Role of Fetal Hemoglobin in Regulation of Platelet Activation.

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Abstract
During intravascular hemolysis, RBCs gets rupture and hemoglobin release into the plasma which shows effect on vessel endothelial cells and gets platelets activation via interaction with GP1bα. This study is showed to focus on the role of HbF in regulation of platelet activation and demonstrates the effect of HbF on platelets and compared with the effects of HbA. The first objective shows that HbF interact with GP1bα in a concentration dependent manner. GP1bα is the outermost part of glyocalcicin which is present on platelet. The second objective demonstrates the expression of P-Sel and binding of PAC-1 with platelets. In the presence of hemoglobin, platelet P-Selectin expression shows more significance in HbA as comparison to HbF (**p<0.001, ***p<0.0001). In case of PAC-1 binding with platelet, HbA shows more significance than HbF (*p<0.01, **p<0.001). HbF mediated platelet activation and spreading shows by confocal microscopy and live cell imaging.

Introduction
During an intravascular hemolysis, the premature erythrocytes ruptured and hemoglobin comes out into the plasma. But in case of extracellular hemolysis, premature and abnormal erythrocytes destruct and release hemoglobin into the circulation. This hemoglobin dimmers attach with the haptoglobin to form the haptoglobin-hemoglobin complex. This complex scavenged by CD163 which is present on the surface of macrophages. The macrophage scavenges the hemoglobin up to a certain limit. cell free hemoglobin impairs vascular nitric oxide availability and cause oxidative and inflammatory processes. The major effect of free hemoglobin on platelet function is mediated by scavenging of nitric oxide by hemoglobin. Nitric oxide inhibits platelet aggregation and platelet adhesion via cGMP pathway. This shows that plasma hemoglobin indirectly contribute in the regulatin of platelet activation. Platelets are small and enucleated cell and they are in moving state in the circulation till no damage has been occurred to the endothelium. They perform three functions – activation, adhesion and aggregation. Platelets are activated by the release out of ADP from rupture red blood cells during an injury. Activated platelet starts to adhere with endothelium at the injury site due to release of some coagulation factors like VWF, collagen, fibrinogen and thrombin. These factors are binding on the glycoprotein surface receptor of platelet as a ligand and initiate the platelet activation for binding. GP1b-IX-V complex is the major receptor which binds collagen with VWF and initiate platelet adherence. Adhered platelet secretes calcium and ADP granules for platelet aggregation. Platelet aggregates via glycoprotein IIb/IIIa forms the primary hemostatic plug. Thus platelets play an important part in controlling excessive blood loss.

Methodology
Isolation of washed platelets: PRP was collected from anti-coagulant blood of normal individuals. pellet was resuspended in Tyrode hepes buffer pH 6.5 and gel filtered in column of Sepharose 2B, and eluted by calcium free Tyrode hepes buffer pH 7.

ELISA (Enzyme Linked Immunosorbent Assay): The various concentration of HbF and HbA were added into the prefixed platelet. HRP substrate enzyme was added to measure the absorbance at 492 nm.

Preparation of HbF and HbA coated lab-tech chamber slides: FITC conjugated anti-P-selectin (CD62P) antibody (R&D systems) was added washed platelet (10 µg/ml) into the wells of lab tech chamber slide, immobilized with BSA (Bovine Serum Albumin) and incubated at 37°C for 40 min. After incubation, Slides were visualized under a DM1600B Confocal microscope (TCS-SP8, Leica Microsystems, and Germany) for real time imaging.
Flow Cytometry – Fluorescence Activated Cell Sorting (FACS): P-selectin and PAC – 1 were added with washed platelet platelets. FITC labeled PAC-1 was used to recognize activated glycoprotein IIB/IIIa, and analyzed by flow Cytometry (Becton Dickinson, San Jose, USA).

Hemoglobin binding with Glycocalcin

Graph 1: ELISA results for HbF and HbA binding with glycocalcin. Different concentration of HbF (0µM, 0.15µM, 0.5µM, 0.75µM, 1.5µM, 3µM, 6µM, 9µM) and HbA (9µM) were added to the respective wells, the binding of HbF and HbA were detected with Anti-rabbit IgG HRP tagged (secondary antibody).

Platelets activate by hemoglobin F shown by live cell images with the help of confocal microscopy.

Fig 1: Platelets spreading on immobilized HbF. Figure A is showing fluorescence image. The image stained with P-selectin. Figure B is showing bright field image. Figure C is showing merged image of A& B.
Platelets activate by hemoglobin A shown by live cell images with the help of confocal microscopy.

**Fig 8:** Platelets spreading on immobilized HbA. Figure A is showing fluorescence image. The image stained with P-selectin. Figure B is showing bright field image. Figure C is showing merged image of A & B.

**Hemoglobin activated Platelet by FACS.**

**Graph 2:** Platelet P–selectin expression in the presence of hemoglobin. Washed platelets were incubated with HbA and HbF both, labeled with FITC P-selectin antibody and analyzed by flow Cytometry. **p<0.001, ***p<0.0001.

**Graph 3:** PAC-1 binding with platelet in the presence of hemoglobin. Washed platelet were incubated with HbA and HbF both. The PAC-1 binding was measured using flow Cytometry. *p<0.01 and **p<0.001 (compare to control).
Discussion

The present study evaluates the role on fetal hemoglobin (HbF) in platelet activation. This study described the effects of HbF on platelets and compared with the effects of HbA. Our first objective demonstrates that HbF bound to platelet via GP1bα (outermost part of glyocalcin). This Hb – GP1bα interaction initiated granule secretion, change in platelet shape and inside out signaling process. The HbF binding to GP1bα induced platelet activation in a concentration dependent manner. Our data also showed the expression of P-selectin on platelets induced by both HbF and HbA irrespective. HbF induced the PAC-1 binding to platelets. To validate further the platelet activation we used confocal microscopy and live cell imaging which further provided evidences showing HbF mediated platelet activation and spreading. However further studies are in progress to delineate the role of HbF in platelet activation and its correlation with the pathogenesis of fetal diseases.

References:


