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Platelets regulate vascular endothelial stability: assessing the storage lesion and donor variability of apheresis platelets

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Abstract

BACKGROUND—In current blood banking practices, platelets (PLTs) are stored in plasma at 22°C, with gentle agitation for up to 5 days. To date, the effects of storage and donor variability on PLT regulation of vascular integrity are not known.

STUDY DESIGN AND METHODS—In this study, we examined the donor variability of leukoreduced fresh (Day 1) or stored (Day 5) PLTs on vascular endothelial barrier function in vitro and in vivo. In vitro, PLT effects on endothelial cell (EC) monolayer permeability were assessed by analyzing transendothelial electrical resistances (TEER). PLT aggregation, a measure of hemostatic potential, was analyzed by impedance aggregometry. In vivo, PLTs were investigated in a vascular endothelial growth factor A (VEGF-A)-induced vascular permeability model in NSG mice, and PLT circulation was measured by flow cytometry.

RESULTS—Treatment of endothelial monolayers with fresh Day 1 PLTs resulted in an increase in EC barrier resistance and decreased permeability in a dose-dependent manner. Subsequent treatment of EC monolayers with Day 5 PLTs demonstrated diminished vasculoprotective effects.

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AUTHOR CONTRIBUTIONS

All authors participated in manuscript preparation. SP, MC, PS, and APC planned the experiments, interpreted the results, and contributed to writing the paper; GB and BM performed the animal experiments, in vitro experiments, the statistical analysis, and preparation of the figures and interpreted the data; MM performed and analyzed the flow cytometry experiments; DP provided experimental assistance, prepared figures, and performed data analysis; and SG and RB analyzed data.

CONFLICT OF INTEREST

The authors have disclosed no conflicts of interest.

Donor variability was noted in all measures of PLT function. Day 1 PLT donors were more variable in their effects on TEER than Day 5 PLTs. In mice, while all PLTs regardless of storage time demonstrated significant protection against VEGF-A–induced vascular leakage, Day 5 PLTs exhibited reduced protection when compared to Day 1 PLTs. Day 1 PLTs demonstrated significant donor variability against VEGF-A–challenged vascular leakage in vivo. Systemic circulating levels of Day 1 PLTs were higher than those of Day 5 PLTs

CONCLUSIONS—In vitro and in vivo, Day 1 PLTs are protective in measures of vascular endothelial permeability. Donor variability is most prominent in Day 1 PLTs. A decrease in the protective effects is found with storage of the PLT units between Day 1 and Day 5 at 22°C, thereby suggesting that Day 5 PLTs are diminished in their ability to attenuate vascular endothelial permeability.

In massively transfused trauma patients, balanced ratios of plasma to platelets (PLTs) to red blood cells (RBCs) have been shown to be associated with a survival benefit. [1–6] These findings have formed the basis and principles behind damage control resuscitation (DCR). [3, 4] To make DCR practical, trauma centers typically maintain thawed fresh-frozen plasma (FFP) units for emergency transfusion. We have demonstrated that storage of FFP at 4°C for 5 days results in a diminished potential of the plasma to inhibit endothelial permeability for some donors.[7] Recent work by a number of groups indicates that plasma-based resuscitation mitigates vascular injury and endothelial permeability.[3, 7–10] Preclinical studies in small and large animals have demonstrated that FFP inhibits acute lung injury, lung vascular permeability, and pulmonary inflammation associated with hemorrhagic shock and trauma.[3, 7–10] FFP has also been shown to decrease blood–brain barrier permeability and cerebral edema in a swine model of traumatic brain injury.[11, 12] The mechanism of action of PLTs in DCR, aside from hemostasis, has yet to be determined. Since PLTs have been known to modulate vascular integrity, it is possible that synergy may exist between plasma and PLTs leading to improved outcomes in DCR.[7] However, the clinical impact of donor variability and the storage lesion of apheresis PLTs in traumatic injury is largely unknown. Retrospective studies have suggested that PLT transfusion is directly associated with improved survival in severe trauma and that transfusion of stored PLTs is associated with poor outcomes.[13] There are no prospective clinical studies to date on the age of stored PLTs and outcomes.

