Primary and secondary hemostatic functionalities of rehydrated, lyophilized platelets

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BACKGROUND: The rehydrated, lyophilized (RL) platelet (PLT) is being developed as a hemostatic infusion agent for the control of active bleeding. The key to the method for preparing RL PLTs is a mild aldehyde stabilization that allows for freezing and lyophilizing without cellular rupture. RL PLTs have been shown to be effective at rapidly controlling bleeding in animal models of cardiopulmonary bypass induced PLT dysfunction and washout thrombocytopenia, yet the rehydrated cells have proved to be safe with respect to induction of pathologic intravascular coagulation.

STUDY DESIGN AND METHODS: In vitro and in vivo studies were performed to better understand the differential effect of the RL PLT manufacturing method on primary and secondary hemostatic processes. The functionality of the von Willebrand factor (VWF) receptor (glycoprotein Ib) complex, the PAR receptors, integrin-mediated aggregation (inside-out signaling), and surface membrane prothrombin to thrombin conversion systems were investigated.

RESULTS: RL PLTs were found to retain native VWF-mediated adhesion and surface thrombin generation functions. In contrast, the coupling of thrombin receptors to integrin inside-out signaling was largely inhibited.

CONCLUSION: These results suggest that RL PLTs may stop bleeding by forming primary hemostatic plugs and providing a localized source of thrombin for secondary hemostatic processes, yet do not build up occlusive pathologic clots possibly because integrin functions for forming PLT-PLT aggregates are partially inhibited.

Bleeding that results from platelet (PLT) dysfunction and thrombocytopenia (PLT-responsive bleeding) is a major cause of morbidity and mortality. The human PLT is the logical replacement therapeutic for PLT-responsive active bleeding, and the development of methods for liquid storage of this cell has lead to millions of PLT transfusions per year. PLT blood banking practices, however, have not been perfected to the extent that full functionality is preserved,[1] microbial contamination issues are resolved,[2] and logistical supply and demand factors are completely managed. The rehydrated, lyophilized (RL) PLT has been developed to resolve some of these difficulties.

The key to the technology for preparing RL PLTs is a mild aldehyde stabilization that allows for freezing and lyophilizing without cellular disintegration.[3] RL PLTs effectively controlling hemorrhage in rabbit,[4] swine,[5] and dog[6] bleeding models without inducing complications related to intravascular coagulation. In contrast to normally

ABBREVIATIONS: GP = glycoprotein; RL = rehydrated, lyophilized (platelets).

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THF, APB, MSR, and JKR own stock in Entegrion, Inc. JKR is a full-time employee of Entegrion, Inc. Entegrion is commercially developing products related to rehydrated, lyophilized platelets.

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liquid-stored PLTs, which are used primarily on a prophylactic basis indications,[7-9] RL PLTs are being developed as a hemostatic agent for active (therapeutic) bleeding. An important question concerns how RL PLTs can effectively provide hemostasis without pathologic intravascular coagulation. Two observations have been reported that address this safety question. First, RL PLTs are *activateable*; this is a basic key element for the safety of all hemostatic systems. The incorporation of RL PLTs into forming fibrin gels results in an irreversible activation response that involves shape change, pseudopodia extension, granule centralization and secretion, and cytoskeletal rearrangements.[10] Exposure of RL PLTs to thrombin has been shown to activate multiple intracellular signaling pathways, including protein kinase C and myosin light chain kinase systems. Second, RL PLTs are cleared from the free circulation by the reticuloendothelial system in minutes ($t_{1/2}$ ~10 min).[11] Infused RL PLTs that do not localize to sites of vascular injury for hemostasis are phagocytized by reticuloendothelial system macrophages through mechanisms that involve Fc and scavenger receptor systems. Thus, the potentially prothrombotic therapeutic does not persist in the free circulation for prolonged periods.

