Platelet CD36 surface expression levels affect functional responses to oxidized LDL and are associated with inheritance of specific genetic polymorphisms

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CD36 modulates platelet function via binding to oxidized LDL (oxLDL), cell-derived microparticles, and thrombospondin-1. We hypothesized that the level of platelet CD36 expression may be associated with inheritance of specific genetic polymorphisms and that this would determine platelet reactivity to oxLDL. Analysis of more than 500 subjects revealed that CD36 expression levels were consistent in individual donors over time but varied widely among donors (200-14 000 molecules per platelet). Platelet aggregometry and flow cytometry in a subset of subjects with various CD36 expression levels revealed a high level of correlation (r² = 0.87) between platelet activation responses to oxLDL and level of CD36 expression. A genome-wide association study of 374 white subjects from the Cleveland Clinic ASCLOGEN study showed strong associations of single nucleotide polymorphisms in CD36 with platelet surface CD36 expression. Most of these findings were replicated in a smaller subset of 25 black subjects. An innovative gene-based genome-wide scan provided further evidence that single nucleotide polymorphisms in CD36 were strongly associated with CD36 expression. These studies show that CD36 expression on platelets varies widely, correlates with functional responses to oxLDL, and is associated with inheritance of specific CD36 genetic polymorphisms, and suggest that inheritance of specific CD36 polymorphisms could affect thrombotic risk. (Blood. 2011;117(23):6355-6366)

Introduction

CD36 is an 88-kDa glycoprotein belonging to the Scavenger Receptor Type B family. It was identified initially as a protease-resistant platelet surface glycoprotein and named glycoprotein IV for its migration on sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Since then, work in our laboratory and others has shown that CD36 is expressed on a broad array of other cell types, including microvascular endothelial cells, vascular smooth muscle cells, erythroid precursors, epithelia of breast, gut, and kidney, and cardiac and skeletal muscle. It is expressed on most phagocytic cells, including dendritic cells, microglia, monocytes/macrophages, and retinal pigment epithelium where it plays an important role in mediating recognition and uptake of oxidized phospholipids, apoptotic cells, and certain microbial cell wall components. Other well-characterized functions of CD36 include mediating endothelial cell antiangiogenic responses to thrombospondin-related proteins; fatty acid transport in gut epithelial cells, myocytes, and adipocytes; and regulating oxidant stress.

Although CD36 was recognized as a major platelet glycoprotein more than 3 decades ago, its role in platelet physiology has only recently been appreciated based on work by our group and others. In collaboration with Podrez et al., we identified platelet CD36 as a receptor for oxidized low-density lipoprotein (oxLDL) and showed that oxLDL induced platelet activation in a CD36-dependent manner. Using apo-e null mice fed a high fat “Western” diet as a model of hyperlipidemia, we showed that cd36 gene deletion abrogated the associated prothrombotic state and platelet hyper-reactivity. This work defined CD36 as providing a mechanistic link between oxidant stress, hyperlipidemia, and thrombosis. Subsequently, we showed that platelet CD36 also functions as a receptor for cell-derived microparticles and thereby contributes to thrombus formation in settings of vascular injury and inflammation where microparticles are generated. Mechanistically, platelet CD36 engagement by oxLDL triggers a specific signal transduction pathway involving the src family kinases fyn and lyn and the MAP kinase jnk. CD36 also participates with CD47 in TSP-1 mediated pathways involving adenyl and guany1 cyclases. Based on these findings, we hypothesized that the level of CD36 expression on platelets would modulate platelet reactivity; therefore, we set out to define the variability of expression in human subjects, relate it to platelet activation responses to oxLDL, and determine whether it is associated with inheritance of specific single nucleotide polymorphisms (SNPs) in the CD36 gene or in genes known to be involved in atherothrombotic risk.

