The 9p21.3 Locus:

Platelets Enter the Fray

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A series of reports in 2007 established that single nucleotide polymorphisms (SNPs) in the 9p21.3 locus are associated with coronary heart disease (CHD), both in patients with acute myocardial infarction and chronic atherosclerosis.[1–4] Subsequently, many additional studies used a candidate locus approach to replicate the 9p21.3-CHD association. Intriguingly, other phenotypes have also been found to be associated with this genomic region, including type II diabetes, stroke, malignant melanoma, aortic aneurysms, cerebral aneurysms, and periodontitis. Unfortunately, neither the causative variants nor genes have been established. Although genetic associations may permit better disease risk stratification, without the knowledge of the genes and variants we lose opportunities to improve our basic understanding of the disease process and hence the potential for targeted therapies.

Many cellular pathways in multiple tissues contribute to the pathogenic processes resulting in CHD. There is value in using intermediate phenotypes as outcomes in genetic association studies because there is enhanced power to detect gene associations when the number of genes potentially responsible for the phenotype is reduced, thereby increasing the fraction of the variance explained by any single factor or gene. In addition, intermediate traits are usually easier to define (have less heterogeneity) than clinical disease. Somewhat surprisingly, 9p21.3 has not been associated with measures believed to represent the chronic process of atherosclerosis or endothelial cell reactivity,[5] suggesting that this locus may contribute to CHD by an alternate pathophysiologic mechanism. There is abundant pathological and clinical evidence demonstrating the vital role played by blood platelets in acute coronary syndromes, which result from the formation of occlusive platelet thrombi in coronary arteries at the sites of ruptured atherosclerotic plaques,[6,7] and antiplatelet agents

have become a mainstay of therapy in patients with acute coronary syndromes. In addition to the acute occlusive thrombosis mediating CHD events, there is evidence that platelets participate in the early initiating events of atherosclerosis. Platelets are important delivery vehicles for inflammatory chemokines, such as CD40L, RANTES, interleukin-1β, macrophage chemotactic peptide-3, and so forth, and for growth factors such as platelet-derived growth factor and transforming growth factor-β. Platelets do not interact with healthy, quiescent endothelial cells but adhere to activated endothelium via platelet GPIIb-IIIa (integrin aIIbβ3) and other cell adhesion molecules.[8,9] Platelets also bind to monocytes and mediate monocyte recruitment to inflamed endothelium.[10]

The above rationale provides great interest for testing the 9p21.3 locus for associations with platelet reactivity, which was the goal of the work by Musunuru et al[11] in this issue of *Circulation: Cardiovascular Genetics*. Important for any genetic association study, these authors had previously shown a strong heritability component to the marked interindividual variation in platelet reactivity.[12,13] The primary cohort for the platelet genetic study was 788 largely healthy Amish adults (the Heredity and Phenotype Intervention [HAPI] Heart Study) who had had assessment of platelet reactivity and genotyping with the Affymetrix 500K Array set. The authors focused on SNPs in a 175-kb region of 9p21.3 and analyzed 29 SNPs that had minor allele frequencies >0.02 and an excellent genotyping call rate. These 29 SNPs formed 2 haplotype blocks, and all 12 SNPs in block 1 were associated ($P<0.001$) with the platelet aggregation in response to 0.5 μg/mL collagen in diluted whole blood. SNPs rs10965212, rs7049105, rs10965215, and rs10965219, which are in strong linkage disequilibrium (pairwise $r^2<0.97$), displayed the strongest associations ($P=0.0001$ to 0.0002). Notably, higher concentrations of collagen (expected to induce a greater extent of aggregation) lost statistical significance. SNPs in block 2 were not associated with platelet reactivity but were associated with coronary artery calcification (CAC) scores, a measure of atherosclerotic burden. CAC scores did not correlate with platelet aggregation. Furthermore, the strength of the association between rs10965219 and platelet reactivity was not substantially altered when adjusting for CAC score or traditional CHD risk factors, further suggesting that the genetic variant(s) at 9p21.3 directly influences an intrinsic property of this platelet phenotype. A replication study used the Framingham Heart Study (FHS), which had performed platelet aggregation assays and genomewide genotyping using the Affymetrix 500k array on 2364 healthy subjects. Using the threshold aggregation response to epinephrine in platelet-rich plasma, the FHS replicated ($P=0.001$) the association with rs10965219. A second replication study was performed with the Genetic Study of Aspirin Responsiveness (GeneSTAR) study, using 327 families (1169 generally healthy adults). The samples from this study had been genotyped using the Illumina 1M/L panel, and SNP rs10965219 was imputed using MACH. The GeneSTAR replication showed a trend to significance ($P=0.087$) between the imputed rs10965219 allele and 1 μg/mL collagen-induced whole blood platelet aggregation. Meta-analysis was also performed using the 3 studies, and $P=0.0002$ for SNP rs10965219 was obtained.

Platelets are the only cell whose function is routinely studied ex vivo in a clinical setting, and there are unique challenges to these assays. Platelets cannot be frozen for "batch" analysis, must be processed carefully, and activity must be measured within 2 to 3 hours of phlebotomy. The gold standard for assessing platelet function ex vivo is platelet aggregation,

but this assay is quite labor-intensive. In addition, there are numerous other variables affecting the ex vivo assessment of platelet aggregation, including the use of multiple different platelet agonists with different agonist concentrations. Because of these challenges, there are only a few well-characterized cohorts with sufficient numbers of subjects available for genetic studies. For all these reasons, Musunuru et al are to be commended for a huge amount of high-quality phenotyping and combining their phenotypes into a coherent analysis.

Why study platelet aggregation in healthy individuals instead of using patients with established coronary disease? The short answer is that we presume genetic variants that contribute to platelet hyperreactivity in people without CHD will be relevant to patients with the disease. The somewhat less satisfying answer is that it is the most practical approach. Most patients with established CHD are taking aspirin or clopidogrel, such that native platelet responsiveness cannot be measured. The alternative—to follow a large healthy cohort for many years for incident coronary disease—would be both expensive and time-consuming. The good news is that both the FHS and GeneSTAR have been designed in such a way that should allow future analyses of genetic variants associated with incident CHD and aspirin pharmacogenetics.

It was especially interesting to note that the lower concentration of collagen in HAPI, a concentration predicted to better identify a hyperreactive platelet phenotype, had a stronger association with rs10965219 than the higher concentrations used in HAPI and in GeneSTAR. The basic biology of platelet aggregation to low-dose agonist concentrations probably has some fundamental differences compared with high-dose agonist concentrations. First, more signaling pathways (hence more genes) are involved in high doses. Second, the low doses will define the "hyperreactive" platelets, which we believe defines the subjects at highest risk for myocardial infarction and stroke.[14]

There were some differences among the platelet phenotypes used in this report. Platelet aggregation was assessed by maximal aggregation to 0.5 μg/mL collagen in 1:1 buffer-diluted whole blood (HAPI), the threshold aggregation response to epinephrine in platelet rich plasma (FHS), and maximal aggregation to 1.0 μg/mL collagen in undiluted whole blood (GeneSTAR). It would have been useful to know the genotype associations with collagen and with a "hyperreactive" epinephrine phenotype (aggregation to 0.1 μmol/L or 0.5 μmol/L) in the FHS. On the other hand, at least for this genomic region, the use of 1:1 buffer-diluted whole blood versus platelet-rich plasma (largely free of red and white blood cells) made no difference in the outcome. Taking this one step further, these findings suggest that 9p21.3 regulates a shared platelet signaling pathway downstream of the two major pathways of platelet activation, since epinephrine activates platelets though several G protein coupled receptors and collagen primarily activates platelets through an immunoreceptor tyrosine-based activation motif associated with glycoprotein VI. The FHS and GeneSTAR groups have recently reported results from a genomewide association study using platelet phenotypes from the same subjects,[15] but SNPs in the 9p21.3 locus were not identified as associated with platelet reactivity. Most likely, the SNPs in the 9p21.3 region did not meet accepted levels of genomewide significance in their prior work. The GeneSTAR replication did not meet statistical significance in the current work, perhaps because of imputation of

SNP data, demographic differences in the cohort, and/or because of the more rigorous adjusting for confounders.

Nevertheless, Musunuru et al provide the first evidence that a platelet phenotype is associated with 9p21.3 in European Americans. In fact, this is one of the first studies to show a significant association between the 9p21.3 locus and any intermediate phenotype for CHD. The pleiotropism of this locus is likely to remain bewildering until causative genes are identified. Although there are no known expressed genes in this region, strong evidence indicates this region has enhancer activity.[16,17] The laboratory of Pennacchio (Visel et al[17]) knocked out the corresponding region in the mouse genome and found that the cardiac expression of *Cdkn2a* and *Cdkn2b* was markedly reduced via a distal *cis*-acting mechanism, and aortic smooth muscle cells exhibited excessive proliferation. The broad expression of these cyclin-dependent kinase inhibitors could modulate multiple tissue phenotypes. An intriguing *trans*-acting mechanism has also been suggested, wherein the expression of the noncoding RNA *ANRIL*, located in the 9p21.3 region, correlates with different alleles.[16] Noncoding RNAs may use a variety of mechanisms to alter the expression of multiple genes, which could also account for multiple phenotypes. The accurate definition of the pathophysiologic processes associated with 9p21.3 generates more optimism that disease-causing molecular genetic mechanisms may soon be identified.

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