The functionality of primary (initial adhesion via glycoprotein [GP]Ib/V/IX–von Willebrand factor [VWF]-mediated mechanisms) and secondary (thrombin-mediated fibrinogen polymerization and integrin function) hemostatic systems on RL PLTs is an important consideration in understanding the prothrombotic safety profile of RL PLTs. Excess function of secondary hemostatic systems is associated with pathologic intravascular coagulation for vessel occlusion and ischemia. Based on ICD-9 statistics, more than 1 million patients are affected per year in the United States by pathologic intravascular coagulation.[12] Thrombophilic defects in primary hemostasis are less frequent, the most common defect of this type being thrombotic thrombocytopenia purpura, which affects approximately 1 in 100,000 individuals in the Unites States.[13] Thus, the preponderance of pathologic cases of intravascular coagulation is associated with secondary as compared to primary hemostatic processes. These public health statistics indicate that the potential pathologic involvement of RL PLTs with secondary hemostatic processes (e.g., thrombin activation, fibrinogen polymerization) are of paramount concern.

We report here experiments that were carried out to better understand the differential effect of the RL PLT manufacturing method on primary and secondary hemostatic processes. The functionality of the VWF receptor (GPIb) complex, the PAR receptor, and integrin inside-out signaling system was investigated. These results provide a possible explanation for the observed safety profile of RL PLTs with respect to pathologic intravascular coagulation: The freeze-dried cells provide hemostasis through VWF-mediated clot formation and then, due to the partial inhibition of secondary hemostatic processes, do not grow occlusive fibrin clots.

MATERIALS AND METHODS

Fresh PLT isolation

Fresh PLTs were prepared from venous blood drawn from healthy volunteers in accordance with institutional guidelines. Whole blood (42.5 mL blood to 7.5 mL anticoagulant) was drawn into a syringe containing citrate (3.2% by weight, pH 7.4). PLT-rich plasma was obtained by centrifugation ($200 \times g$, 20 min) at 25°C. PLTs were removed from unbound plasma proteins by subjecting the PLT-rich plasma to chromatography on a Sepharose CL-4B column (2.5×18 cm) that was preequilibrated in Tyrode's buffer (5.55 mmol/L glucose, 136 mmol/L NaCl, 2.7 KCl, 12 mmol/L $NaHCO_3$, 10 mmol/L Hepes). Turbid (void volume) fractions were pooled, and then PLT concentrations were measured with a hematologic analyzer (Heska, Loveland, CO). The final PLT concentration was adjusted to 1.0×10^8 PLTs per mL by diluting with Tyrode's buffer. The cells were then immediately used for the experimentation detailed below.

RL PLT preparation

RL PLTs were prepared as previously described with centrifugal[3,14] or tangential-flow diafiltration[15] cell separation steps. Briefly, PLT-rich plasma was collected as described above for fresh PLT isolation, and then the cells were subjected to three centrifugal washings or a single tangential-flow filtration to separate the cells from plasma proteins. Washed PLTs were incubated in 1.8 percent paraformaldehyde for 1 hour at 25°C. After aldehyde stabilization, the PLTs were subjected to an additional three centrifugal washings or a single tangential-flow filtration to separate the cells from soluble components of the aldehyde stabilization reaction mixture. PLT concentration was adjusted to 1×10^9 cells per mL in 5 percent human serum albumin (HAS) and lyophilized. Freeze-dried PLTs were stored at –80°C until use.

In preparation for in vitro measurements, the RL PLTs were rehydrated with 1 mL of imidazole buffer (84 mmol/L imidazole, pH 7.35), pelleted by centrifugation ($1000 \times g$ for 8 min) at 25°C and resuspended in citrated saline. After a centrifugal wash in citrated saline (5.376 mmol/L citrate, 146 mmol/L NaCl, pH 7.4), the RL PLT pellet was suspended for a cell concentration of 1.0×10^8 PLTs per mL in Tyrode's buffer.

Ristocetin agglutination and thrombin aggregation measurements

In preparation for ristocetin agglutination assays, PLT-poor plasma was isolated by centrifuging fresh PLT-rich

plasma (prepared as described above) for 10 minutes at $1000 \times g$. RL PLTs were rehydrated and centrifugally washed as described in the previous paragraph. The final RL PLT pellet was suspended at 10^5 cells per mL in PLT-poor plasma (obtained by centrifuging PLT-rich plasma at $10,000 \times g$ for 30 min). PLT-rich plasma was prepared with 10^8 cells per mL by diluting PLT-rich plasma with PLT-poor plasma. A quantity of 450 μL of RL or fresh PLTs was placed in a whole-blood aggregometer (Chronolog Corp., Havertown, PA) cell at 37°C, and then the agglutination reaction was initiated by diluting ristocetin (Sigma Diagnostics, St. Louis, MO) 1/10 into the PLTs for a final ristocetin concentration of 1.0 mg per mL. The turbidity time course was then recorded.