The human CD36 gene is very large, extending up to 309.7 kb on band q11.2 of chromosome 7 and consisting of 15 or more exons of which 12 are coding. All but one of the 20 putative CD36 transcripts span a much smaller distance of 77.1 kb. The CD36 gene has been most extensively studied in Asian populations, including in Japan, Korea, Indonesia, Thailand, and China, where
Table 1. Demographic and clinical phenotypes of study populations used for assessment of platelet CD36 expression levels and genetic analyses

<table>
<thead>
<tr>
<th>Variable</th>
<th>Cardiac cath patients, n (%)</th>
<th>ASCLOGEN white patients, n (%)</th>
<th>ASCLOGEN black patients, n (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, y (mean ± SD)</td>
<td>62 ± 9</td>
<td>63 ± 10</td>
<td>58 ± 9</td>
</tr>
<tr>
<td>Sex</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>313 (62.7)</td>
<td>304 (81.3)</td>
<td>18 (72)</td>
</tr>
<tr>
<td>Female</td>
<td>186 (37.3)</td>
<td>70 (18.7)</td>
<td>7 (28)</td>
</tr>
<tr>
<td>Tobacco use</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Present</td>
<td>62 (12.9)</td>
<td>59 (15.8)</td>
<td>9 (36)</td>
</tr>
<tr>
<td>Past</td>
<td>338 (70.3)</td>
<td>243 (65.0)</td>
<td>20 (80)</td>
</tr>
<tr>
<td>History</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Atherosclerosis</td>
<td>271 (54.6)</td>
<td>374 (100)</td>
<td>25 (100)</td>
</tr>
<tr>
<td>MI</td>
<td>249 (51.2)</td>
<td>168 (44.9)</td>
<td>13 (52)</td>
</tr>
<tr>
<td>CABG/PCI</td>
<td>221 (44.3)</td>
<td>235 (62.8)</td>
<td>15 (60)</td>
</tr>
<tr>
<td>Stroke</td>
<td>37 (9.8)</td>
<td>18 (4.8)</td>
<td>1 (4)</td>
</tr>
<tr>
<td>Hypertension</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BP &gt; 120/90 mmHg</td>
<td>412 (85.3)</td>
<td>311 (83.2)</td>
<td>23 (92)</td>
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<td>Diabetes</td>
<td>140 (29.4)</td>
<td>111 (29.7)</td>
<td>11 (44)</td>
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<tr>
<td>Serum cholesterol ≥ 200 mg/dL</td>
<td>104 (23.4)</td>
<td>57 (20.1)</td>
<td>6 (31.6)</td>
</tr>
<tr>
<td>LDL ≥ 120 mg/dL</td>
<td>86 (19.9)</td>
<td>68 (23.9)</td>
<td>6 (33.3)</td>
</tr>
<tr>
<td>HDL ≤ 50 (M) or ≤ 60 (F) mg/dL</td>
<td>279 (63)</td>
<td>221 (81.0)</td>
<td>15 (88.2)</td>
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<td>Triglyceride ≥ 200 mg/dL</td>
<td>80 (18)</td>
<td>65 (23.0)</td>
<td>3 (15.8)</td>
</tr>
<tr>
<td>Glucose ≥ 120 mg/dL</td>
<td>108 (23.6)</td>
<td>85 (23.4)</td>
<td>7 (29.2)</td>
</tr>
<tr>
<td>Platelet count ≥ 150 × 10^3/μL</td>
<td>37 (8.1)</td>
<td>27 (7.3)</td>
<td>1 (4.0)</td>
</tr>
<tr>
<td>BMI &gt; 30</td>
<td>228 (44.3)</td>
<td>NA</td>
<td>NA</td>
</tr>
</tbody>
</table>

MI indicates myocardial infarction; CABG/PCI, coronary artery bypass graft/percutaneous coronary intervention; BP, blood pressure; BMI, body mass index; and NA, not applicable.

3% to 8% of persons lack platelet CD36. This deficiency was initially defined in Japan as the Naka-negative (Naka−) phenotype. Approximately 90% of Naka− subjects lack CD36 expression only in platelets; the remaining 10% are presumably CD36 null (type I deficiency) and to not express CD36 in any cells or tissues. Five mutations have been reported to be associated with type I deficiency in Asians. The type I Naka− phenotype is also common in African populations, although the associated null mutations are different from those reported in Asia.