The hemostatic function of PLTs has been known since the late 1800s. For the past 60 years it has been known that PLTs regulate immune function and vascular stability. The generation of an activated PLT plug at the site of vascular trauma seals the lesion to achieve hemostasis. The membrane-oriented processes of subendothelial adhesion of PLTs, granule release, cohesion, aggregation, and plug stabilization involve several well-characterized ligand dependent processes. Aside from these highly regulated processes, PLTs maintain vascular integrity by the constitutive release of proangiogenic cytokines and growth factors (trophogens).[14, 15] These molecules, which PLTs store within granules, bind to specific receptors on the surface of endothelial cells (ECs), thereby eliciting intracellular signaling that stabilizes the vascular–endothelial cadherin complex at intercellular adherens junctions. When PLTs numbers decrease dramatically, molecular disassembly of adjacent intercellular endothelial junctions occurs which leads to vascular fragility and a propensity to bleed. In

addition to these effects, PLTs have multiple regulatory functions on endothelial progenitor cell development as well.[14] Taken together, PLTs are potent regulators of endothelial stability, development and function.

Currently in blood banking practice, PLTs are stored in incubators in plasma at 22°C, with gentle agitation for up to 5 days.[16] The storage lesion of PLTs has been characterized as including changes in pH, decreases in PLTs numbers, morphologic changes, loss of aggregation response to agonists, enhanced procoagulant activity, and increased release of PLT microparticles.[17–19]

In this study we sought to elucidate the storage lesion of apheresis PLTs on vascular endothelial stability between Day 1 and Day 5 of storage in plasma. We furthermore sought to determine the efficacy of apheresis PLTs on vascular stability amongst different donors. We hypothesized that PLTs would demonstrate considerable donor variability in vitro and in vivo and that these effects would be altered by storage for 5 days at 22°C. To test our hypothesis we investigated the potential of PLTs to inhibit vascular permeability and restore endothelial barrier function in vitro and in vivo. Furthermore, we also hypothesized that these protective effects would depend on the donor and diminish as PLTs are stored for 5 days at 22°C.

MATERIALS AND METHODS

Cell culture

Human umbilical vein endothelial cells (HUVECs) were grown in endothelial growth medium (EGM-2, Lonza Walkersville, Inc., Walkersville, MD) at 37°C and 5% CO₂ in a humidified incubator. Experiments were conducted using Passages 4 to 6. Cells were starved for 1 hour in EBM-2 (Lonza Walkersville, Inc.) before the treatments were applied to normalize the cell cultures and cell growth to a baseline of activation. This synchronizes the cell cycle to produce uniform responses between wells.

Preparation of human PLTs

Leukoreduced apheresis PLTs (Trima Accel automated component collection system, Terumo BCT, Inc., Lakewood, CO) were used fresh (Day 1 PLTs) within 24 hours after collection from normal blood donors or stored 5 days (Day 5 PLTs) in plasma in plastic bags (PVC, SOLMED, Terumo BCT) in the incubator (PC3200, Helmer, Noblesville, IN) at room temperature (22°C) with horizontal agitation according to standard procedures (Blood Centers of the Pacific, San Francisco, CA). All PLTs were tested for bacterial infection (Bac-T testing) by the blood bank (Blood Centers of the Pacific) and found to be negative. PLTs were washed in phosphate-buffered saline (PBS) by centrifugation at $1600 \times g$ for 20 minutes, resuspended in EBM-2, supplemented by addition of CaCl₂ (1 mmol/L) for 30 minutes at 37°C before treatment, and counted in an automatic hematology analyzer (Model XE-2100D, Sysmex, Mundelein, IL). Use of washed human PLTs separated from plasma in the absence of anticoagulants is essential for the study of intrinsic PLT properties since we have shown in our past studies that plasma itself has effects on the vascular endothelium that could confound the results.[7, 20]