Thrombin aggregation measurements were performed by placing fresh or RL PLTs at 10^8 cells per mL in Tyrode's buffer in the aggregometer cell. The aggregation time course was initiated by diluting 100× stock solutions of calcium to a final concentration of 3 mmol per L and human thrombin (Sigma Chemical Corp., St. Louis, MO) to 1 NIH unit per mL. The aggregation time course was then followed.

Receptor surface density and ligand-binding measurements

The PLT surface density of GPIb was measured with a monoclonal antibody (MoAb) SZ2 (Coulter, Hialeah, FL) and labeled with ^{125}I (iodobead iodination reagent, Pierce, Rockford, IL) according to the manufacturers' directions. Fresh and RL PLTs (prepared with centrifugational cell separation steps) at 9.0×10^7 PLTs per mL in Tyrode's buffer were incubated for 30 minutes at 25°C with increasing concentrations of ^{125}I-labeled GPI MoAb SZ2 at 25 μg per mL (determined to be above the stoichiometric saturation value) in the presence of 1 mg per mL ristocetin. After incubation, an aliquot of each sample was transferred to a microfuge tube, layered over 20 percent sucrose solution in Tyrode's buffer, and centrifuged at $7000 \times g$ for 5 minutes. Radioactivity of the PLT pellet was measured. Nonspecific binding was defined as the amount of labeled anti-GPIb that could not be displaced from PLTs by the addition of threefold excess of unlabeled anti-GPIb. Specific anti-GPIb binding was calculated by subtracting the nonspecific binding from the total labeled anti-GPIb bound. A ratio of one anti-GPIb molecule to one GPIb receptor site was assumed.

The binding of VWF to fresh and RL PLTs was measured in a manner similar to the binding of the anti-GPIb detailed in the previous paragraph. VWF was isolated from normal human plasma as described by Mazurier[16] and then labeled with ^{125}I with the Iodobead method. The total multimeric VWF binding to PLTs was determined by incubating PLTs for 30 minutes at 25°C with increasing concentrations of the radioiodinated protein in the presence of 1 mg per mL ristocetin. The PLTs were separated from unbound VWF as in the previous paragraph. Nonspecific VWF binding was determined by the amount of labeled VWF bound to fresh or RL PLTs in the absence of ristocetin. Specific VWF binding was calculated by subtracting the nonspecific binding from the total labeled VWF bound.

The density of the integrin $\alpha_{IIb}\beta_3$ complex was measured with ^{125}I-labeled MoAb 10E-5, at a concentration of 20 μg per mL (determined to be above the stoichiometric saturating value[17]). ^{125}I-fibrinogen binding to fresh and RL PLTs was compared as detailed elsewhere.[10]

Thrombin generation kinetics

Thrombin generation kinetics was analyzed by following the hydrolysis of the thrombin substrate D-Phe-Pro-Arg-ANSNH-C_6H_{11} (Haematologic Technologies, Inc., Essex Junction, VT) to yield a fluorescent reaction product. Experiments were performed in 96-well Costar tissue culture plates that had been incubated for 24 hours at 37°C with 5 percent HSA in citrated saline to block sites of spontaneous contact activation initiation. The serum albumin solution was removed and 100 μL of normal citrated plasma was then added to each well. Substrate hydrolysis was followed by measuring the fluorescence at 480 nm every minute in a fluorescent plate reader (Polarstar Galaxy, BMG Laboratory Technologies, Inc., Durham, NC). Triplicate relative fluorescence values were averaged to obtain the time-course curves presented in this article. The lag time for substrate generation was defined as the time point when the fluorescence increased 10 percent over the initial baseline value. The amount of thrombin that is generated was estimated by comparing the maximum slope of the fluorogenic curve to fluorogenic time courses that were recorded with known amounts of human thrombin (in the same plasma). Thrombin activities are expressed as NIH units per mL with a molecular activity of 10^{11} NIH units per mole thrombin.[18,19]