CD36 is highly polymorphic; in addition to the null mutations mentioned in the preceding paragraph, data from Ensembl Variation Build 60 (which is based on dbSNP Release 131), describe 2935 common genetic variants in or within 5 kb of the gene, all but one of which are SNPs. Some involve putative transcription factor binding sites or sites in the 5′-untranslated region, which are of potential significance because translational efficiency of the CD36 mRNA and thereby CD36 protein expression levels have been shown to be regulated by the 5′-untranslated region.

Although the functional impact of CD36 deficiency has been well studied in different mouse and rat models, the impact of CD36 null mutations and polymorphisms in human biology are not well characterized. The cd36 null mouse strain generated in our laboratory shows dyslipidemia, defective fatty acid uptake in heart and muscle, alterations in insulin responsiveness, and protection from diet-induced atherosclerosis, thrombophilia, insulin resistance, and adipose inflammation. A small number of studies from Japan have implicated type I CD36 deficiency with increased risk for developing insulin resistance or cardiomyopathy, but these have not been consistently replicated. More than a dozen gene association studies have been reported linking CD36 SNPs to various phenotypes, including circulating high-density lipoprotein (HDL), LDL, and triglyceride levels, risks of metabolic syndrome and obesity, and risk of acute myocardial infarction (AMI) or stroke. Unfortunately, only a small number of these studies related CD36 SNPs or haplotypes to CD36 expression level. Love-Gregory et al have analyzed a cohort of black subjects and found associations of 5′ SNPs with metabolic syndrome and with HDL level. In a small subset of these subjects, they reported association of CD36 SNPs with peripheral blood monocyte CD36 levels. Interestingly, they found a negative correlation between monocyte CD36 levels and circulating HDL levels and a positive correlation with very low-density lipoprotein and apoB.

In the current study, we show that the level of platelet CD36 surface expression is highly variable among persons from a heterogeneous population. We also demonstrate that this variability affects the functional response of platelets to oxLDL and that the variability is associated with inheritance of specific genotypic polymorphisms at the CD36 locus in both whites and blacks.

Methods

Study populations

The initial study population included 32 healthy human volunteers who were students or employees at the Cleveland Clinic. We then recruited 500 patients through the Cleveland Clinic cardiac catheterization laboratory for the study of CD36 expression measurement and 440 patients from the Cleveland Clinic ASCLOGEN (ASpirin and CLOpidogrel: GENotype vs Platelet Function Phenotype in Clinical Response) Trial for a gene association study. ASCLOGEN is a prospective clinical observational study of genotype-phenotype associations in patients undergoing percutaneous coronary intervention, who are on dual antiplatelet therapy. The study was designed to determine the influence of genotype on patient response to aspirin and clopidogrel therapy and on clinical end points. The study population included men and nonpregnant women at least 18 years old who had undergone percutaneous coronary intervention at the Cleveland Clinic. Subjects were excluded if they had taken ticlopidine, dipyriramole, steroids, drugs, or COX-2 inhibitors during 7 days before enrollment or glycoprotein IIb/IIIa inhibitor within 5 days before enrollment or during percutaneous coronary intervention. Patients with known history of platelet disorders, with platelet counts <150 000/μL, or with hemoglobin <10 g/100 mL or hematocrit <30% were also excluded, as were those with a known allergy to aspirin or
clopidogrel or who had a major surgical procedure in the week before enrollment. Clinical and demographic data on the 2 patient cohorts are shown in Table 1. All studies were approved by the Cleveland Clinic Institutional Review Board with informed consent from each subject in accordance with the Declaration of Helsinki. For platelet studies, blood samples were collected in 3.9% citrate buffer. Platelet-rich plasma (PRP) and washed platelets were isolated by sequential centrifugation. DNA was isolated from whole blood using the Puregene DNA purification kit (Gentra Systems).

Quantitative assay of platelet CD36 expression

PRP was incubated with phycoerythrin (PE)-conjugated anti-CD36 monoclonal antibody (clone 185-1G2; Santa Cruz Biotechnology) or isotype-matched control IgG, and mean fluorescence intensity (MFI) was then quantified by flow cytometry. A standard curve relating PE fluorescence intensity to PE surface density was generated using a mixture of PE-Quantibrite Beads (BD Biosciences) tagged with 4 defined amounts of PE ranging from 200 to 70,000 molecules per bead. The number of bound antibody molecules on each platelet was calculated from the standard curve using the platelet MFI and the molar ratio of PE to anti-CD36 IgG (supplied by the manufacturer). The stoichiometry of anti-CD36 binding to CD36 was assumed to be 1:1 for these studies.

