The Influence of Platelet Collagen Receptor Polymorphisms in Hemostasis and Thrombotic Disease

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Abstract—Extracellular collagens modulate the rate of platelet activation and thereby markedly influence hemostasis and thrombosis. Platelet receptors for collagens, such as the integrin α2β1, platelet glycoprotein (GP) VI or, indirectly, the GPIb complex, are unexploited targets of pharmacological control, and polymorphisms of these receptors have recently become factored into the genetic risk for thrombosis. Seemingly contradictory findings already exist with regard to the contribution of GPIbα and integrin α2 polymorphisms, but these discrepancies will be resolved once there is better standardization of clinical studies. There is already substantial evidence that GPIbα VNTR A or B alleles, the GPIbα-5C allele, and integrin α2 allele 1 (T807) each contribute to increased risk for morbidity in thrombotic disease. However, larger, prospective genetic and epidemiological studies are needed to clarify the role of each of these polymorphisms, the contribution of other platelet receptor polymorphisms, and the synergistic effects of combinations of these factors. In addition, in vitro studies that establish the functional relevance of these polymorphisms will provide sound biological explanations for the results of clinical correlation studies. (Arterioscler Thromb Vasc Biol. 2002;22:14-20.)

Key Words: platelet ■ collagen ■ integrin ■ GPVI ■ thrombosis

The importance of thrombosis as a health hazard cannot be overstated. Ischemic heart disease and cerebrovascular disease are the leading causes of morbidity and mortality among both men and women in the developed western world.1–4 Recent evidence indicates that the incidence of this disease is steadily increasing among Asian populations,5 and cardiovascular disease is now the leading cause of death among native American Indians.6 Epidemiological studies indicate that these diseases result from complex interactions between genetic susceptibility factors, chronic environmental influences (for example, hormonal imbalance, smoking, or obesity), and established, intercurrent disorders (such as diabetes, hypertension, dyslipidemia, or hyperhomocysteinemia). The most devastating complication of these disorders is acute myocardial infarction, resulting from the formation of an occlusive thrombus at the site of a ruptured atherosclerotic plaque. The critical role of platelets in this process is now well-accepted.7

At the same time, efficient platelet cohesion is necessary for the life-saving process of hemostasis. The rate of platelet activation is one important variable that contributes to the selection of these very different outcomes. Collagen is an important modulator of platelet activation through its stimulation of at least three platelet receptors. Platelets have two major primary receptors for collagens, the integrin α2β1 and the platelet-specific receptor glycoprotein (GP) VI. A third receptor that figures prominently at the very onset of adhesion, the GPIb complex, is not usually thought of as a collagen receptor. Nonetheless, it is collagen that captures the von Willebrand Factor (VWF) molecule by binding to most likely its A3 domain, thereby localizing it and somehow altering its conformation to make it bind via its A1 domain with greater avidity to the GPIb complex. This interaction is relatively weak and short in duration, resulting in a slowing of platelet motion and a tethering or rolling of the platelet across the thrombogenic collagen-rich surface. It is at this point that the two major receptors, α2β1 and GPVI, become involved. GPVI binds to collagens, albeit weakly, and plays an important role in transduction of signals leading to platelet activation. The more avid stable platelet attachment afforded by α2β1 leads to a stable monolayer of activated platelets that serves as a nidus for prothrombin conversion and thrombus formation.

There are a number of additional aspects of the platelet-collagen interaction that are relevant. Like many integrins, α2β1 can also undergo activation-dependent increases in avidity for collagens.8 Because the binding of VWF to the GPIb complex is known to activate another platelet integrin, αIIbβ3, it is conceivable that it may also activate α2β1. In addition, an overly simplistic model wherein GPVI functions in signal transduction while α2β1 merely mediates attachment would overlook the important contribution of α2β1 to signal transduction in the absence of GPVI.9–11

Plaque rupture and/or endothelial damage leads to exposure of collagens, retention of VWF, and the adhesion of circulating platelets to the damaged vessel wall, largely via...
the receptors described above. In the process, the platelet is activated leading to a conformational change of a second integrin α5β3 that facilitates fibrinogen binding and platelet aggregation. Thrombin generated at the blood-plaque interface converts fibrinogen to fibrin, which stabilizes thrombus growth. Therefore, any genetic differences that might alter surface expression or activity of the primary collagen receptors could influence risk for adverse outcomes as a result of the hemostatic process. In the last five years, there has been a rapid accumulation of literature concerning the relationship between genetic variations in platelet glycoproteins and risk for coronary heart disease.

