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Mechanisms of hemolysis-associated platelet activation

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Summary

Background—Intravascular hemolysis occurs after blood transfusion, in hemolytic anemias and other conditions, and is associated with hypercoagulable states. Hemolysis has been shown to potentially activate platelets in vitro and in vivo and several mechanisms have been suggested to account for this including (1) direct activation by hemoglobin, (2) increase in reactive oxygen species (ROS), (3) scavenging of nitric oxide by released hemoglobin, and (4) release of intraerythrocytic ADP.

Objective—The aim of the current study is to elucidate the mechanism of hemolysis-mediated platelet activation.

Methods—We used flow cytometry to detect PAC-1 binding to activated platelets for in vitro experiments and a Siemens' Advia 120 hematology system to assess platelet aggregation using platelet counts from in vivo experiments in a rodent model.

Results—We show that Hb does not directly activate platelets. However, ADP bound to Hb can cause platelet activation. Furthermore, platelet activation due to shearing of RBCs is reduced in the presence of apyrase which metabolizes ADP to AMP. Use of ROS scavengers did not affect platelet activation. We also show that cell free Hb does enhance platelet activation by abrogating the inhibitory effect of NO on platelet activation. In vivo infusions of ADP and purified (ADP-free) Hb as well as hemolysate result in platelet aggregation as evidenced by decreased platelet counts.

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ADDENDUM

C. C. Helms, M. Marvel and R. Vest performed experiments; W. Zhao performed animal surgery; C. C. Helms wrote first draft of the manuscript; M. Stahle and C. C. Helms coordinated volunteers; M. Stahle provided laboratory assistance; C. C. Helms, R. R. Hantgan, G. J. Kato, J. S. Lee, G. Christ, M. T. Gladwin and D. Kim-Shapiro contributed to experiment conception and design; D. Kim-Shapiro supervised the research. All authors reviewed and commented on the draft and approved the final manuscript.

Conclusion—Two primary mechanisms account for red blood cell hemolysis-associated platelet activation: ADP release which activates platelets and cell-free hemoglobin release which enhances platelet activation by lowering NO bioavailability.

Keywords

hemoglobin; hemolysis; nitric oxide; platelets; red blood cells

Diseases involving hemolysis are often associated with hypercoagulability and increased baseline platelet activation [1–3]. For example, platelet activation is present in hemolytic uremic syndrome and sickle cell disease [4, 5]. In addition, Villagra et al. reported a correlation between platelet activation and markers of hemolysis in sickle cell disease [6]. However, the mechanisms contributing to hemolysis-associated platelet activation are not well defined. Studies propose a role of shear stress, endothelial damage, hyposplenism, reactive oxygen species (ROS) production by hemoglobin (Hb), NO scavenging by cell free Hb and ADP release from damaged red blood cells (RBCs) in platelet activation occurring in disease states [5, 7–10]. Here we investigate the role of hemolysis associated hemoglobin, ROS, ADP and NO scavenging on platelet activation.

In 1960 Hellem discovered a small molecule in RBCs that was responsible for platelet adhesion to glass [11]. Shortly thereafter this small molecule was identified as ADP [12]. Hemolysis, permanent RBC damage, RBC deformation and shear stress all cause RBCs to release ADP [9, 13, 14]. Once in the blood stream, ADP can cause platelet activation. Studies involving ADP infusions in rats and rabbits show reversible platelet aggregation [15, 16]. In addition, activated platelets release granules containing ADP which further promotes activation. Furthermore, ex vivo studies of blood from transfusion recipients have shown increased platelet activation and aggregation attributed to ADP-release from red blood cells [17]. The identification of platelet ADP receptors P2Y₁ and P2Y₁₂ led to the development of a class of antiplatelet drugs based on platelet ADP receptor antagonist [18, 19]. However, ADP can also interact with ectoADPases on endothelial and white blood cells, converting the platelet agonist to AMP which does not activate platelets. Furthermore, ADP can bind P2X receptors on endothelial cells and promote NO production [20–22]. While ADP infusions have shown a transient decrease in platelet count, they have also shown an increase in bleeding time attributed to NO production and platelet desensitization [15, 23].