Platelet aggregation and activation studies

oxLDL was prepared by incubation of LDL with 5 mM copper sulfate at 37°C for 6 hours as previously described.20 PRP was incubated with 50 μg/mL oxLDL or native LDL as control for 30 minutes, and then a low concentration of adenosine diphosphate (ADP; 1 or 2 μM) was added.
Platelet aggregation was assessed turbidometrically using a dual-channel aggregometer (Chronolog). Platelet activation was also analyzed by flow cytometry with PE-conjugated anti–P-selectin IgG (BD Biosciences) to detect granule secretion.

Genotyping
Genotyping was performed using the Illumina Human Cardiovascular Disease (CVD) array, a gene-centric 50,000 SNP array designed to assess potentially relevant loci across a range of cardiovascular, metabolic, and inflammatory diseases. Twenty additional SNPs in the CD36, CYP2C19, and PR2Y12 genes, which are not on the array but previously reported in the literature as potentially relevant to the ASCLOGEN study goals, were genotyped by the allelic discrimination (TaqMan) assay as previously described.

Genotype calling, annotation, and filtering of Illumina human CVD arrays
Genotypes were called using the Genotyping Module (Version 3.3.7) from Illumina’s BeadStudio program (Version 3.1.3). Called genotypes were exported to text files and imported into R (Version 2.11.1) using the GenABEL R package (Version 1.6-5) for quality filtering and statistical analysis. Unique National Center for Biotechnology Information (NCBI) Build 37/hg19 chromosome and base pair locations were obtained from Ensembl Variation Build 59 (which is based on dbSNP Release 131) using the biomaRt R package (Version 2.6) for 48,090 of 49,111 measured genetic markers, 48,960 of which had rs identifiers and unique positions in NCBI Build 36/hg18. Gene associations and SNP types for each SNP were also obtained from Ensembl. Quality filtering by SNP was performed separately for the white and black subsets. For the white subset, the following criteria for SNP inclusion were used: call rate $\geq 0.97$, minor allele frequency $\geq 0.01$, and false discovery rate value $\leq 0.01$ from test of Hardy-Weinberg equilibrium. Because of the smaller sample size of the black subset, we used a higher minor allele frequency cutoff of 0.10 and lower SNP call rate threshold of 0.90.

Subject filtering
Subjects were included using the following criteria: per-subject SNP call rate $\geq 0.95$, no sex mismatch, autosomal heterozygosity false discovery rate value $> 0.01$, inbreeding coefficient $< 0.2$, and Identical by state (IBS) score $< 0.95$. In addition, outliers defined as more than 6 SDs for whites or...
more than 4 SDs for blacks along the genetic principal components used to adjust for population stratification/substructure were dropped (n = 51).

Single SNP statistical analysis

For each SNP that passed our filtering step within each subset of samples, we fit an additive linear regression model with untransformed CD36 expression levels (MFI) as the response variable and predictor variables sex, first genetic principal component score,38 and SNP. SNP was coded as the number of copies of the coded allele. The first genetic principal component scores were calculated separately in the white and black subsets to help account for racial-specific population stratification. CD36 MFI values were restricted to those observed between 0 and 10, eliminating a few extreme values in the upper tail of the MFI distribution. After truncation, the CD36 MFI distribution was approximately symmetric and unimodal in both subsets. Fitted regression coefficients, truncation, the CD36 MFI distribution was approximately symmetric and unimodal in both subsets. Fitted regression coefficients, test, and genomic control-adjusted SNP values to assess genome-wide significance and significance of the preceding paragraph and recorded the minimum P value across all tested SNPs (either all filtered SNPs or all CD36 filtered SNPs). This was repeated 1000 times, generating 1000 of 10000 minimum P values. Then we calculated the min-p P value for each SNP as the fraction of times the minimum permutation P value was greater than the observed P value.

