (200 $\mu$g/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 $\mu$g/ml) has no effect; 3) the collagen-inhibitory antibody 6F1 (10 $\mu$g/ml) blocks platelet adhesion to collagen I but not to decorin; and 4) purified IgG preparations (50 $\mu$g/ml) from two 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 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 rector 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 $\alpha_2\beta_1$. 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 histologic backgrounds (23, 24).

Indirect evidence for an effect of the immunogenic ITGA2 SNP rs1801106 on the pathobiology of $\alpha_2\beta_1$ 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 his recurrence of stroke (2).

Our results represent the first evidence of a physiologic effect of HPA-5 alleles and provide important clues regarding the nature of the decorin binding site on $\alpha_2\beta_1$, 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 $\alpha_2\beta_1$ binding site for decorin and other proteoglycans and its relationship to hemostasis and thrombosis is clearly warranted.
AUTHORSHIP AND DISCLOSURES

TJK designed the studies, wrote the paper, and conducted statistical analyses. SAW and DJN performed genotyping, DD performed experiments, and RWF provided key peptide reagents. SAW, DD, DJN and RWF critically evaluated the data and the written manuscript. The authors report no potential conflicts of interest.
REFERENCES


### Table 1. Comparison of key subject parameters.

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<th></th>
<th>GG</th>
<th>GA+AA</th>
<th>p*</th>
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<td>Males/Females</td>
<td>7/3</td>
<td>6/4</td>
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<tr>
<td>Age (years)</td>
<td>35.4±7.6†</td>
<td>36.6±7.8</td>
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<td>MPV¶ (fL)</td>
<td>6.8±1.5</td>
<td>6.1±0.8</td>
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<td>Platelet Count (x 10^3/µl)</td>
<td>211.3±49.7</td>
<td>197.5±42.9</td>
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<td>GPIbα (AP1) §</td>
<td>19.4±5.5</td>
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<td>GPVI (HY101) §</td>
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<td>Integrin α2β1 (8C12) §</td>
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<td>Integrin αIIbβ3 (AP2) §</td>
<td>280.4±55.6</td>
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**Candidate gene alleles**

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*p-value: GG vs. GA+AA; †Mean ± 1 Standard Deviation; ¶Mean Platelet Volume
§Receptor (Monoclonal Antibody): Geometric Mean Fluorescence Intensity
FIGURE LEGENDS

Figure 1. Platelet adhesion to (A) collagen I or (B) decorin. Each data point represents the mean ±1 SD. GG (black) represents ten donors homozygous for ITGA2 1600G; GA+AA (white) are one donor homozygous for 1600A and nine heterozygous donors 1600GA. Asterisks denote a significant difference (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 nonimmune IgG (50 µg/ml).
Figure 2

A) Collagen I

- None
- 6F1
- GFOGER
- GPP(10)
- Ab1
- Ab2
- IgG

B) Decorin

- None
- 6F1
- GFOGER
- GPP(10)
- Ab1
- Ab2
- IgG

Absorbance (405nm)