NO reduces platelet activation through a pathway in which NO binds sGC leading to a downstream inhibition of calcium mobilization [24]. The effect of endothelial-derived relaxing factor (NO) on platelet activity was demonstrated in 1986 by Azuma and coworkers where the effluent from perfused acetylcholine-treated aorta inhibited arachidonic acid induced platelet aggregation [25]. The platelet agonist ADP can bind endothelial cells and increase NO production. In 1997, Wollny et al showed prolonged bleeding times in rats and rabbits after a low dose ADP infusion which was abrogated by administration of L-NAME, a NO synthase [23]. The need for basal NO was demonstrated by Schafer et al in a study where blocking nitric oxide synthase led to increased platelet activation [26].

Hemolysis of red blood cells releases hemoglobin, a potent NO scavenger, into the blood stream. Oxygenated hemoglobin (oxyHb) rapidly reacts with NO with a rate constant of $5 \times 10^7 \text{ M}^{-1}\text{s}^{-1}$ [27–29]. When Hb is confined within the RBC the reaction between Hb and NO is limited by cell membrane permeability, an unstirred layer surrounding the RBC and a pressure gradient pushing the RBCs toward the center of the vessel creating a cell free zone near the NO producing endothelium [30–34]. Therefore, Hb released during hemolysis scavenges NO at a rate 1000 times more effectively than that encapsulated in the red cell [35]. Hemolysis releases both ADP, a mediator of NO production and cell free Hb a

scavenger of NO yet, diseases associated with hemolysis show a decrease in NO bioavailability [36–38]. Villagra et al showed in vitro that NO, known to decrease platelet activation, fails to do so in the presence of Hb [6].

In addition to elucidating the role of the reaction of Hb with NO in platelet activation, Villagra et al showed direct platelet activation by Hb [6]. We hypothesized platelet activation by Hb could be attributed to direct platelet apoprotein interactions, reactive oxygen interactions or endogenous NO scavenging. Work by Iuliano et al showed platelets “primed” with low, non-aggregating concentrations of collagen or arachidonic acid aggregate when Hb is added and they attributed the platelet aggregation to ROS generated by iron in Hb [8].

The combination of ADP and Hb release during hemolysis make it difficult to distinguish individual effects of hemolysis on platelet activation. Here we examine the interactions of platelets with individual elements of hemolysis such as ADP and Hb as well as combinations involving ADP, Hb and NO. Furthermore, we investigate mechanisms of hemolysis-mediated platelet activation in vivo in a rodent model.

METHODS

Materials

Hemoglobin was prepared from leukoreduced packed RBCs obtained from Interstate Blood Bank (Memphis, TN). Superoxide dismutase (SOD) was purchased from Enzo Life Sciences (Plymouth Meeting, PA). PBS, hemin, myoglobin, catalase, FeCl₃, potassium ferricyanide, formaldehyde and apyrase grade VII were purchased from Sigma-Aldrich (St. Louis, MO). AR-C 66096 tetrasodium salt and MRS 2500 tetraammonium salt were purchased from Tocris Bioscience (Bristol, UK). PerCp-CD61 and FITC-Pac-1 were purchased from Becton Dickinson (San Jose, CA). ADP, arachidonic acid and collagen were purchased from Bio Data Corporation (Horsham, PA). Sephadex G-25 columns were obtained from GE Healthcare.

Mechanical hemolysis

Blood was drawn from healthy volunteers with approval from Wake Forest University Health Sciences Institutional Review Board (IRB). Red blood cells were separated from plasma by sedimentation and added back to the plasma at the desired hematocrit. Hemolysis samples were prepared at 20% and 0% hematocrit in 50% platelet rich plasma (PRP) in PBS, pH 7.4. 2 U/ml apyrase was added as indicated to samples. Samples were stirred between 70 and 110 rpm at 37°C for 30 minutes. Aliquots of the samples were taken prior to and after stirring to test for platelet count and hemolysis. Platelet aggregation was assessed by examining the % decrease in counts of individual platelets as employed previously [39, 40]. Platelet counts were run in the core lab at Wake Forest Baptist Medical Center. Aliquots for hemolysis measurements were spun at 1000 g for 5 minutes to separate RBCs; then the supernatant was spun a second time at 10000 g for 7 minutes to remove platelets. The second supernatant was tested for hemolysis by absorption spectroscopy from 700 – 450 nm.

Hemolysate/Hb preparation

Packed RBCs were washed 3 times in PBS by centrifugation. To prepare hemolysate, washed RBCs were vortexed and frozen at –80°C to lyse the cells. The lysed RBCs were centrifuged at 17,211 RCF to pellet cell membranes and the supernatant was collected. For hemoglobin preparation washed RBCs were hypotonically lysed 5:1 and centrifuged at 17,211 RCF for one hour to remove cell membranes. The supernatant was dialyzed extensively to prepare dialyzed-hemoglobin (D-Hb). D-Hb was passed sequentially through

two Sephadex G-25 columns to prepare G25-Hb. To form methemoglobin (metHb), potassium ferricyanide was added 5:1 to D-Hb, rocked for 10 minutes then passed through two Sephadex G-25 columns and dialyzed to remove the potassium ferricyanide.

In vitro platelet activation

Blood was obtained from volunteers with IRB approval and informed consent, collected into sodium citrate (BD San Jose, CA), and centrifuged at 120 g for 12 minutes to obtain PRP. PRP was diluted 1:10 in PBS (pH 7.4) in the presence or absence of platelet agonist, incubated for 10 minutes at room temperature, transferred into PAC-1 and CD61 antibodies for 15 minutes in the dark, then diluted 1:50 in 1% formaldehyde. Samples with 5 U/ml apyrase were incubated at 37°C for 60 minutes prior to addition of PRP.

A BD FACS Calibur flow cytometer and Cell Quest Pro software were used for data collection and analysis. The activation threshold was set so 99% of baseline platelets were beneath the threshold. Significance was determined using student's t-test.

ADP detection

ADP concentration was determined using an Enzyglight ADP Assay Kit obtained from BioAssay Systems (Hayward, CA). Luminosity was measured using a Synergy H1 microplate reader and Gen5 software (BioTek, Winooski, VT).

In vivo platelet function

Female Sprague-Dawley rats 260 – 420 g, were anesthetized and the femoral vein was catheterized for blood draw and drug infusion, as approved by the ethics committee for animal subjects use at Wake Forest University Health Sciences. 250 – 500 µl of blood was drawn from the catheter into EDTA microtainers for platelet counts. Next, hemolysate, G25-Hb, ADP or buffer was infused through the catheter. Rats were infused at a rate of approximately 120 ml/kg/hr for 1 minute until the free hemoglobin concentration in the blood reached 500 µM or the ADP concentration reached 5 µM, assuming a blood volume of 64 ml/kg body weight. Assuming a RBC concentration of 200 µM ADP and 20 mM Hb, a target concentration of 5 µM ADP was chosen because it corresponds to hemolysis with a release of 500 µM Hb. Two minutes after the infusion, 250 – 500 µl of blood was drawn from the left ventricle into EDTA microtainers for platelet counts. A ratio of platelet count to RBC count was recorded before and after infusion to control for any dilution due to the infusate. Platelet aggregation was assessed by examining a % decrease in platelet count to RBC ratio [39, 40]. Whole blood counts were performed by the Wake Forest Institute for Regenerative Medicine using a Siemens' Advia 120 hematology system. Significance before and after infusion was determined using a paired homoscedastic student's t-test. Outliers were determined using a Dixon's q-test and were eliminated if there was confidence of 90% or more that it was an outlier. None of the eliminated outliers affected the significance of the data.

RESULTS

In vitro platelet activation by hemoglobin

In vitro platelet activation was determined using fluorescent PAC-1 binding and flow cytometry. We found red blood cell hemolysate effectively activates platelets (Figure 1A). Dialysis of lysed RBCs (which removes small molecules not bound to Hb) to produce dialyzed-Hb (D-Hb) reduced but did not eliminate platelet activation (Figure 1A, D-Hb). To determine if the mechanism by which D-Hb activates platelets is due to an interaction of the globin we tested activation by metHb and ferrous Mb. Neither metHb nor ferrous Mb activated platelets (Figure 1A), which also argued against a mechanism involving

scavenging of endogenous NO produced by platelets (since Mb scavenges NO) and effects of the ferric heme of metHb. To test whether ROS are involved, we added catalase and SOD to the D-Hb and found no effect (Figure 1A). D-Hb effluent, obtained after passing the D-Hb through a 10 kDa centrifugal filter that removes Hb but passes small molecules like ADP, caused platelet activation (Figure 1B). Platelet activation by D-Hb and D-Hb effluent was abrogated by the addition of apyrase, platelet ADP receptor antagonists P2Y1, or P2Y12 (Figure 1B and C) indicating ADP was responsible for platelet activation. After dialysis the ADP concentration in the D-Hb was found to be 100 nM per 100 μ M Hb as measured using luminosity (data not shown). Dialysis only partially removed ADP from Hb formed from red cell lysate due to binding of ADP to Hb. A G-25 size exclusion column removed ADP from D-Hb (Figure 1C).

In vitro mechanical hemolysis

PRP and buffer with and without RBCs and/or apyrase were incubated at 37°C under mechanical shear. In support of previous works, we found an increase in hemolysis produced in vitro by mechanical shear led to increased platelet aggregation. Hemolysis was determined by cell free hemoglobin concentration and platelet aggregation was determined by a decrease in platelet count [41]. An increase in platelet aggregation correlated with an increase in cell free hemoglobin concentration, and incubation with apyrase (2 U/ml) attenuated this relationship between hemolysis and platelet aggregation (Figure 2).

In vitro Hb effect on NO abrogation

Once again platelet activation in vitro was determined by Pac-1 binding and flow cytometry. We found Hb abrogates the inhibition of platelet activation by NO as shown by Villagra et al and verified in Figure 3 using ADP free G25-Hb.[6] Importantly, we show that metHb (which does not effectively scavenge NO) does not abrogate the inhibition of platelet activation by NO.

In vivo infusion

The femoral veins of Sprague-Dawley rats were infused with red cell hemolysate containing 500 μ M cell-free Hb, 5 μ M ADP, 500 μ M G25-Hb or buffer. All infusates except buffer significantly increased platelet aggregation in a rodent model, as evidenced by a reduction in platelet count (Figure 4, $p = 0.01$, $n = 5$; $p = 0.04$, $n = 5$; $p = 0.04$, $n = 5$; respectively). Hemolysate infusions showed a 19 % change in platelet count, the largest percent change in platelet count of all the infusates (ADP = 17 % and G25-Hb = 9 %) however the percent change in platelet count between the groups did not reach significance. Infusion of buffer did not result in a significant decrease in platelet count ($p > 0.1$).

DISCUSSION

ADP was first recognized as the platelet adhesive factor located inside the RBC in 1961 by Gaarder et al [12]. Since their discovery, shearing experiments like those in Figure 2 have shown shear induced ADP release and platelet aggregation. Further confirmation of the role of ADP in these experiments is given by the removal of ADP using apyrase. As depicted by the data in Figure 2, using this in vitro hemolysis model the effect of ADP release on platelet aggregation was seen at hemolysis levels ranging from 10 to 20 μ M hemoglobin, within the physiological range of hemolysis seen in sickle cell disease [42].

In addition to ADP, Hb is also released during hemolysis. Our in vitro results demonstrate Hb does not directly activate platelets, as previously proposed [6], but ADP bound to Hb during Hb preparation is responsible for platelet activation. Similar to 2,3 diphosphoglycerate, dialysis was not sufficient in removing ADP from hemolysate due to

binding of ADP to Hb [43]. However, a G-25 size exclusion column removed ADP (Figure 1C) and tests run with the purified Hb (G-25Hb), which was passed through the column, did not cause platelet activation.

The complexity of the in vivo system makes it difficult to determine how ADP released during hemolysis affects platelet activation. As discussed above, ADP is a known platelet activator. However, ADP is reduced to AMP and adenosine by ADPases located on endothelial cells, RBCs and white blood cells. Additionally, ADP stimulates NO production by endothelial cells, while Hb released during hemolysis scavenges available NO. Because of the interdependency of these interactions, in vivo studies were carried out looking at platelet aggregation.

Infusions of hemolysate equivalent to 500 μ M cell free Hb, 5 μ M ADP and 500 μ M G25-Hb in rats lead to an increase in platelet aggregation as measured by a decrease in platelet count after infusion. It is of interest to note purified Hb does not produce platelet activation in vitro but in vivo induces a decrease in platelet count (Figures 1B and 4). This could be due to Hb scavenging of endogenous NO, as Schafer et al showed the necessity of NO to maintain normal platelet function in vivo [26]. Infusion of hemolysate equivalent to 500 μ M cell free Hb showed a larger decrease in platelet count than ADP and G25-Hb. However, the difference in the decrease in platelet count due to hemolysate, ADP and G25-Hb when compared with one another was not significant. The trend of a larger change in platelet count with hemolysate infusion could be due to the combination of both ADP release and cell free Hb scavenging of NO. In vivo infusions of hemolysate, ADP and Hb offer some understanding of hemolysis associated platelet activation but these measurements are limited due to the complexity of the system as other mechanisms such as cell-free arginase (by diminishing NO[44]), free iron [45, 46], endothelial activation or white blood cell activation may contribute to hemolysis associated platelet activation as well.