In addition to performing the genome-wide association study (GWAS) analysis, we also performed a more focused “cis” analysis looking only at the 72 available SNPs in or near the CD36 locus (supplemental Table 1, available on the Blood Web site; see the Supplemental Materials link at the top of the online article). Of these, 55 were common to the white and black sets, 14 were unique to the white set, and 3 were unique to the black set. These SNPs span 85.74 kb ranging from position 80,220,608 to 80,306,350 and fully cover 19 of the 20 known CD36 transcripts. The partially covered transcript had not yet been identified when our study was designed and initiated.

Multivariate SNP analyses

To jointly test the genetic association of all SNPs uniquely mapped to a given gene with CD36 MFI, we fit a least-squares kernel machine (LSKM) model41 using a weighted IBS kernel, with untransformed CD36 MFI as the response variable and predictor variables sex, first genetic principal component score, and an unspecified function of the joint vector of SNP genotypes mapped to a given gene as predictors. SNPs were coded in the kernel as the number of copies of the coded allele. The kernel weights were proportional to the inverse of the square root of the minor allele frequency of the SNP. Association of the set of SNPs from a gene with CD36 MFI was assessed using the GenABEL function r2fast.

Results

Platelet CD36 surface expression varies widely among persons

Among 32 normal healthy volunteers in the initial set, platelet CD36 surface expression levels ranged from 0 to 14,000 molecules
per platelet (Figure 1A-B) with a mean of 6005, a median of 5766, and an SD of 1484. From this group of 32, a representative subgroup of 4 individual donors were studied repetitively over time periods as long as 3 years and showed consistent levels of CD36 expression on at least 5 analyses with coefficients of variance in all donors < 9%. Analyses of the same subjects at different times in the day were also consistent (not shown). These results strongly suggest that a genetic component determines expression levels. As reported by others, we found that when platelets were activated CD36 levels increased by approximately 20% (not shown). This group of volunteers, however, were healthy nonsmokers with no history of cardiovascular disease and thus highly unlikely to have increased numbers of circulating activated platelets. In addition, we found no significant difference in the percentage increase in CD36 expression after ex vivo platelet activation among donors regardless of resting levels of expression, suggesting that the wide variability in platelet CD36 expression cannot be accounted for by differences in degrees of in vivo or ex vivo platelet activation among donors.

To replicate these findings, we studied platelets from a larger sample of 500 successive subjects recruited through the Cleveland Clinic Cardiac Catheterization Laboratory. Demographic and clinical data on this population are shown in Table 1. As with the normal volunteers, this group also showed a wide variability in platelet CD36 expression (Figure 2A) with a mean of 7876 ± 1924 molecules per platelet and a median of 7611 molecules per platelet. There was no significant difference in CD36 expression levels between male and female subjects (Figure 2B; \( P = .31 \)). The narrower range of expression among this cohort compared with our original 32 subjects results from original group containing several persons of Asian ancestry who were platelet CD36 null (Naka).

**CD36 expression levels correlate with platelet reactivity to oxLDL**

To assess the functional consequences of the variability in CD36 expression, we studied the effect of oxLDL on platelet aggregation and \( \alpha \)-granule release in response to low-dose ADP (1-4 \( \mu \)M) from donors with a wide range of CD36 expression levels. oxLDL induced a significant increase in the rate and extent of platelet aggregation (Figure 3A top left panel) in PRP from high CD36-expressing subjects but not from CD36 null donors (Figure 3A right lower panel). The maximal extent of aggregation to 2 \( \mu \)M ADP increased from 21% to 44% when oxLDL was added (\( P < .05 \)). Donors with intermediate (lower left) and low (upper right) CD36 expression levels showed correspondingly lower levels of response to oxLDL, increasing the maximum extent of aggregation by 5% to 15%. Similar results were seen using 4 \( \mu \)M ADP or low doses of collagen (50 \( \mu \)g/mL) as an agonist. At maximum agonist concentrations (eg, 20 \( \mu \)M ADP), oxLDL did not induce further increases in the extent of aggregation (not shown). We then assessed platelet P-selectin expression by quantitative flow cytometry in a series of donors and showed that platelet activation in response to low-dose ADP plus oxLDL correlated well (\( r^2 = 0.87 \)) with the level of CD36 surface expression (Figure 3B).