Transendothelial electrical resistance

The integrity of HUVEC monolayers was measured using an electric cell-substrate impedance sensing system (ECIS 1600, Applied BioPhysics, Troy, NY). An increase or decline in transendothelial electrical resistance (TEER) across the cell monolayers indicated, accordingly, decreased or increased endothelial paracellular permeability. HUVECs were grown on l-cysteine-reduced, fibronectin-precoated 96-well plates containing electrodes in each well. Cells were treated with PLTs ($50 \times 10^9/\text{L}$ in 200 μL of EBM-2) and, in some experiments, subsequently challenged after 1 hour by vascular endothelial growth factor (VEGF-A165, R&D Systems, Minneapolis, MN), a common inducer of permeability, at a concentration of 50 ng/mL. Monolayer resistance at 4/16/64 kHz was analyzed in 5-minute intervals. Data were normalized to the mean resistance of cell monolayers before the treatments. PLT concentrations tested were chosen based on concentrations that have been previously shown to attenuate endothelial permeability in the ECIS assay system.[21]

Impedance aggregometry

Using a multiplate analyzer (Dynabyte, Munich, Germany), we examined individual-donor PLT aggregation response to stimulation by the agonists as adenosine diphosphate (ADP), collagen, thrombin receptor-activating peptide-6 (TRAP), and arachidonic acid (ASPI) for six PLT donors who were tested in our in vitro assays of EC function. Briefly, donor PLTs were diluted with a baseline whole blood at a concentration of $500 \times 10^9/\text{L}$ and then added to the test wells. Whole blood was used as a medium for dilution since the multiplate aggregometer does not function properly with PLTs alone or with plasma and PLTs. RBCs are required for consistent reading. The whole blood added to the PLTs before measurement on the multiplate was from the same donor and the hematocrit was used as a reference point to normalize the readings and variability between runs which took place at different times. For the ADP, collagen, and TRAP tests, the PLTs were incubated 1:1 with a 0.9% NaCl, 3 mmol/L CaCl_2 solution for 3 minutes. For the ASPI tests, the PLTs were incubated 1:1 with just 0.9% NaCl. After incubation, agonists were added in concentrations of 6.5 $\mu\text{mol/L}$ ADP, 3.2 $\mu\text{g/mL}$ collagen, 32 $\mu\text{mol/L}$ TRAP, 0.5 mmol/L ASPI. Measurements were recorded for 6 minutes. Significance was determined by paired t tests.

Vascular permeability assay in vivo

The modified Miles assay[22, 23] was performed in 8- to 10-week-old NOD.Cg-*Prkdc*^{scid} *Il2rg*^{tm1Wjl}/SzJ (NSG) mice that were purchased from Jackson Laboratories (Sacramento, CA). They were bred at our institution with approval of the Institutional Animal Care and use Committee at PMI Preclinical (San Carlos, CA). Isoflurane-anesthetized mice were treated with Day 1 PLTs or Day 5 PLTs through the tail vein (3×10^8 in 200 μL of PBS per animal. The control mice received the same volume of PBS alone. Thirty minutes after PLT or PBS treatment 50 μL of VEGF-A (2 ng/ μL), an equal volume of PBS was administered intradermally into the dorsal skin on the right and left side, respectively. The PBS on the left side controls for vascular leak induce by the injection trauma and infusion of the fluid. Subsequently, 100 μL of a 0.5% Evans blue dye (Sigma-Aldrich, St Louis, MO) was administered in the retro-orbital sinus. Two hours posttreatment with PLTs mice were photographed ($\times 200$, 1 NIKKOR, Nikon, Melville, NY) and euthanized. A vascular leak (the

area of blue skin) was assessed by the accumulation of Evans blue dye into the injected side. For quantitative measurement, the 5 mm of biopsied tissues (Tru-Punch, Sklar Instruments, West Chester, PA) was placed into formamide at 37°C for 48 hours. The absorbance of the extracts was determined at a wavelength of 620 nm on microplate reader (SoftMax Pro 5, Molecular Devices, Sunnyvale, CA). Day 1 PLTs and Day 5 PLTs groups were compared using unpaired t tests. Variation between donors was determined by post hoc Tukey tests after a one-way analysis of variance.