This review will focus mainly on the nature of GPIbα and integrin α2 polymorphisms and how they might contribute to the regulation of hemostasis and thrombosis. It will also briefly touch on the diversity in expression of GPVI, and how this might be related to expression of α2. However, none of these platelet receptors acts in a vacuum in vivo, and the synergy between genetic differences in these and other key receptors needs to be considered.

Clinical Relevance of GPIb Polymorphisms

The GPIb complex is a heptamer composed of four distinct gene products: two molecules of GPIbα, two of GPIβ, two of GPIX, and one of GPV. VWF is directly bound by the GPIbα subunits, each of which is disulfide-linked to a GPIββ subunit. To date, all of the potentially relevant polymorphisms of the GPIb complex have been associated with the GPIbα subunit.

The function, expression, and immunogenicity of the GPIb complex is influenced by each of three GPIbα polymorphisms (Figure 1). The first involves a variable number of tandem repeats (VNTR) within the mucin-like macroglycopeptide region of GPIbα, resulting in the duplication of a 13-amino acid sequence once (VNTR D), twice (VNTR C), thrice (VNTR B), or four times (VNTR A). This produces a polypeptide length of 610, 623, 636, or 649 amino acids, respectively.12,13 Each repeat could add up to 32 angstroms to the length of the GPIbα extracellular domain, because these repeats are rich in proline, serine, and threonine (Thr) and can become glycosylated.12 In a simple model, yet to be proved, this could extend the GPIbα binding sites for VWF and thrombin further above the plane of the plasma membrane, increasing the avidity for these ligands and accounting for the observed increased risk for acute coronary artery disease associated with the longer variants.14,15

The second GPIbα polymorphism, a Thr/methionine (Met) substitution at amino acid 145, lies within the region of the ligand-binding, leucine-rich motifs (LRM),16,17 and is the basis of the HPA-2 (Ko) platelet alloantigen system (Figure 1). The gene frequencies of each allele are: A (0.01), B (0.07), C (0.82), and D (0.11). Another dimorphism, the Met/Thr at residue 145 (nucleotide T/C substitution), gives rise to the Koα and Koβ alloantigens and is linkage disequilibrium with the VNTR polymorphism. Thus, the VNTR A and B alleles express only Met-145, whereas the VNTR C and D alleles express only Thr-145. Bottom, In GPIbα, a C/T substitution at a position five nucleotides upstream from the ATG start codon influences an adjacent Kozak sequence and thus the rate of translation of the mRNA transcript by the cell machinery. On average, the relationship of GPIbα levels to genotype at this position is: T/T = 1.0; T/C = 1.3; C/C = 1.5.

and risk for coronary artery disease14,15 or stroke15,18 in younger individuals. However, there has not been a universal confirmation of this finding, based on several additional reports.19–22 These inconsistencies may be explained by the unaccounted for contribution of additional polymorphisms.

The final polymorphism of GPIbα represents a T/C substitution in the region of the translation start site, at position 5 nucleotides upstream (−5) from the initiator codon (ATG) (Figure 1). In platelets,28,29 the presence of the −5C allele (gene frequency of ~0.15 in various western populations) increases the mean level of GPIbα copies on the platelet plasma membrane (roughly, a 50% increase in homozygous individuals, and a 33% increase in heterozygous individuals) presumably because it enhances the interaction of the cellular protein translation machinery to the adjacent Kozak sequence.28 Although a number of clinical studies initially failed to show an association of the −5C allele with increased risk for development of acute coronary artery disease, more recent reports paint a different picture. There may well be an association between −5C and the severity of negative outcomes after acute myocardial infarction in younger individuals (~6 years old).30 Moreover, a recent study has documented a synergistic effect of −5C and Met-145 that results in an increased risk for stroke in younger individuals.31

