LEUKOCYTE MEDIATED PLATELET AGGREGATE STABILIZATION UNDER SHEAR FLOW

Eric Y.H. Park and Michael B. Lawrence

Department of Biomedical Engineering
University of Virginia
Charlottesville, VA

INTRODUCTION

Deep vein thrombosis (DVT) is an important health problem affecting 600,000 patients each year. At least 50,000 patients will die each year from pulmonary embolism associated with DVT. There is increasing evidence of an interplay between thrombosis and inflammation and that the development of one pathology will activate the other. During an inflammatory response, tethering and rolling of circulating leukocytes on the endothelium is mediated by the selectin family of receptors. The initial engagement of selectin receptors is followed by β2 integrin mediated arrest and transmigration of leukocytes to the endothelium [1].

P-selectin is pre-formed in secretory granules stored in platelets and endothelial cells and are rapidly mobilized to the membrane surface of each respective cell upon activation. E-selectin is expressed on endothelial cells after de novo synthesis. Both selectins will bind to P-selectin glycoprotein ligand (PSGL-1) which is constitutively expressed on many leukocyte subsets, including monocytes [2]. Along with P-selectin, another important platelet adhesion receptor is the integrin αIIbβ3 which can mediate platelet aggregation via binding to fibrin(ogen). Leukocyte Mac-1 (CD11b/CD18) can also bind to fibrin(ogen) and may be able to mediate adhesion to platelets through the αIIbβ3 receptor [3].

In the present study, our aim was to determine which receptor-ligand pairs are crucial in the ability of platelets to aggregate and stabilize over adherent monocytes under flow conditions. We hypothesized that the fast kinetics of the P-selectin binding to PSGL-1 is responsible for the initial attachment of platelets to adherent leukocytes while the slower kinetic binding of the platelet integrin αIIbβ3 will mediate the stabilization of the thrombotic structure.

METHODS

Human monocytes were obtained from heparin (1,000 U/ml) anti-coagulated whole blood and were isolated by centrifugation over monocyte isolation density medium. In order to obtain platelet rich plasma (PRP), whole blood was drawn in acid-citrated dextrose (ACD) in a 1:10 ratio and centrifuged for 10 minutes at 350g. The PRP was collected and supplemented with platelet antagonists ACD (1/2 vol), prostacyclin (0.3 mg/ml), and indomethacin (1 mg/ml) before labeling with 5μM calcein AM for 30 minutes in the dark. The PRP was then centrifuged at 600g for 20 minutes and washed twice with ACD, prostacyclin, and indomethacin before resuspension in HEPES-based buffer. Platelet and monocyte solutions were supplemented to 1 mM $CaCl_2$ and 1mM $MgCl_2$ prior to experimentation.

Anti-CD18 monoclonal antibody (mAb) TS1/18, anti-P-selectin mAb G1, and anti-PSGL-1 mAb KPL-1 were obtained as described previously [4]. Anti-CD11b mAb LPM19c was purchased from Dako. mAb concentrations used in the platelet accumulation experiments were 5 μg/ml unless otherwise noted.

P-selectin and ICAM-1 were co-immobilized over polystyrene plates with a site density of $10/\mu m^2$ and $20/\mu m^2$, respectively. The protein slides were blocked against non-specific adhesion by incubation with 0.5% Tween-20. Human umbilical vein endothelial cells (HUVEC) were plated and were allowed to reach confluence on glass slides. HUVEC were stimulated with IL-1β (10 ng/ml) for 3 hours prior to experimentation. The slides were attached to a parallel plate flow chamber and the chamber was mounted over an inverted differential interference contrast microscope for observation. Monocytes resuspended to 0.5×10^6 were initially perfused over the HUVEC or P-selectin/ICAM-1 substrate for 5 minutes at 1 dyne/cm^2 wall sear stress. A solution of assay media or blocking antibody was washed through for 1 minute. Platelets were resuspended to 1×10^8 and were perfused into the flow chamber at a venous wall shear stress of 1 dyn/cm^2 unless otherwise indicated.

RESULTS

To assess the possible extent that an inflammatory event of endothelial cell origin could trigger platelet recruitment and aggregation, flow chamber experiments were conducted on HUVEC activated with IL-1β. The presence of rolling or adherent monocytes over HUVEC visibly directed the attachment and localization of platelets to the endothelial surface. Individual platelets could be seen tethering to the

adherent monocyte and rolling on the surface of the leukocyte. The rolling platelets would reach the downstream end of the monocyte whereupon they would accumulate and form thrombotic bodies within 5 minutes of platelet flow (Figure 1A). Negligible platelet attachment to HUVEC was seen in areas without monocytes. Likewise, platelet flow over IL-1β stimulated HUVEC without the inclusion of monocytes displayed very little platelet deposition (Figure 1B).