Circulation of transfused PLTs in immunodeficient NSG mice

The capacity of the human PLTs to circulate was measured after transfusion into immunodeficient mice. Equal numbers of human PLTs were transfused into age-, sex-, and weight-matched NSG mice and then identified by their expression of human CD41 and CD42b. The initial measurements of circulating human PLTs took place 5 minutes after transfusion followed by 1- and 2-hour sampling. PLT surface levels of CD62P (P-selectin) were compared using the mean fluorescence intensity of expression. The frequencies of human PLTs were identified by double staining of CD41+CD42b+ or CD62P PLTs among all events not stained with propidium iodide, which stains nucleated cells. All events were analyzed with computer software (FlowJo, Version 9.7, Tree Star, Inc., Ashland, OR).

Statistical analyses

Measures of PLT adhesion, TEER, impedance aggregometry, and vascular permeability were analyzed using unpaired t tests (Stata 13.1, StataCorp, Dallas, TX). Levene's robust test was applied to the analyses of TEER data to determine the variability in the groups. Briefly, the Levene's test is an inferential statistic used to assess the equality of variances for a variable calculated for two or more groups. Some common statistical procedures assume that variances of the populations from which different samples are drawn are equal. Levene's test assesses this assumption. It tests the null hypothesis that the population variances are equal. If the resulting p value of Levene's test is less than typically 0.05, the obtained differences in sample variances are unlikely to have occurred randomly and it is concluded that there is a difference between the variances in the population. Differences in the numbers of human PLTs transfused into mice were evaluated using a two-tailed Mann-Whitney U test (Aabel NG, Gigawiz Ltd. Co., Redwood City, CA). Differences between groups were considered significant with p values of not more than 0.05.

RESULTS

Day 1 PLTs strengthen the endothelial barrier in a dose-dependent manner

To determine the biologic effects of PLTs on endothelial cell (EC) permeability, we utilized a novel assay that allows us to evaluate the resistance and integrity across endothelial monolayers treated with PLTs. This assay is run using an ECIS (see Materials and Methods). In these studies we found that the addition of fresh human PLTs (Day 1) to EC monolayers produced a rapid increase in TEER (decreased permeability) that is expressed as normalized resistance, for each of the two donors tested (Fig. 1A). Increased TEER is directly correlated with decreased EC paracellular permeability.[24, 25] There was evidence of dose-dependent increases in resistance with increasing dose of PLTs ($p < 0.001$, nonparametric test for trend).

Mean increases were 9.0, 13.7, and 18.0% for Donor 1 and 10.7, 15.5, and 17.0% for Donor 2, corresponding to PLT treatment dosages of 10×10^9 , 25×10^9 , and 50×10^9 /L, respectively (Figs. 1A and 1B). The highest concentration of 50×10^9 PLTs/L, which was maximally effective, was used in the remaining experiments in vitro.