Thrombin generation assays to investigate the effect of PLT concentration on prothrombin to thrombin conversion were performed by diluting fresh donor PLTs or RL PLTs in the plasma of the fresh PLT donor. PLT-rich plasma was isolated from blood drawn into citrate as in the first paragraph of this section, and then PLT-poor plasma was isolated. PLT-rich plasma was diluted with the PLT-poor plasma to obtain samples with various fresh PLT concentrations. RL PLTs were prepared in the same donor's plasma by rehydrating and centrifuging the freeze-dried cells as under "RL PLT preparation" above and then suspending the RL PLTs in the donor's plasma at varying concentrations. Samples were then analyzed for thrombin substrate hydrolysis as described in the previous paragraph. The normal plasma pools from more than 1000

individuals were from Innovative Research (Southfield, MI).

The acceleration of thrombin generation by RL PLTs in the plasma from different donors was measured in a manner similar to that described in the previous paragraph. RL PLTs were rehydrated, centrifugally washed, and then suspended at 10^8 RL PLTs per mL in the plasmas from individual donors or pooled plasma from more than 1000 normal donors (Innovative Research). The thrombin generation time and activity was then measured.

RESULTS

The experiments presented here were designed to evaluate the effect of aldehyde stabilization and lyophilization on the activation responses of RL PLTs. Each of the experiments presented in Figs. 1 through 3 were performed three independent times with a single representative data set being presented in each figure. Primary (GPIb and VWF-mediated responses) and secondary (thrombin responses for fibrin polymerization and integrin function) hemostatic systems were directly compared in the analysis presented in Figs. 1 and 2. The top two panels in Fig. 1 depict the agglutination responses of RL (top left panel) and fresh (top right panel) PLTs to ristocetin, an antibiotic that interacts with the PLT GPIb receptor to induce an active conformation for VWF binding. Ristocetin-mediated agglutination time courses were similar for fresh and RL PLTs. In contrast, thrombin-mediated aggregation was largely abolished by the RL PLT manufacturing process. The results presented in the bottom left panel in Fig. 1 show that RL PLTs undergo minimal thrombin-induced PLT-PLT aggregation.

The more detailed comparison of primary and secondary processes that is presented in Fig. 2 shows that RL and fresh PLTs have similar surface densities of active GPIb. This measurement was performed with the MoAb SZ2, which binds to an epitope on GPIb (the anionic sulfonated domain) that does not strongly affect ristocetin function.[20] Similarly, RL and fresh PLTs bind equivalent amounts of VWF when activated with ristocetin. In contrast, both the integrin $\alpha_{IIb}\beta_3$ complex surface density, as judged with the MoAb 10E-5, and the fibrinogen-binding ability of RL PLTs are approximately half that of fresh PLTs. These results indicate that RL PLTs retain approximately native primary hemostatic function. In contrast, the integrin-mediated aggregation responses to thrombin are largely abolished with RL PLTs. These studies indicate that the mode of hemostatic action of RL PLTs principally involves primary hemostatic mechanisms. This result is supported by the finding (data not shown) of a statistical trend between age of PLTs before initiation of the RL PLT manufacturing process, reduced ristocetin agglutination of both the input material and the resulting RL PLTs, and diminished vessel coverage by the RL PLTs upon Baumgartner analysis.