**CD36 expression level is associated with inheritance of specific CD36 SNPs**

Our targeted genome-wide scan across 34 088 filtered SNPs for association with platelet CD36 expression level in whites (\( n = 374 \)) identified 24 SNPs with \( q \) values (false discovery rate) < 0.05 (Table 2). rs2058703, located in the \( 3' \)-untranslated region of \( BCL11A \), was the only non-CD36 SNP to meet this statistical
criterion. Six of these 24 SNPs, all within the CD36 locus, met the Bonferroni genome-wide significance threshold of 0.10 divided by 34,088/11005/2.93/11003/106. The genome-wide min-p P value for the strongest SNP rs3211870 was 0.057. Figure 4 shows a Manhattan plot for the data with the Bonferroni threshold shown as a horizontal black line. The estimated genomic control λ-inflation factor for the scan was 1.021.

We also conducted a gene-based genome-wide scan on the white set using a one-degree of freedom LSKM score test. The LSKM test is a multi-SNP semiparametric test that jointly tests the association of all SNPs in a gene with the phenotype while accounting for the linkage disequilibrium among SNPs. It is a powerful intermediate approach between single SNP tests and multiple degrees of freedom haplotype tests. Figure 5 is a Manhattan plot of the gene-based scan across 1706 genes with at least 3 filtered SNPs. CD36 had the lowest gene-based P value, 4.55 × 10^{-5}, for genetic association with platelet CD36 expression. This P value was considerably smaller than the smallest CD36 SNP P value. The FAF1 gene on chromosome 1 had the next smallest gene-based P value (6.19 × 10^{-4}), but only CD36 met the Bonferroni threshold of 0.05 divided by 1706 = 2.93 × 10^{-5} or the q value threshold of 0.05.

A focused “cis” analysis considering only the SNPs located in or near the CD36 locus (listed in supplemental Table 1) identified 31 SNPs with min-p P values < .05 for genetic association with platelet CD36 expression levels (Table 3). Seven of the top 10 CD36 SNPs identified by this analysis have also been associated with various CD36-relevant phenotypes in other published studies,26-33 strengthening the validity of our findings. Lastly, we estimated linkage disequilibrium (LD), measured by R^2, of each tested SNP with the strongest SNP (rs3211870). Most of the top SNPs were in strong LD with rs3211870 as indicated by the dark red color of their plotting symbols in Figure 6, although the observed CD36 LD structure was not very block-like. The statistical associations with CD36 expression levels quickly weakened just upstream of rs3211870, where the LD with rs3211870 also diminished.

We also analyzed genetic associations with platelet CD36 expression levels in the smaller black cohort (25 subjects; 58 SNPs). As shown in Table 3, of the top 31 SNPs identified in the cis analysis of the white subset, 19 also showed evidence of genetic association at P < .05 in the black cohort. Importantly, directions of the estimated effects were consistent between analyses for all of the SNPs, and 6 of the 10 most strongly associated CD36 SNPs in

Figure 5. Manhattan plot of gene-CD36 MFI associations from the LSKM genome-wide scan from the white cohort. Chromosome and gene position information from hg19. Dark gray horizontal line indicates the 0.05 Bonferroni threshold.

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Table 3. *CD36* SNPs showing strongest association with platelet *CD36* expression level

<table>
<thead>
<tr>
<th>SNP</th>
<th>Position</th>
<th>A1/2</th>
<th>CAF</th>
<th>$\beta$</th>
<th>$P^*$</th>
<th>PP†</th>
<th>CAF</th>
<th>$\beta$</th>
<th>$P^*$</th>
<th>PP†</th>
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<tbody>
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<td>0.55</td>
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<td>1.30</td>
<td>0.005</td>
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<td>0.92</td>
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<td>0.53</td>
<td>0.0000218</td>
<td>&lt; 0.0001</td>
<td>0.38</td>
<td>1.62</td>
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<td>1.62</td>
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<td>rs1358337</td>
<td>20.208</td>
<td>G/A</td>
<td>0.43</td>
<td>0.52</td>
<td>0.00000344</td>
<td>&lt; 0.0001</td>
<td>0.50</td>
<td>1.54</td>
<td>0.0000456</td>
<td>0.014</td>
</tr>
<tr>
<td>rs1761667</td>
<td>63.654</td>
<td>A/G</td>
<td>0.47</td>
<td>0.51</td>
<td>0.00000417</td>
<td>&lt; 0.0001</td>
<td>0.54</td>
<td>1.83</td>
<td>0.0000276</td>
<td>0.011</td>
</tr>
</tbody>
</table>

