Measurement of Platelet Collagen Receptor Density in Human Subjects

To the Editor:

We are writing to provide data on a novel approach for measuring platelet collagen receptors in human subjects. Immediately after vascular injury, circulating platelets are exposed to collagen, a matrix protein that both supports platelet adhesion and activates platelets through platelet collagen receptors.1 Collagen signals generated at a site of arterial vessel injury are therefore likely to be among the first platelet activating signals that generate the coronary thrombi responsible for myocardial infarction. The molecular basis of platelet collagen responses has recently been elucidated by the identification and characterization of two platelet collagen receptors: the immune receptor homologue glycoprotein VI (GPVI)2 and the integrin α2β1.3 GPVI is required for platelet activation in response to collagen,4,5 whereas α2β1 plays an accessory role to support platelet responses to immobilized collagen in the setting of high shear forces.6

Preliminary findings from both laboratory and clinical studies suggest that the surface density of the platelet collagen receptors GPVI and α2β1 may regulate the degree of platelet activation by collagen and its clinical outcome.7-11 Despite these preliminary findings, the precise role of platelet collagen receptor density as a risk factor for MI remains poorly understood, with the few reported studies yielding apparently contradictory results. A study examining the correlation of platelet GPVI receptor polymorphisms with surface receptor density in normal individuals found one common allele (associated with the coding region polymorphism T683C) that correlated with lower GPVI receptor density and reduced collagen-mediated platelet aggregation.8 In contrast, a study examining GPVI polymorphisms in a population with vascular disease found the presence of this allele to correlate with an increased risk of MI, although these investigators did not directly measure platelet GPVI receptor levels.12 The discrepant findings of these studies highlight the limitations associated with using common polymorphisms as clinical predictors, rather than biological expression, such as actual platelet collagen receptor density.

In the present study we assessed the feasibility of directly measuring the density of platelet collagen receptors GPVI and α2β1 in human subjects, by using a novel anti-GPVI monoclonal antibody and a previously characterized anti-α2β1 monoclonal antibody.

The anti-human GPVI mouse monoclonal antibody HY101 was produced by injection of GPVI R272L-expressing 3T3 fibroblasts derived from BALB/c mice as described.7 Generation of fluorescein isothiocyanate (FITC)-HY101 has also been described.7 The ability of FITC HY101 to accurately measure the surface level of GPVI receptors on platelets and on GPVI-expressing RBL-2H3 cells was established by comparing the FITC-conjugated HY101 antibody to 125I-HY101.7 These studies used 3 stable GPVI-expressing cell lines whose GPVI-receptor densities differed by 2-fold and 5-fold, to demonstrate that flow cytometric quantification of GPVI density matched that obtained using studies of surface GPVI measured with radiolabeled HY101 and studies of total cellular GPVI measured using standard western blotting.7 FITC-conjugated anti-human α2 antibody (AK-7) was purchased from Pharmingen, and has been shown to reproducibly distinguish between α2β1-expressing cell lines that differ in receptor density by <2-fold.12

A total of 154 subjects were enrolled from outpatient practices at the Philadelphia Veterans Administration (VA) Medical Center between January 2002 and January 2003. The study was approved by the local institutional review board, and all subjects provided signed informed consent before participating in the study. All subjects donated 5 mL of whole blood into a heparinized tube, which was taken immediately to the laboratory for measurement of GPVI and α2β1 receptor density.

To measure collagen receptor density, 1 mL of platelet-rich plasma obtained by centrifugation of heparinized whole blood was mixed with 10 μL of FITC-conjugated anti-GPVI monoclonal antibody, and a second sample was mixed with 10 μL of FITC-conjugated anti-α2β1 antibody (final concentration in both cases was 1 μg/mL). Samples were incubated for 30 to 60 minutes at room temperature to allow antibody binding and then diluted with saline solution and immediately studied by flow cytometry to measure mean fluorescence. The fluorescence measurements were calibrated for each run using FITC-conjugated bead standards (DAKO PTY, LTD; catalog #K0110). Receptor densities are shown as mean fluorescence (MEF). The coefficient of variation for all bead standards was <2%. The inter-sample coefficient of variation, based on 30 samples run in duplicate, was 4.8%. The use of flow cytometry for this assay results in a consistent number of platelets in the analysis chamber, and thus is not affected by an individual’s total platelet count. Because there is great variability in platelet size, we established a uniform forward- and side-scatter gate for all measurements, to ensure that only platelets of similar size were measured in each individual.

We found ~16-fold variation in GPVI levels among enrolled subjects (mean 3136±1514 MEF, range 505 to 7953), and ~23-fold variation in α2β1 levels (mean 2972±1438, range 398 to 9077). Both receptor levels were normally distributed among the population. There was a significant correlation between GPVI and α2β1 levels (r=0.68 by Pearson correlation, P<0.0001, Figure).

These findings demonstrate the feasibility of directly measuring the density of platelet collagen receptors GPVI and α2β1 in human subjects, through the use of a novel anti-GPVI monoclonal antibody and a previously characterized anti-α2β1 monoclonal antibody. We found that the measurement of both receptor densities could be determined efficiently and reproducibly, and with relatively few steps when compared with genotyping.

Although numerous platelet receptor genotypes have been identified, and some associated with an increased risk of MI,13,14 surprisingly few specific platelet phenotypes have been characterized that alter platelet function and affect the risk for MI accordingly. Both in vitro data and human studies support an important role for the density of platelet collagen receptors on the risk of atherothrombotic events. In cells engineered to express GPVI and α2β1, higher receptor levels confer increased collagen responses.15 In normal human platelets, the level of GPVI correlates directly with the strength of platelet activation in response to collagen.8 Recent clinical studies have identified a common polymorphism of the α2β1 receptor associated with higher receptor density and an increased risk of MI.10,11,16 and stroke.1 A single nucleotide polymorphism in GPVI associated with an amino acid change (S219P) has also been reported to correlate with an increased risk of MI in older individuals.12 These studies indirectly support the concept that higher platelet collagen...
receptor levels on platelets may increase the risk of MI or stroke, but have not directly tested this hypothesis.

Our study confirms a previous observation indicating that levels of GPVI and α2β1 receptors correlate closely in individuals. We and others have recently demonstrated that these receptors function in an interdependent reciprocal manner to activate platelets in response to collagen. It could therefore be speculated that both receptors are also linked by shared regulatory elements that control their expression in platelets. The correlation between the density of these two receptors should not be related to platelet number, because flow cytometry for this assay results in a consistent number of platelets in the analysis chamber, nor should the correlation between the density of the two receptors be related to platelet size, because we applied a uniform forward- and side-scatter gate for all measurements to ensure that only platelets of similar size were measured for each individual.

In conclusion, we have developed an efficient and reproducible method for measuring platelet collagen receptor density in human subjects. A large definitive trial based on this method would be feasible and clinically important in determining whether collagen receptor density serves as a novel risk factor for MI.

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