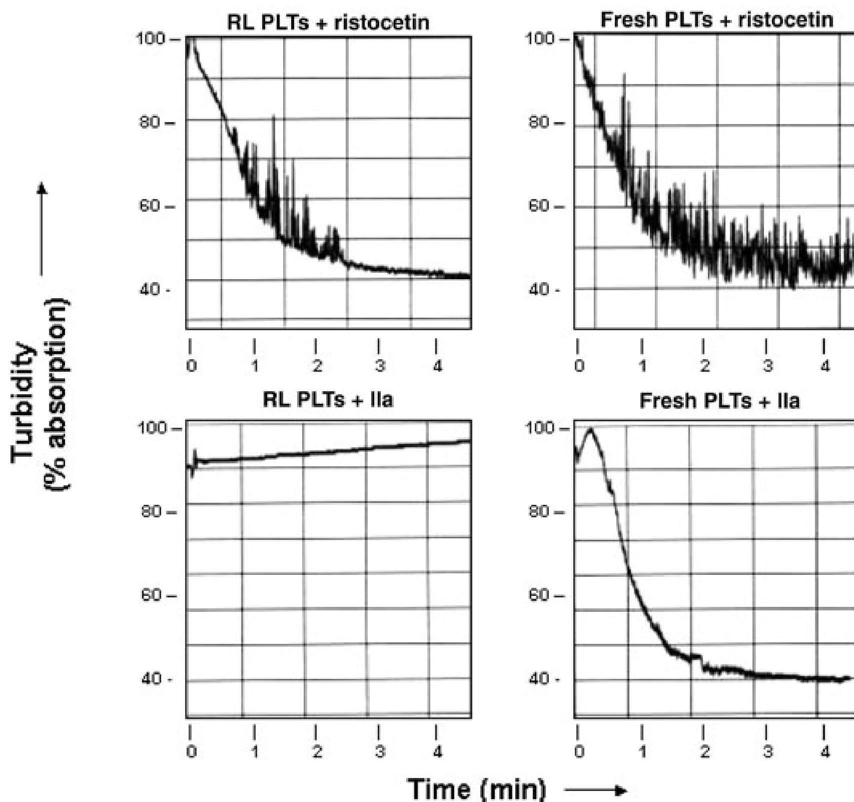

Fig. 1. Aggregometry with ristocetin and thrombin. Fresh and RL PLTs were prepared in normal plasma for ristocetin agglutination and in Tyrode's buffer for thrombin aggregation. Assays were initiated by adding ristocetin or thrombin, and then turbidity time courses were measured as detailed under Materials and Methods.

The ability of RL PLTs to catalyze prothrombin to thrombin conversion was investigated with in vitro and in vivo experiments that assess the prothrombotic safety of RL PLTs. First, the lag time for initiation of thrombin generation and the amount of generated thrombin was measured with a fluorogenic substrate in normal plasma that contained varying amounts of RL and fresh PLTs. The data depicted in Figs. 3A and 3B were representative comparisons of a single normal donor's fresh PLTs with RL PLTs that were manufactured with tangential-flow filtration from five pooled double-apheresis units (from five donors). Both the fresh and the RL PLTs were assayed in the plasma

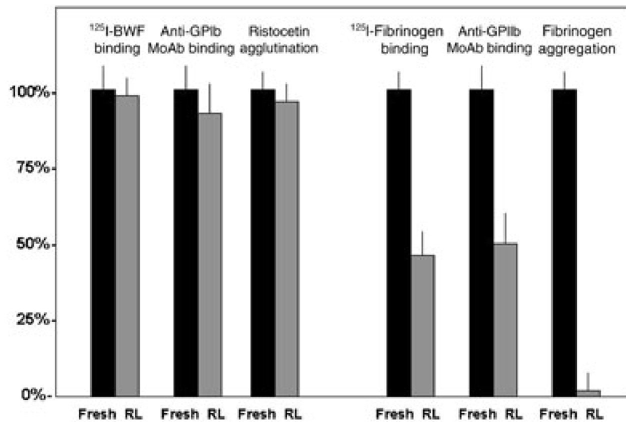

Fig. 2. GPIb-VWF and integrin functions of RL and fresh PLTs. Metrics for VWF binding, anti-GPIb MoAb SZ2 binding, initial ristocetin agglutination slope, fibrinogen binding, anti-GPIIb MoAb 10E-5 binding, and initial thrombin aggregation slope were obtained as detailed under Materials and Methods for fresh PLTs (black bars) and RL PLTs (gray bars). Parameters were normalized to a value of 100 percent for fresh PLTs. Each measurement is the mean of triplicate values obtained with the same fresh and RL PLT determination. The error bars depict the upper arm of ±SDs for the three values.