All *CD36* SNPs with PP values ≤ 0.05 using the white subset ordered by PP value.

Position indicates base pair location using NCBI Build 37.p2, dbSNP Build 131; A1/2, allele 1/allele 2; CAF, coded allele, allele 2, frequency; $\beta$, SNP estimated regression coefficient from the linear regression of *CD36* MFI on sex, first genetic principal component, and SNP, where SNP was coded as number of copies of allele 2; and NA, not applicable (SNP was dropped during QC filtering).

*Genomic control-adjusted P value from F test of $\beta = 0$.

†Min-p permutation $P$ value of $\beta = 0$ based on 10 000 permutations restricted to *CD36* SNPs. Regressions fit separately for white and black subsets.

...the black cohort have also been associated with various *CD36*-relevant phenotypes in other published studies.26,27

Although the size of the black cohort was very small, we were able to perform a genome-wide scan (28 446 SNPs) of the cohort and identified rs7803660, an intronic SNP in the *CD36* gene, as the sole SNP to meet the Bonferroni significance threshold; the estimated genomic control-$\lambda$ inflation factor for the scan was 1.087.

Discussion

This study represents the first systematic examination of platelet *CD36* expression in normal human subjects and reveals a surprisingly wide range. Among those persons who are not Nak+ (ie, platelet *CD36* null), expression on resting platelets ranged from approximately 2000 to 14 000 molecules/platelet. Importantly, this wide range of expression was also observed in a larger population of subjects of mixed age, sex, and ethnicity who presented to the Cleveland Clinic Cardiac Catheterization Laboratory. *CD36* surface expression has been reported to increase in response to platelet activation.43 Because *CD36* is expressed on the membranes of $\alpha$-granules and the open canalicular system,44 this is probably the result of increased accessibility of this pool of *CD36* to antibodies after platelets are activated. Indeed, we also observed an approximately 20% increment in anti-CD36 antibody binding after platelet activation. This effect was similar among donors regardless of their basal level of *CD36* expression (data not shown); thus, variations in platelet activation in vivo or during blood processing ex vivo cannot account for the large degree of individual *CD36* expression variance observed. Platelet *CD36* expression levels were consistent in individual donors over time periods as long as 3 years and did not vary significantly with the time of day of phlebotomy.

Although these data cannot define the degree of heritability of *CD36* expression levels, they strongly suggest that a significant component of the population variability is genetically determined. *CD36* is a large and highly polymorphic gene, yet systematic studies relating its genetic polymorphisms to protein expression are extremely limited. In one of the few such reports, Love-Gregory et al analyzed a cohort of 74 black subjects and found associations of $5^\prime$-SNPs with total level of *CD36* protein in peripheral blood...
monocytes. We now, for the first time, report strong associations of SNPs in the \( \text{CD36} \) gene with platelet surface CD36 expression levels. We used multiple statistical methods to analyze data obtained from 440 subjects from the Cleveland Clinic ASCLOGEN study. Both SNP-based and gene-based GWAS in the white cohort (Figures 4, 5) identified the \( \text{CD36} \) locus as highly associated with platelet CD36 expression levels with significant \( P \) values, even after rigorous Bonferroni correction. In addition, a more highly powered parallel analysis looking only at the available SNPs in or near \( \text{CD36} \) also showed that multiple \( \text{CD36} \) SNPs were strongly associated with CD36 expression in both white and black cohorts (Table 3). Although we did not measure monocyte CD36 expression levels, our data are concordant with the smaller study of Love-Gregory et al (Table 4) in that minor alleles in 4 \( \text{CD36} \) SNPs that they reported to be associated with monocyte CD36 expression in blacks were associated with the same direction of platelet CD36 expression in our student in both the white and black populations. Their study also included a small subset of 57 black subjects in whom total platelet CD36 was assessed by semiquantitative Western blot. Mostly weak associations with \( \text{CD36} \) SNPs were found in that analysis, including some identified in both our white and black cohorts.