Stable Adhesion to Collagens Mediated by Integrin α2β1 and GPVI

The Human α2 Gene

The integrin α2 subunit is a single chain transmembrane polypeptide that pairs exclusively with the β1 subunit. In
humans, a single copy of the \( \alpha_2 \) gene (ITGA2) is present in the haploid genome, located on the short arm of chromosome 5 (5p11.12). An earlier study had incorrectly located the gene on the long arm of this chromosome at 5q23 to 31. It is now also apparent that ITGA2 is situated very close to and immediately downstream from the integrin \( \alpha_1 \) gene (ITGA1). This is important to our understanding of the transcriptional regulation of both of these genes. As depicted in Figure 2, ITGA1 (\( \sim 170 \) kb in length) and ITGA2 (\( \sim 110 \) kb in length) are separated by only 32 kb. Three independently defined chromosomal tags are situated in this same locus: D5S2092 is located roughly 44 kb upstream from the ITGA1 start codon; D5S623 is located at the very beginning of ITGA2 intron 1; and D5S2037 is situated about 420 kb downstream from the ITGA2 stop codon. The ITGA2 cDNA sequence published by Takada and Hemler\(^{34} \) was nearly complete, lacking the bulk of the final exon 30 and a polyadenylation site. We have subsequently completed the \( \alpha_2 \) C DNA sequenced and located the polyadenylation site.\(^{32} \) These findings establish that ITGA2 exon 30 is unusually large (\( \sim 4.1 \) kb), larger than the combined size of the first 29 exons. The initial published signal sequence of human ITGA1 is incorrect, albeit 100% identical to the rat ITGA1 signal sequence.\(^{35} \) We have subsequently reported the correct human ITGA1 signal sequence.\(^{32} \)

### \( \alpha_2 \) Polymorphisms

There is, on average, a 4-fold range in platelet \( \alpha_2 \beta_1 \) density among randomly selected individuals, and these differences correlate directly with platelet adhesiveness to type-I or type-III collagens. This relationship cannot be explained by differences in mean platelet volume, because these vary by no more than 20%.\(^{36,37} \) On the other hand, platelet \( \alpha_2 \beta_1 \) density does correlate with and likely results from the inheritance of different alleles of the \( \alpha_2 \).\(^{38,39} \) Three common \( \alpha_2 \) alleles can be defined. Allele 1 (807T/1648G/2531C) is associated with increased levels of \( \alpha_2 \beta_1 \), whereas Allele 2 (807C/1648G/2531C) and Allele 3 (807C/1648A/2531C) are each associated with decreased levels of this receptor. The gene frequencies of these three alleles in a typical white population are: allele 1 = 0.36; allele 2 = 0.56; and allele 3 = 0.08. Right, The relative level of platelet \( \alpha_2 \beta_1 \) is controlled by the inheritance of these three \( \alpha_2 \) alleles. For example, individuals who are homozygous for \( \alpha_2 \) allele 1 (1,1) express, on average, 4-fold the amount of this receptor that would be found on platelets of individuals who are homozygous for \( \alpha_2 \) allele 2 (2,2).

The continuum of differences in platelet \( \alpha_2 \beta_1 \) density among normal individuals belies a simple gene dosage effect and suggests the influence of additional, unlinked genetic factors. Not surprisingly, we have identified inherited, single-base substitutions at two positions, C\(_{\alpha_2} \)T and C\(_{\alpha_2} \)G, within the proximal 5'-regulatory region (within \( \sim 1096 \) to +48) of the human integrin \( \alpha_2 \) gene.\(^{45,46} \) The T\(_{\alpha_2} \) and G\(_{\alpha_2} \) sequences have a gene frequency of 0.35 and 0.15, respectively, in a typical white population. The substitutions T\(_{\alpha_2} \) and G\(_{\alpha_2} \) independently attenuate gene transcription, whereas the combination G\(_{\alpha_2} \)T\(_{\alpha_2} \) has an additive negative influence, in transfected human megakaryocytic cell lines. Thus, the natural dimorphisms C\(_{\alpha_2} \)T and C\(_{\alpha_2} \)G within the proximal 5'-regulatory region of the human integrin \( \alpha_2 \) gene contribute to the regulation of integrin \( \alpha_2 \beta_1 \) expression on megakaryocytes and blood platelets and must thereby modulate collagen-related platelet functions in vivo.