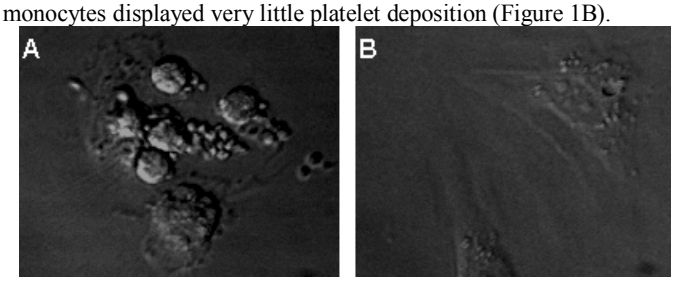

Figure 1. Thrombi formation on HUVEC

Measurements of platelet accumulation around adherent monocytes were conducted over purified P-selectin & ICAM-1 plates. At the first time point measurement of 5 minutes after platelet entrance, the average platelet aggregate size that attached to each monocyte was 6 μm^2. This measurement increased to 16 μm^2 and 19 μm^2 for 10 minutes and 20 minutes of platelet flow, respectively. Supplementation with 200 μg/ml of soluble fibrinogen resulted in variable accumulation of platelets. Addition of a small dose of thrombin (0.05 U/ml) to the platelet and fibrinogen mixture caused the platelets to attach to the monocytes more rapidly and in greater numbers. The aggregate sizes per monocyte for these thrombi started out at 23 μm^2 at 5 min and rose to 29 μm^2 at 20 min. Monoclonal antibody blockade of P-selectin or PSGL-1 resulted in the near elimination of platelet attachment to monocytes with thrombi sizes averaging no more than 1-2 μm^2 per monocyte throughout the experimental run. TS1/18 blockage of the CD18 (the β chain of LFA-1 and Mac-1) did not appear to have any effect on thrombi buildup. mAb blocking of CD11b (Mac-1), however, did significantly reduce the ability of platelets to accumulate around monocytes with thrombi sizes remaining around 11μm^2 for 10 minutes and 20 minutes post-entrance.

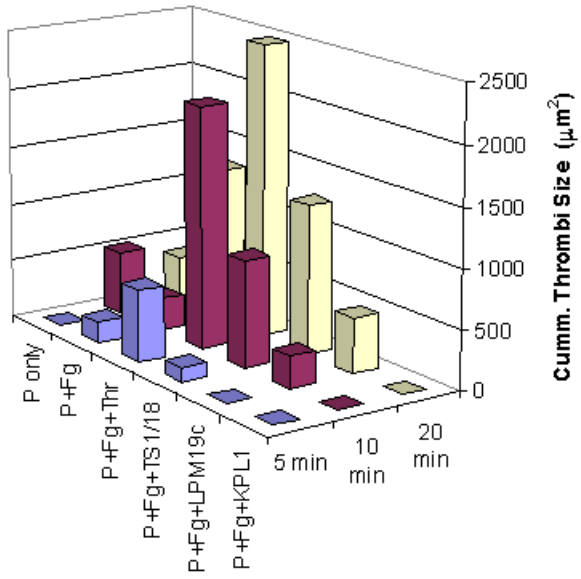

Figure 2. Cummulative size of large (>50 μm^2) aggregates

Analysis of the size distribution histogram of each platelet aggregate did not reveal any significant differences among the experimental groups except for the KPL-1 mAb set. Excluding the anti-PSGL-1 experimental group that displayed few platelet clusters, the remainder of the set were able to generate numerous small clusters (<15 μm^2) of platelets. Selective examination of thrombi that were 50 μm^2 or larger revealed significant differences based on the presence of soluble fibrinogen and between the mAb groups (Figure 2). Fibrinogen supplementation with the flowing platelets allowed more than three times as many platelets to aggregate around each monocyte compared to platelets alone at 20 minutes. TS1/18 blockade did not display any ability to abrogate the formation of large platelet thrombi. In contrast, Mac-1 inhibition by LPM19c was able to reduce large aggregate formation to the levels found in the platelet alone set.

DISCUSSION

Our data demonstrates that individual monocytes are able to capture flowing platelets and localize platelet aggregates to the endothelium under venous flow conditions. The traditional view of thrombogenesis entails vessel wall damage and exposure of the procoagulant subendothelial surface to the circulating blood, whereupon the platelets first recruited to the disrupted endothelium would become activated and attract leukocytes or other platelets. In this study, we introduce an alternative perspective for the origin of venous thrombosis. The inflammatory response of an intact endothelium not only triggers leukocyte rolling and arrest but these surface bound leukocytes would also be able to act as procoagulant sites for platelet accumulation.

Reconstituting the essential adhesive components of activated endothelial cells onto clear polystyrene slides enabled the quantitation of platelet aggregates forming around the attached monocytes. Platelet P-selectin binding to leukocyte PSGL-1 is a requirement for the initial interaction to bring flowing platelets into contact with bound monocytes. The fast kinetic association rate of the selectin molecules allows the platelets flowing in circulation to attach to the surface of leukocytes. The availability and its ability to bridge the binding between platelet integrin αIIbβ3 and leukocyte integrin Mac-1 stabilizes platelet aggregates around leukocytes. The absence of fibrinogen or blockade of Mac-1 has no effect on the formation of small platelet clusters, which is mainly dependent on the P-selectin/PSGL-1 interaction. However, the availability of fibrinogen to bridge the binding to leukocyte Mac-1 appears to amplify the formation of large aggregates greater than 50 μm^2.

REFERENCES

1. Springer, TA, 1994, "Traffic signals for lymphocyte recirculation and leukocyte emigration: the multistep paradigm," Cell 76:301-314.
2. Moore, KL, Patel, KD, Bruehl, RE, Fugang, L, Johnson, DA, Lichenstein HS, Cummings, RD, Bainton, DF, and McEver, RP, 1995, "P-selectin glycoprotein ligand-1 mediates rolling of human neutrophils on P-selectin," J. Cell Biol. 128:661-671.
3. Altieri DC, Plescia J, Plow EF, 1993, "The structural motif glycine 190-valine 202 of the fibrinogen gamma chain interacts with CD11b/CD18 integrin (alpha M beta 2, Mac-1) and promotes leukocyte adhesion," J. Biol. Chem. 268:1847-53.
4. DiVietro, JA, Smith, MJ, Smith, BRE, Petruzzelli, L, Larson, RS, and Lawrence, MB, 2001, "Immobilized IL-8 triggers progressive activation of neutrophils rolling in vitro on P-selectin and intracellular adhesion molecule-1," J. Immunol. 167:4017-4025.