samples from the fresh PLT donor. When RL or fresh PLTs were mixed in plasma with the thrombin substrate D-Phe-Pro-Arg-ANSNH-C_6H_{11}, the resulting fluorogenic response was characterized by a lag time, as the intrinsic coagulation cascade turns over, and then an increase in fluorescence (see the inset curves in Fig. 3A, which depict triplicate measurements of RL PLTs at 4000 cells/μL). The amount of thrombin that is generated was estimated by comparing the maximum slope of the fluorogenic curve to time courses that were recorded with known amounts of human thrombin in the same plasma. At the thrombocytopenic to physiologic PLT concentrations that were investigated in these studies, RL PLTs generated amounts of thrombin (see Fig. 3A) similar to those of fresh PLTs. There was a trend for RL PLTs to generate less thrombin than fresh PLTs at the lower PLT concentrations (see the right three points in Fig. 3A). This trend, however, was within the standard deviations (SDs) of the measurements and thus might be judged as insignificant at lower PLT levels. The lag time for thrombin generation was a shallow function of PLT concentration, and values for RL and fresh PLTs were within the SD of triplicate measurements. In the absence of RL or fresh PLTs, thrombin was not generated (data not shown). Thrombin generation was inhibited in a dose-dependent manner by corn trypsin inhibitor (data not shown), indicating that factor XIIa–mediated turnover of the intrinsic (contact) arm of the coagulation pathway was the process probed with this assay system.

Substantial inter- and intraindividual variation in humoral coagulation system status is a well-documented phenomenon (e.g., see Monroe et al.[21]). Experiments were thus performed with RL PLTs at 10^8 RL PLTs per mL in plasma samples obtained from 70 randomly selected normal donors to determine whether there was a subgroup of individuals that displayed a hyperthrombogenic response to RL PLTs. Donors were randomly selected men and women who reflected the racial composition of the United States and were not on anti-PLT or anticoagulation therapeutics. Figure 4 shows that the histogram of the thrombin generation times in the donor plasmas was approximately bell-shaped with a mean value of 39 minutes, a minimum value of 14.8 minutes, and a maximal value of 75 minutes. The thrombin generation time in a normal plasma pool of more than 1000 donors (obtained from Innovative Research) was 45 minutes. Intraindividual variation was tested in 12 donors who were tested twice with 2 weeks between donations. Large intraindividual variations in RL PLT–mediated thrombin generation times were not measured (see the inset in Fig. 4). An important result of these analyses is that a group of individuals with a highly thrombophilic response to RL PLTs was not found.

DISCUSSION

An important concern in the development of infusion therapeutics for hemostasis is that the product be both effective at inducing coagulation at wound sites and safe with respect to pathologic intravasular coagulation. RL PLTs have proven not only effective at controlling hemorrhage but safe with respect to inducing overt clinical symptoms of pathologic intravascular coagulation when infused into rabbits,[4] dog,[3,22] pigs,[5] and baboons.[23] The findings reported in this article detail processes that underlie the favorable safety and efficacy profile of the rehydrated cells. Primary hemostatic processes, which support initial wound closure through GPIb and VWF-mediated mechanisms, are retained by RL PLTs. In contrast, secondary hemostatic processes involving thrombin coupling to integrins are largely eliminated, and the fibrinogen-binding activity of RL PLT integrins is reduced by approximately half. The differential effect of the RL PLT manufacturing process on primary and secondary hemostatic functions of the rehydrated product is related to three observations. First, RL PLTs are not more prothrombotic than fresh PLTs with respect to turnover of the intrinsic coagulation cascade in the fluorogenic thrombin substrate assays reported in Fig. 3. Second, based on the distribution study with normal volunteers depicted in Fig. 4, there does not seem to be a subpopulation of individuals that are hyperresponsive to RL PLTs with respect to thrombin generation. Finally, Valeri and coworkers[23] found that the infusion of RL PLTs into baboons did not elevate surface markers PLT activation on the animals intrinsic PLTs beyond the level of negative controls. These findings, when considered with the observation (e.g.,

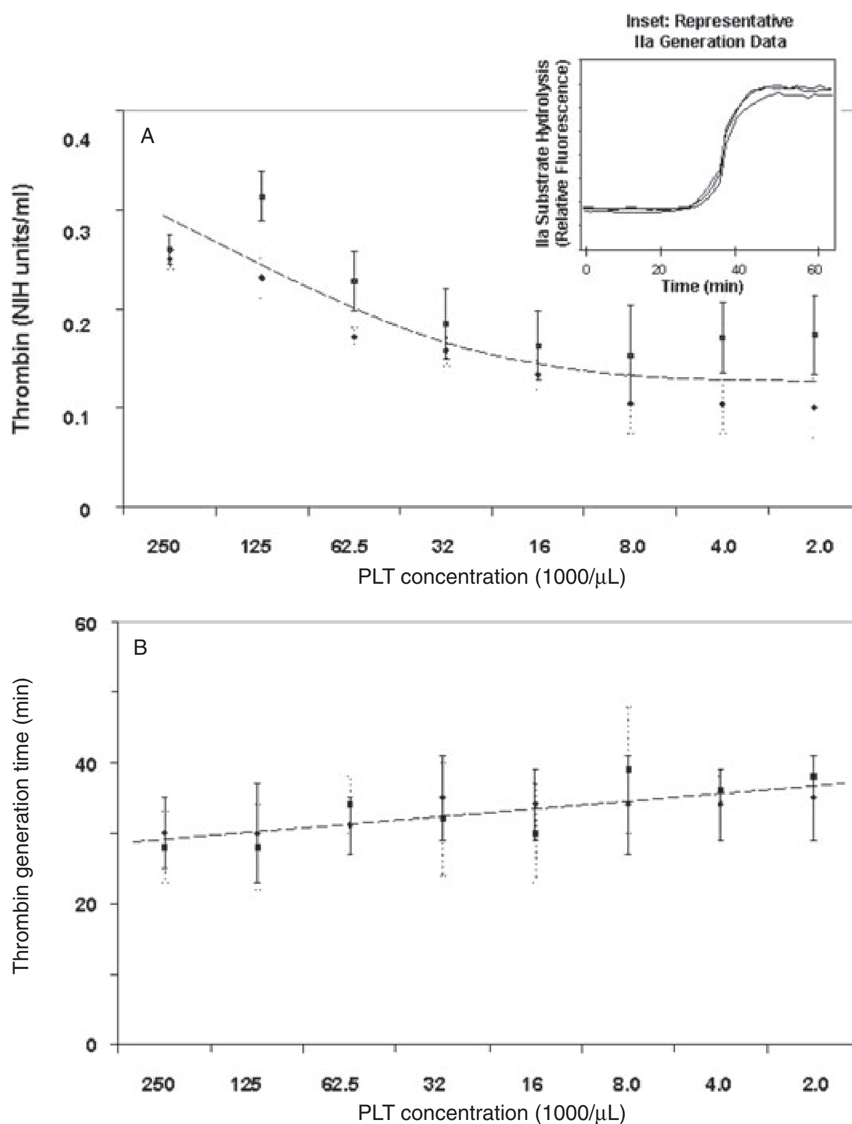

Fig. 3. Thrombin activity (A) and generation time (B) with RL (◆) and fresh (■) PLTs. Fresh or RL PLTs were analyzed in the plasma of the normal fresh PLT donor at PLT concentrations indicated on the horizontal axis as detailed under Materials and Methods. (A, inset) Representative triplicate fluorogenic thrombin substrate generation curves for RL PLTs at 4000 cells per µL. Each measurement is the mean of triplicate values obtained with the same fresh and RL PLT determination. The error bars depict SDs for the three values.

based on ICD-9 statistics in the United States[12]) that it is secondary hemostatic processes for fibrin thrombus formation that are associated with the vast majority of vascular occlusions in cardiovascular diseases, and support the hypothesis that RL PLT should prove safe when infused into humans.

In addition to partial inhibition of secondary hemostatic responses, there are two other mechanistic factors that potentially contribute to the prothrombotic safety profile of RL PLTs. First, RL PLTs must be turned on to be active, the key to the safety of hemostatic systems in general. RL PLTs are capable of a degree of intracellular stimulus response coupling whereby intracellular protein kinases such as protein kinase C and myosin light chain kinase are stimulated by PLT agonists.[24] The result of activation-dependent intracellular signaling is that RL PLTs provide positive feedback amplification with respect to turnover of intrinsic PLTs and the humoral coagulation. Lyophilized PLTs degranulate for the secretion of coagulation factors and small molecules that recruitment of additional PLTs. RL PLTs also generate thromboxanes and provide a procoagulative surface for the catalysis of prothrombin to thrombin conversion.[24] RL PLTs adhere to denuded endothelium in vitro in Baumgartner analysis in a GPIb-VWF–dependent manner, but bind to the subendothelium with approximately half the surfaced density of fresh PLTs.[25] The reduced density of RL PLTs on the denuded endothelium might be a consequence of inhibited integrin function for fibrin clot stabilization after the initial primary hemostatic event. The overall picture that has emerged is that RL PLTs provide hemostasis by adhering to wound sites through "primed" VWF-dependent mechanisms and provide a catalytic surface for thrombin generation.