In the very common, but symptomatically mild, type 1 von Willebrand disease (VWD), platelet adhesive functions are dimorphisms is causally involved in inherited expression differences. The rate of platelet attachment to type I collagen in whole blood under conditions of high shear rate (1,500/s) is proportional to the density of \( \alpha_2 \beta_1 \) receptors on the platelet surface, as determined by differential inheritance of alleles 1, 2 or 3 (Figure 3). These initial findings suggested that \( \alpha_2 \) alleles could influence risk of thrombosis or bleeding in relevant disease states.
impaired because of the decrease in VWF levels in plasma and platelets. DiPaola et al. have demonstrated that the low-density α2 allele 2 increases the risk for bleeding in type 1 VWD. The frequencies of α2 alleles in symptomatic patients with five types of VWD were measured (type 1, n = 78; type 2A, n = 25, type 2B, n = 14; type 2 mol/L, n = 10; and type 3, n = 20). Compared with the normal group, no significant difference in allele frequencies was observed among individuals with types 2A, 2B, 2 mol/L, or 3 VWD. However, the frequency of allele 2 among type 1 VWD patients (0.71) was significantly higher than that of the normal population (P = 0.007). Also, in patients with VWD type 1 and borderline to normal ristocetin-cofactor (VWF:RCo) activity values, collagen receptor density correlates inversely with closure time in a high shear stress system, the Platelet Function Analyzer (PFA-100; Dade-Behring). Thus, low platelet α2β1 density results in less efficient primary platelet adhesion and may result in increased tendency to bleed, as evidenced by the high frequency of this polymorphism in patients with type 1 VWD compared with normal individuals. In addition, this may account for the variability between patients with similar levels of VWF antigen, but strikingly different bleeding histories.

Santos et al. investigated the relationship of the α2 alleles to the risk of coronary artery disease (CAD) and myocardial infarction (MI; Figure 4). DNA samples from 2237 male patients who underwent coronary angiography on account of coronary heart disease as verified illness or presumptive diagnosis were genotyped. A strong association between allele 1 and nonfatal MI was found among individuals younger than the mean age of 62 years (n = 1057; odds ratio, 1.57; P = 0.004), and an even stronger association existed among individuals within the youngest 10% of the study sample (<49 years; n = 223; odds ratio, 2.61; P = 0.009). In contrast, no evidence of an association between α2 alleles and CAD was found.

The significance of the C807T dimorphism to arterial disease has been evaluated in several other studies (Figure 4). The initial reports described a correlation between allele 1 (T807) (high receptor density) and risk for MI. These have been confirmed by Roest et al., who found that allele 1 (T807) was associated with increased vascular mortality in women who are heavy, chronic smokers. In addition, a significant association was also found in younger patients with stroke and in patients with diabetic retinopathy. On the other hand, some studies were unable to establish an association between allele 1 (T807) and MI. Conversely, in two of these studies, the frequency of the homozygous TT genotype among the study controls was higher than that reported among normal white or Asian populations, respectively, and it may have markedly influenced the conclusions. It is evident that patient and control selection bias needs to be eliminated from clinical studies of this kind by careful consideration of ethnic and racial influences on allele frequencies.

The Lys551Glu amino acid substitution located in the cation-binding domain of α2 is responsible for HPA-5b (Br) and HPA-5a (Br) epitope formation. In a large study, Kroll et al. found an association between HPA-5 dimorphism in low-risk patient subgroup with CAD. In this population, the frequency of Br homozygous individuals was over-represented. This finding suggests that allele 3 (807C; Br) may increase risk for thrombotic disease through a qualitative effect on α2β1 function that is independent of genetic effects on expression levels.

In contrast, the density of platelet α2β1 has not been found to be a risk factor for venous thrombosis. This lack of influence on thrombosis on the venous side is a typical finding for all of the platelet glycoprotein dimorphisms that are currently under study.

### Platelet GPVI

GPVI is a major platelet glycoprotein (60- to 65-kDa) that had been considered a putative receptor for collagen since the identification of a patient with a mild bleeding disorder whose platelets lacked GPVI and exhibited defective collagen-induced responses. Collagen binding to GPVI induces platelet activation through a pathway that involves phosphorylation of the FcγR chain followed by the binding of Syk and the phosphorylation-dependent activation of PLCy2. The sequence of collagen that is recognized by GPVI has been identified as Gly-Pro-Hyp, and a synthetic collagen-related peptide (CRP) based on the triple helical form of this tripeptide sequence is a GPVI-specific platelet agonist. The GPVI-Fcγ complex will transduce outside-in signals by an immune receptor-like mechanism that involves Syk and the phosphorylation-dependent activation of PLCy2. The C-type lectin convulxin (CVX), from the tropical rattlesnake Crotalus durissus terrificus, is a multimeric protein that binds specifically to GPVI and induces platelet activation through the clustering of GPVI. CVX is a ready tool for the detection and quantitation of GPVI that also proved invaluable for the isolation and eventual sequencing of this receptor. GPVI cDNA has an open reading frame of 1017 base
pairs coding for a protein of 339 amino acids including a putative 23–amino acid signal sequence and a 19–amino acid transmembrane domain between residues 247 and 265. By sequence homology, GPVI is a member of the immunoglobulin superfamily, and its sequence is closely related to FcεR and the natural killer receptors. It contains two Ig-C2-like domains formed by disulfide bridges. The cytoplasmic tail, consisting of 51 amino acids, is atypical of and shows little homology with the C-terminal part of the other members of this protein family.