Day 1 PLTs attenuate endothelial permeability, an effect that diminishes more than 5 days of storage

In standard blood banking practice, PLTs are stored for 5 days, shaking gently at 22°C. To assess the role of PLT storage time on endothelial barrier function, PLTs of varying storage time were added to the EC monolayers, and their effects on TEER were measured. In an experiment with three storage time groups (Day 1, Day 3, and Day 5 PLTs), and three different donors per group, a significantly larger mean percent increase in TEER (decreased endothelial permeability) was observed in groups treated with Day 1 PLTs compared to Day 5 (36.7 ± 2.5 vs. 24.3 ± 2.2 , $p=0.002$, respectively; Figs. 1C and 1D). In this experiment Day 1 PLTs induce a slightly larger, but not significant, mean resistance increase over baseline than Day 3 PLTs (33.1 ± 2.4 , $p=0.073$), and there was stronger evidence that Day 3 PLTs demonstrate an intermediate effect (Day 3 vs. Day 5, $p=0.005$; one-tailed t-tests, mean $\pm$ SD). Taken together these data suggest a progressive loss with storage in the PLT's ability to regulate endothelial permeability.

Day 1 PLTs display interdonor variability in their effects on endothelial monolayer permeability

To determine the level of donor variability present among PLTs, we compared two different sets of six donors (Fig. 2B). There was evidence that Day 1 PLT donors were more variable in their effects on TEER than Day 5 PLTs (Figs. 2B and 2C; $p=0.013$ by Levine's robust test—see Materials and Methods). The mean increase in TEER was also significantly higher for Day 1 than Day 5 in this experiment PLTs. It is of interest to note that the TEER changes in Day 1 PLTs were between 25.7 and 40.5%, giving a range of 14.8%, compared to a range of 2.6% associated with Day 5 PLTs with minimum to maximum of the TEER values 10.8% to 13.4%, accordingly (Fig. 2C). These results confirm a storage lesion of PLTs between Day 1 and Day 5 of storage and also demonstrate considerable variability among donors at Day 1 of testing.

Day 1 PLTs attenuate EC barrier permeability induced by VEGF stimulation, which is diminished in Day 5 PLTs

To determine the relative strength of the protective effects of the PLTs on endothelial monolayer integrity, we challenged the ECs with VEGF-A, a known established inducer of vascular permeability. Our goal was to test if we could tease out differences between the donors by determining how they respond to a "stressor" or challenge such as VEGF.[26] Treatment of EC monolayers with Day 1 versus Day 5 PLTs was associated with a smaller percentage decrease in resistance that is associated with increased permeability induced by VEGF-A (Day 1 9.8 ± 0.7 vs. Day 5 12.7 ± 0.3 vs. control 13.6%; Fig. 2D). Controls were not treated with PLTs.

The decline after VEGF treatment was larger for Day 5 PLTs compared to Day 1 PLTs ($p=0.005$, two-sample, one-tailed t test). In an experiment with PLTs using multiple donors stored up to 5 days, Day 1 PLTs ($p=0.035$) were superior to Day 3 PLTs and to Day 5 PLTs ($p=0.016$, data not shown). These data suggest that Day 1 PLTs are more potent than Day 3 or Day 5 PLTs at withstanding challenges that induce vascular leak.

PLT aggregation declines over 5 days of storage and displays interdonor variability

Although our focus in this article is PLT–endothelial interactions and function, we sought to characterize and determine the effects of PLT storage on functional in vitro measures of PLT aggregation related to clot formation. We measured the aggregation response of freshly isolated PLTs to the agonists ADP, collagen, TRAP, and ASPI. We tested six donors who were the same as those tested in Fig. 2. After 5 days of storage, these same PLTs were evaluated again. Compared to Day 1 PLTs, Day 5 stored PLTs significantly lost the aggregation response to collagen, TRAP, and ASPI. However, when stimulated by ADP, the aggregation response was similar between Day 1 PLTs and Day 5 PLTs (Fig. 3A). The rate at which the aggregation occurred also declined significantly when stimulated by collagen, TRAP, and ASPI, but not ADP (Figs. 3A and 3B). Examination of Day 1 PLTs by individual donor revealed a broad variation in activation response to these agonists (Fig. 3C) thereby demonstrating an inherent variability among donors. Stimulation with ADP resulted in activation units ranging from 100.5 to 36.8, collagen ranges from 172.1 to 96.0, TRAP ranges from 204.4 to 185.6, and ASPI ranges from 170.3 to 144.0 (Fig. 3C). Interestingly, in Day 5 PLTs variation in response widened significantly with TRAP, collagen, and ASPI stimulation (Fig. 3D). Stimulation of Day 5 PLTs with ADP resulted in activation ranges from 89.5 to 37.9, collagen ranges from 113.2 to 47.8, TRAP ranges from 168.4 to 106.2, and ASPI ranges from 115.1 to 53.5 (Fig. 3D).

In vivo, Day 1 PLTs are superior inhibitors of VEGF-induced vascular permeability and display interdonor variability

Since we demonstrated that PLTs modulate endothelial permeability in vitro, we sought to determine if our findings would translate in vivo in an established mouse model of VEGF-A–induced vascular permeability in mice. Using a modified Miles assay in NSG mice, vascular permeability is assessed in response to VEGF-A stimulation. Control untreated mice display large blue patches of Evan's blue dye extravasation indicative of vascular leak whereas injection of PBS (the control) on the opposite side of the mouse resulted in minimal dye extravasation caused by the trauma from the needle insertion, thereby indicating effective induction of vascular leak by VEGF-A administration (Fig. 4B, left). VEGF-A–induced permeability was decreased in both treatment groups (Day 1 PLTs and Day 5 PLTs, $p<0.001$) compared to control mice receiving PBS vehicle alone (Fig. 4B center, right). However, quantification of Evans blue extravasation showed a lower amount of dye in the skin of Day 1 PLT mice (mean $\pm$ SD, 0.003 ± 0.001 ; Fig. 4C) compared to Day 5 PLTs treated mice (mean $\pm$ SD, 0.022 ± 0.006 ; $p<0.001$). These data indicated that Day 1 PLTs protect vascular barrier function more potently than Day 5 PLTs in vivo. Additionally, Day 1 PLTs exhibit significant ($p<0.05$) interdonor variability in their protective effect on VEGF-A–induced vascular leakage (see Fig. 4D).

Day 5 PLTs are cleared more rapidly from circulation than Day 1 PLTs

The survival of Day 1 versus Day 5 PLTs in mice was evaluated in the blood of the mice. CD41+CD42b+ PLTs were analyzed by flow cytometry after transfusion into NSG mice (Fig. 5A). Two hours after infusion the frequency of Day 1 PLTs in circulating in the blood was significantly higher than Day 5 PLTs (Fig. 5B). Although there was a trend toward lower numbers of Day 5 PLTs in the spleen at this time point, the difference was not significant (data not shown). An analysis of the kinetics of PLT clearance showed a rapid decrease in both fresh and aged human PLTs within 1 hour of transfusion, which is likely due to the constraints of testing of human PLTs in mice (Fig. 5C).

DISCUSSION

The storage lesion of PLTs has indeed been described by other groups in multiple measures of function and outcome.[17],[27–32] In this study we present data that support the premise that the regulation of vascular endothelial integrity and permeability by Day 1 fresh PLTs is superior to that of Day 5 PLTs stored at 22°C in plasma. Our data suggest that the storage lesion is progressive over the 5 days with the largest changes occurring between Day 3 and Day 5. Furthermore our studies demonstrate that there is interdonor variability among donors in these measures of function as well as in PLT aggregometry, which potentially indicates the ability of the PLTs to form clots. This is the first report evaluating donor PLT variability and effect of storage on PLT regulation of vascular permeability. Using a novel method for evaluation of endothelial permeability (the ECIS assay), we demonstrate that EC resistance increases with Day 1 PLTs and decreases over 5 days of storage. This drop is also found in PLT aggregation with storage at 22°C and also in vivo in the Miles model of vascular leak.

It is of interest to note that Day 1 PLTs are superior compared to Day 5 PLTs in their ability to protect against vascular leakage in the presence of a challenge such as VEGF-A (Fig. 2D). One can speculate that this could translate into a diminished ability of Day 5 PLTs to attenuate vascular leak in the context of a disease challenge, such as in inflammatory diseases, cancer chemotherapy, and coagulopathic shock. Our studies indicate that extension of storage time impairs the barrier protective effects of the PLTs. It will be of interest to understand in future studies what the clinical impact is of donor variation and storage to the vasculoprotective effects of transfused apheresis PLTs in human disease.

We found that Day 1 PLTs demonstrate the greatest variability in their protective effects on endothelial permeability. We hypothesize that the Day 1 PLTs are the closest functionally and phenotypically to the PLTs circulating within the donors. Their function and biologic effects are likely still affected by interactions with the donor cells and donor tissues after collection, thus resulting in greater interdonor variability. After a period of 5 days we hypothesize that the PLTs come to a set point of diminished function, that is, that they “age” at different rates but still end up at a common level of diminished function after 5 days. We hypothesize that this may be secondary to the lack of some constituent or interaction in vivo in the blood or tissue of the donor that is required for the maintenance of their protective function. It will be of interest to better understand in future studies the molecular mechanisms and aging process of PLTs on endothelial stability.

Limitations of this study include our inability to determine if the variability found in apheresis PLTs is specific to the donated unit or to the donor. To elucidate this aspect further would require repeat donation studies from the same donor, which is worthy of further investigation and may lead to new information that assists in identifying the best donors for PLT donation. The utilization of mouse models used to study human PLTs also poses considerable challenges since the human PLTs are larger than mouse PLTs and are trapped and cleared more rapidly. The ability to evaluate human PLTs in SCID mice has been described by others.[33] We chose to use NSG mice that also lack functional NK cells and potentially allow for a longer circulation times of the infused human PLTs.[33] It is evident from the flow cytometry studies (Fig. 5) that a rapid decline in circulating PLTs occurs after PLT infusion. The location of these cleared PLTs does not appear to be through the spleen and maybe through hepatic macrophages in the liver as described by Hoffmeister and colleagues.[34] However, these mouse models do indeed allow us to study the immediate biologic effects of PLTs such as the regulation of vascular permeability. Our model clearly demonstrates that Day 1 PLTs demonstrate donor variability and superiorly attenuate VEGF-A-induced permeability compared to Day 5 PLTs. These data are in line with previous data regarding progressive reduction in recoveries and survivals of plasma stored PLTs.[17]

Another limitation of the study is that our findings may not apply to PLTs stored in PLT additive solutions (PAS). Our studies were conducted with plasma-stored PLTs and it should be noted that the effects of storage may not be the same on PAS-stored PLTs. Previous work has demonstrated that replacing plasma with PAS results in altered viability, which is better maintained in PAS than in plasma. The lifespan of PLTs stored in PAS was 8.8 days versus 7.2 days in plasma.[18, 35] These results reflect an improvement of PLT quality with modifications of the storage conditions. Ideally one can hypothesize that a storage media can be developed that attenuates the storage lesion of PLTs on vascular function, a topic worthy of future investigation.

In this study we have utilized a combination of in vitro and in vivo assays of vascular endothelial function to elucidate the effects of donor variability and storage of PLTs at 22°C. Using the in vitro ECIS assay and the in vivo assay of vascular permeability in NSG mice, our studies demonstrate the clear effect of storage and donor variability in the vasculoprotective effects of apheresis PLTs. While it has been reported that no in vitro test can adequately predict PLT survival in vivo,[34, 36] our study directly correlates in vitro findings on ECIS with in vivo vasculoprotective effects and the circulation potential of the PLTs in mice. It will be of interest to determine if the noted effects of storage are reproduced in patients and impact patient outcomes.

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ABBREVIATIONS

ASPI	arachidonic acid
CD41	integrin $\alpha 2b$
CD42b	glycoprotein 1b
CD62P	P-selectin
DCR	damage control resuscitation
EC(s)	endothelial cell(s)
ECIS	electric cell-substrate impedance sensing
HUVEC(s)	human umbilical vein endothelial cell(s)
PAS(s)	platelet additive solution(s)
TEER	transendothelial electrical resistance
TRAP	thrombin receptor-activating peptide-6
VEGF-A	vascular endothelial growth factor A

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Figure 1.

Day 1 PLT (Day1-PL) increase TEER while PLTs grow less effective as they age. (A) ECIS bars depict the treatment effects of three doses of Day1-PL of two representative donors on the TEER of HUVEC monolayers. There was a significant trend of increased TEER (decreased endothelial permeability) with increasing PLT dose, $*p < 0.001$ (nonparametric test for trend). (B) Trace is the average of the two donors. (C) Traces and (D) bars show effects on TEER of PLTs stored for three different times: Days 1, 3, and 5. Bars show mean $\pm$ SD, $*p < 0.05$.

Figure 2.

PLT-induced increase in TEER: donor variability. (A) TEER traces show effects of Day 1 PLTs (Day1-PL) from one set of six individual donors. (B) Dots correspond to the TEER maximum six individual donors at Day1-PL and Day5-PL. The line indicates the mean of all donors. (C) TEER traces show effects of Day5-PL from six individual donors. (D) Bars show mean decline in normalized resistance after VEGF-A treatment, adjusted for each PLT storage group by subtracting changes in corresponding pretreated groups without VEGF addition. Values were normalized to baseline before VEGF-A addition. Results are expressed as means $\pm$ SD, * p <0.05.

Figure 3.

PLT aggregation response after 5 days of storage. Day 1 PLTs (Day1-PL) and Day5-PL PLTs were stimulated by the agonists ADP, collagen, TRAP, and ASPI. (A) Total PLT aggregation units and (B) the rate of aggregation (velocity of aggregation) response after stimulation by the respective agonists. *Paired t test ($p < 0.05$) was conducted. Dot plots depicting the donor variation in the aggregation response from (C) Day1-PL and from (D) Day5-PL. Each colored dot represents the aggregation response of an individual donor PLT. The color's identity is conserved throughout the plots.

Figure 4.

Day 1 PLTs (Day1-PL) and Day5-PL differ in protection against VEGF-induced permeability in vivo. (A) Schematic of Miles assay experiment, VEGF-A-elicited vascular leakage is visualized as the accumulation of Evans blue dye into the dorsal skin of mouse after intradermal injection with VEGF-A (right) or PBS (left) as control (n=6 mice/group). (B) Representative photographs of mice treated with vehicle PBS (left), Day1-PL (center), or Day5-PL (right). (C) Evans blue dye extravasation was quantitated from punch biopsy by the spectrophotometric analysis at 620 nm. The results are represented in bar graphs (mean

$\pm$ SD) of (VEGF-A minus corresponding PBS), * $p < 0.001$ for Day1-PL compared to Day5-PL. (D) Comparison of the preventive effect of Day1-PL from five individual donors on VEGF-A-induced vascular leakage. Bars are presented as the percentage of inhibition of leakage compared to control, * $p < 0.05$.

Figure 5.

Circulation of human PLTs in NSG mice. Representative flow cytometric data of blood analyzed for the presence of human PLTs 2 hours after transfusion of PBS or Day 1 PLTs (Day1-PL; A). The frequencies of human PLTs among all live cell events are indicated in each plot. The mean $\pm$ SE of PLT frequencies 2 hours after infusion in the blood of NSG mice are shown. (B). Results are compiled from two experiments and the total number of mice injected are indicated below each column of bars. p values are for the comparison of Day1-

PL and Day5-PL are indicated in each graph. The kinetics of PLT clearance in the blood of individual mice are shown for mice transfused with Day1-PL or Day5-PL (C).

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