The second mechanistic factor that might contribute to the favorable prothrombotic safety profile of RL PLTs involves the reduced circulation half time of the cells. A key to the technology for preparing RL PLTs is a mild aldehyde cross-linking step to stabilize macromolecular structures during freezing and lyophilization; this process modifies the surface with aldehyde adducts that are recognized by receptors on macrophages for phagocytosis.[11] The rehydrated cells are cleared from the free circulation ($t_{1/2}$ of ~10 min) by macrophages of the reticuloendothelial system.[11] Thus, a potentially thrombophilic therapeutic does not persist for long in the free circulation. The short circulation time of RL PLTs underlies the evaluation of these cells by the US Food and Drug Administration as a hemostatic agent and not a PLT substitute. The RL PLT circulation time of several minutes is sufficiently long for multiple passes through the circula-

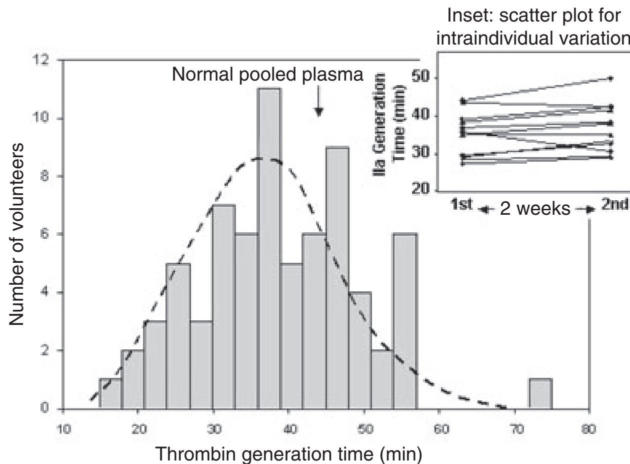

Fig. 4. Thrombin generation time with RL PLTs histogram from 70 randomly selected volunteers. The thrombin generation lag time was measured with a fluorogenic thrombin substrate with RL PLTs at 10^5 per μL as detailed under Materials and Methods as depicted in Fig. 3 with triplicate values being averaged to obtain a thrombin generation time for each donor sample. (Inset) Scatter plot for intraindividual two week variation with 12 randomly selected volunteers.

tory system for wound site localization, but too short to be useful for prophylactic indications.

Although the partial inhibition of secondary hemostatic mechanisms is expected to contribute favorably to the prothrombotic safety profile of RL PLTs, the implication for in vivo functionality of the rehydrated cells is unclear. It is well established that integrin function plays an important role in PLT rolling on, and adhesion to, inflamed and damaged endothelium[26-29] and ultimately stabilization of primary hemostatic plugs with a fibrin network and clot retraction (see Bennett[30] for a recent review). It is thus somewhat surprising that RL PLTs, with minimal coupling between the PAR receptors and integrin inside-out signaling, can provide hemostasis in animal models of cardiopulmonary bypass PLT dysfunction[22,31] and two types of thrombocytopenia,[4,5] whereas normally liquid-stored PLTs with a more active response to thrombin have not performed well in the human clinic for providing immediate hemostasis. Normally liquid-stored PLTs have not demonstrated efficacy, in a statistical sense, as a therapeutic for providing immediate hemostasis to resolve active bleeding associated with cardiopulmonary bypass[32-34] or hemorrhagic shock[35,36]-related PLT dysfunctions. A better understanding of this apparent dichotomy will be obtained when RL and normally liquid-stored PLTs are directly compared in clinical trials. It is reasonable to speculate, however, that RL PLTs are somewhat “primed” for activation and thus might form a primary hemostatic plug upon which endogenous PLTs can bind to form a more native clot. A clinical consequence of the reduced secondary hemostatic function of RL PLTs might be rebleeding, particularly when initial hemostatic plugs are subjected to increased hemodynamic pressures during resuscitation.

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