Increased platelet activation associated with hemolytic conditions support an effect of hemolysis on hemostasis [1]. Here, we have shown that purified Hb in vitro does not directly activate platelets. However, Hb abrogates the inhibitory effect of NO on platelet activation and may have an effect in vivo as previously suggested. [3, 26] Indeed, previous work has suggested an association between hemolysis and loss of NO bioavailability with thrombosis, pulmonary arterial hypertension and death in a murine hemolysis model [10]. Our data showing that purified Hb increases platelet aggregation in vivo support this notion that hemolysis mediated platelet activation is partially due to NO scavenging by cell-free Hb. Our data also suggests that ADP release plays a role. In vivo Hb and ADP infusions decrease platelet count and during hemolysis the combination of ADP and Hb release may cause increased platelet aggregation.

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Disclosures

Drs. Gladwin and Kim-Shapiro are co-authors on patents applications related to hemolysis and delivering NO.

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Figure 1.

Mechanism of platelet activation determined by FITC conjugated PAC-1 fluorescence. Baseline measurements represent platelet activation in PBS. **A)** Hemolysate with 100 μ M Hb and 100 μ M D-Hb caused platelet activation. 100 nM Hemin, 1 μ M FeCl₃, 100 μ M methHb and 100 μ M Mb did not significantly increase platelet activation over baseline ($n = 4$, * $p > 0.1$) and 100 U/ml SOD and Catalase did not significantly reduce activation when compared to D-Hb ($n = 3$, $\dagger p > 0.1$). **B)** Platelet activation by effluent is significantly decreased by addition of 5 U/ml apyrase ($n = 3$, * $p < 0.01$). Effluent volume was equivalent to the volume of 100 μ M D-Hb. **C)** Platelet activation by 100 μ M D-Hb was significantly

diminished after incubation with 5 U/ml apyrase, after passing through a G-25 desalting column, or with the addition of 200 nM platelet P2Y₁₂ receptor antagonist AR-C 66096 or 100 μ M platelet P2Y₁ receptor antagonist MRS 2500 ($n = 3$, $*p < 0.01$). Bars on all graphs represent the average $\pm$ SEM. n is the number of different volunteers.

Figure 2.

Effects of hemolysis on platelet activation. The percent decrease in platelet count increased with hemolysis from whole blood (WB), represented by Hb concentration (black squares) ($r = 0.6659$, $p < 0.01$). Addition of 2 U/ml apyrase prior to shear reduced the change in platelet count due to hemolysis (gray diamonds) ($r = -0.0819$, $n = 8$ different volunteers, tested twice).

Figure 3.

Effects of hemolysis on platelet activation. 2.5 μ M ADP activates platelets and the addition of 20 μ M NO (10 μ M MahmaNONOate) decreased platelet activation (* p < 0.01, comparing ADP + NO vs ADP). 100 μ M G25-Hb abrogates the effect of 20 μ M NO on platelets activated by 2.5 μ M ADP ($^{\dagger}p$ < 0.01, comparing ADP + NO vs ADP + G25-Hb + NO). 100 μ M metHb does not alter platelet activation in the presence of 20 μ M NO and 2.5 μ M ADP ($^{\ddagger}p$ > 0.1, comparing ADP + NO vs ADP + metHb + NO). Platelet activation was determined by FITC conjugated PAC-1 fluorescence. Bars on all graphs represent the average $\pm$ SEM (n = 3 different volunteers).

Figure 4.

Infusions into female Sprague-Dawley rats. Rats were infused with 500 μ M G25-Hb, 5 μ M ADP or hemolysate equivalent to a final blood concentration of 500 μ M cell free Hb and PBS, pH 7.4, at a rate of 120 ml/kg/hr for 1 minute. Blood was collected before and after infusion and the ratio of platelet count to RBC count was recorded. Infusion of G25-Hb, ADP or hemolysate each showed a significant decrease in platelet count after infusion when compared to before ($p = 0.04$, $n = 5$; $p = 0.04$, $n = 5$; $p = 0.01$, $n = 5$; respectively). Buffer infusion did not show a significant decrease in platelet count ($p > 0.1$, data not shown).