To analyze the variability in platelet GPVI content between individuals, we developed a semiquantitative ligand blot assay in which biotin-conjugated CVX binds selectively to GPVI in separated total platelet proteins. We observed a 5-fold range in platelet GPVI content among 23 normal healthy subjects that cannot be accounted for by differences in mean platelet volume between donors, because the latter variation is on the order of 20% to 30%. In addition, we have determined that CVX- or CRP-induced prothrombinase activity is directly proportional to the platelet content of GPVI. In view of the well-documented association of GPVI with platelet procoagulant activity, the variation in GPVI content reported in this study suggests that variation in GPVI content may also contribute to risk for hemorrhagic or thromboembolic disorders.

Among our normal platelet donor panel, we found that the content of GPVI appears to parallel the density of α2β1. This intriguing and statistically significant correlation remains unexplained at this time. It is possible that these two receptors are physically associated at the platelet surface, and that the expression of one is dependent on the presence of the other, the density of the latter under genetic control. However, there is no evidence yet that this is the case. Nonetheless, it is possible that the expression of both receptors is regulated during megakaryocyte development by an independent gene product, perhaps a transcriptional factor, and that the levels of this third gene product are genetically controlled. Consistent with this possibility, Lagrue-Lac-Hal et al identified a 65-kDa receptor that binds uniquely to type I collagen, and a 68- to 72-kDa receptor has been recently identified that binds preferentially to type III collagen. The relative influence of these receptors on collagen-dependent platelet functions in vivo remains to be evaluated. More recently, the exciting possibility was raised that GPV might be involved in collagen responses. The precise contribution of GPV to platelet function has remained elusive, but the establishment of GPV−/− mice may have uncovered an unexpected role for this receptor. Platelets from these mice seem to have a defective response to collagen. This intriguing and unexpected observation warrants further investigation.

Other Collagen Receptors

Additional receptors may contribute to the variability of donor responses to collagens. Early work by Chiang et al identified a 65-kDa receptor that binds uniquely to type I collagen, and a 68- to 72-kDa receptor has been recently identified that binds preferentially to type III collagen. The relative influence of these receptors on collagen-dependent platelet functions in vivo remains to be evaluated. More recently, the exciting possibility was raised that GPV might be involved in collagen responses. The precise contribution of GPV to platelet function has remained elusive, but the establishment of GPV−/− mice may have uncovered an unexpected role for this receptor. Platelets from these mice seem to have a defective response to collagen. This intriguing and unexpected observation warrants further investigation.

Summary

With regard to hemostasis and thrombosis, collagens are among the most important physiological components of the extracellular matrix in their role as platelet activators. Differences in the rate of platelet activation markedly influence normal hemostasis and the pathological outcome of thrombosis. Thus, collagen receptors, such as the integrin α2β1 and, indirectly GPlbα, represent a relatively unexploited target of pharmacological control and are only recently becoming appreciated as potential factors in the genetic risk for thrombosis. In general, the importance of platelet glycoprotein polymorphisms as genetic risk factors for arterial thrombosis is a new area of human genomics that needs to be carefully addressed. As was the case with previously proposed genetic risk factors, controversies and seemingly contradictory findings already exist with regard to GPlbα and the integrin α2. These discrepancies will only be resolved once there is a universal standard for clinical study design. Most of the clinical studies differ by patient population size, ethnicity, bias in the selection of patients and controls, plurality in clinical endpoints and variation of environmental factors. Despite these differences, there is substantial evidence that the GPlbα VNTR A or B alleles, the −5C allele of GPlbα, and allele 1 (T807) of integrin α2 each contribute to the risk for and morbidity of thrombotic disease. There may remain dispute as to the extent of their contribution. However, well-designed, large, prospective, genetic and epidemiological studies are needed to clarify the role of these and other platelet receptor polymorphisms. Additional in vitro studies of the functional relevance underlying these polymorphisms are needed to provide a sound biological explanation for the results of clinical correlations. The opportunity now exists to make significant inroads into the development of strategies for the prevention of thrombotic disease.

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