Although few data have been published linking \( \text{CD36} \) SNPs to expression levels, there are multiple reports associating \( \text{CD36} \) SNPs with phenotypes relevant to the known functions of \( \text{CD36} \). These include serum levels of HDL, LDL, triglycerides, and fatty acids as well as risk of atherothrombotic events, obesity, and metabolic syndrome. The directions of our \( \text{CD36} \) expression associations were consistent with those studies: SNPs associated with higher CD36 are those that have been linked to obesity, metabolic syndrome, and dyslipidemia, whereas SNPs associated with low levels are those linked to low circulating fatty acids and high HDL. Table 4 lists 14 \( \text{CD36} \) SNPs with \( P \) values \( < 0.05 \) that have been associated with other phenotypes in the literature and shows the direction of effect we found on platelet CD36 expression and the direction of other phenotype effects reported in the literature.

The mechanisms by which \( \text{CD36} \) genetic polymorphisms influence platelet CD36 expression remain to be determined. The finding that many of the SNPs associated with platelet CD36 levels have also been reported to be associated with lipid phenotypes suggests that the mechanisms may not be entirely megakaryocyte specific. It is possible that the identified SNPs could be located in genomic areas important to \( \text{CD36} \) gene transcription or mRNA

Figure 6. CD36 SNP \( P \) values for association with CD36 MFI. \( P \) values are listed in supplemental Table 1. CAF indicates coded allele frequency; and R2, estimated \( r^2 \) of SNP with top SNP rs3211870, estimated separately for each subset (white and black). Dark gray horizontal line represents the Bonferroni 0.05 threshold for the CD36 cis analysis. SNPs with dark labels have been associated with lipid, metabolic, or CVD phenotypes in the literature (supplemental Table 2).
GWAS with a loci outside of the CD36 locus in the setting of chronic diseases associated with oxidant stress, hyperlipidemia, or microparticle generation may lower the threshold for platelet activation by other agonists and hence predispose to thrombosis. Our data are also consistent with those of Knowles et al who studied a cohort of subjects presenting with their initial cardiovascular event and found a significant association between a CD36 SNP and those presenting with AMI versus stable angina.32

Acknowledgments

The biologic significance of our findings relates to the potential role of CD36 in promoting thrombus formation. We thus demonstrated, for the first time, that levels of CD36 in healthy subjects correlated closely ($r^2 = 0.87$) with degree of platelet reactivity to oxLDL, a model CD36 ligand, and hypothesize that SNPs or haplotypes that associate with high or low CD36 expression levels might be predictive for thrombotic risk. These data are consistent with our studies of cd36 null mice8,9,11 that showed partial protection from prothrombotic states induced by hyperlipidemia, circulating microparticles, and/or oxidant injury to the vessel wall. We propose that CD36-mediated platelet signals generated in the setting of chronic diseases associated with oxidant stress, hyperlipidemia, or microparticle generation may lower the threshold for platelet activation by other agonists and hence predispose to thrombosis. Our data are also consistent with those of Knowles et al who studied a cohort of subjects presenting with their initial cardiovascular event and found a significant association between a CD36 SNP and those presenting with AMI versus stable angina.32

Similarly, Pellikka et al found an association between a CD36 SNP and risk of fatal prehospitalization AMI.33 A whole genome scan of approximately 9600 subjects enrolled in the Family Blood Pressure Program identified a region of chromosome 7 containing the CD36 locus associated with stroke or AMI.34 These studies are consistent with our hypothesis that CD36 levels could impact thrombotic risk associated with underlying atherosclerosis.

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Platelet CD36 surface expression levels affect functional responses to oxidized LDL and are associated with inheritance of specific genetic polymorphisms

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