In vitro function and phagocytosis of galactosylated platelet concentrates after long-term refrigeration

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BACKGROUND: Short-term refrigeration of platelets (PLTs) in the absence of plasma results in their rapid clearance after transfusion. Blocking β-*N*-acetylglucosamine (β-GlcNAc) residues of glycoprotein Ibα (GPIbα) with galactose prevents binding of refrigerated human and mouse PLTs to macrophages and prolongs the circulation times of refrigerated mouse PLTs. PLT-associated galactosyltransferase efficiently galactosylates chilled PLTs in the presence of its substrate UDP-galactose is added to PLT-rich plasma.

STUDY DESIGN AND METHODS: To characterize the hemostatic function of refrigerated and galactosylated human PLTs processed in the blood bank, PLT aggregation was studied in vitro under static and flow conditions and expression of integrin β3 (CD61), CD62P (P-selectin), GPIbα (CD42b), annexin V binding, and integrin αIIβ3 activation with flow cytometry. Affinity of macrophages for galactosylated refrigerated PLTs was evaluated with THP-1 cells, which recognize and phagocytize refrigerated PLTs.

RESULTS: PLTs refrigerated and galactosylated for 14 days 1) maintained their ability to aggregate when exposed to agonists in a standard aggregometry assay, 2) showed less pronounced changes in surface expression of GPIbα compared with room temperature (RT)-stored PLTs, 3) increased P-selectin expression, and 4) were poorly phagocytized by differentiated THP-1 cells in vitro. In addition, it is shown that refrigeration of PLTs does not affect their adhesive properties under in vitro flow conditions.

CONCLUSION: It is shown that refrigerated human PLTs retain in vitro function better than RT PLTs during storage and demonstrate that galactosylation prevents recognition of stored refrigerated PLTs by macrophages in vitro.

A major advance in medical care half a century ago was the development of platelet (PLT) transfusions for the temporary correction of PLT deficiencies. Since then, PLT utilization has increased significantly. PLTs, unlike other transplantable tissues, do not tolerate refrigeration and disappear rapidly from the circulation of human and other mammalian recipients if subjected to even short periods of chilling. Therefore, for almost five decades PLTs have been prepared and stored at room temperature (RT). RT-stored PLTs have a significant risk for bacterial contamination

ABBREVIATIONS: β-GlcNAc = β-*N*-acetylglucosamine; GPIbα = glycoprotein Ibα; PC(s) = platelet concentrate(s); PPP = platelet-poor plasma; PRP = platelet-rich plasma; RCA I = ricinus communis I lectin; RT = room temperature; S-WGA = succinylated tritium vulgaris-wheat germ agglutinin.

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resulting in bacterial sepsis.[1,2] Storage at RT is favorable for the growth of bacteria and conductive to detrimental changes in PLT structure and function. The estimated total number of PLT units collected in the United States in 2003 is more than 5.9 million, and the current estimated risk for bacterial contamination of PLT units is 1 in 1000 to 1 in 2000.[3] These numbers suggest that more than 2500 bacterially contaminated PLT units are potentially transfused every year. Several methods to screen and decrease the risk of bacterial contamination are currently under development. These methods include bacterial detection and pathogen inactivation.[1,4,5]

We previously demonstrated that transfused murine PLTs subjected to short-term (2-4 hr) refrigeration in the absence of plasma are removed from the circulation primarily by liver macrophages[6] and characterized one receptor-counterreceptor pair that modulates PLT clearance. We proposed that chilling causes the glycoprotein Ib (GPIb)/V/IX receptor complex (VWFR) to cluster on the surface of PLTs bringing exposed β-*N*-acetylglucosamine (β-GlcNAc) residues on N-linked glycans of the GPIbα subunit together, which leads to their recognition by the lectin domain of αMβ2 receptors[7] on phagocytes in the liver. Coverage of exposed β-GlcNAc on the GPIbα chain by galactose (galactosylation) rescues the loss of circulation of murine PLTs refrigerated as described above.[8]

The goals of this study were to test the feasibility of galactosylation of human PLTs under standard blood bank conditions and to assess in vitro functions of refrigerated human PLTs following long-term storage. We show successful galactosylation of PLTs after injection of UDP-galactose into PLT concentrates (PCs). Galactosylation of refrigerated PLTs was stable for at least 14 days, and these PLTs maintained their in vitro response to agonists. Galactosylation markedly prevented phagocytosis of refrigerated PLTs by differentiated THP-1 cells in vitro.

MATERIALS AND METHODS

Preparation of PCs and galactosylation of PLTs

Standard whole-blood donations from healthy volunteers, under informed consent, were collected into triple packs (Baxter Healthcare, Deerfield, IL) and centrifuged at 20 to 24°C for 5 minutes at 1900 × *g* (Jouan, Winchester, VA) to obtain PLT-rich plasma (PRP). The supernatant PRP was transferred to the transfer pack and centrifuged for 5 minutes at 5000 × *g* to pellet the PLTs. The supernatant PLT-poor plasma (PPP) was transferred to a satellite bag, leaving the PLTs in 50 to 60 mL of plasma and is referred to as PC. The PCs were left undisturbed for 1 hour and then placed on an agitator (Helmer Laboratories, Noblesville, IN), running at 60 agitations per minute in a PLT incubator (Helmer) for storage at 22 ± 2°C.

Twenty-four hours after collection and preparation, PLTs were transferred from the hospital blood bank to the research laboratory and placed in an incubator for 1 hour at 37°C, a time point subsequently referred to as Day 0. UDP-galactose (Calbiochem, San Diego, CA) was resuspended in sterile water and filtered with a 0.22 µmol per L sterile filter (Millipore, Billerica, MA). The UDP-galactose stock solution was then injected under sterile conditions into the PLT bags to a final concentration of 800 µmol per L. The PLT bags were incubated with UDP-galactose at 37°C for 40 minutes before refrigeration and storage. The PLT bags were then incubated at either RT with agitation with horizontal flat-bed rotator (New Brunswick Scientific, Edison, NJ) or left undisturbed in the refrigerator at 4°C.

Aggregation-agglutination

PLT aggregation and agglutination experiments were performed with a PLT aggregation profiler (Model PAP-1, Bio/Data Corp., Horsham, PA). PLTs were washed free of plasma by centrifugation (850 × *g*, 5 min) with 5 vol of washing buffer containing 140 mmol per L NaCl, 5 mmol per L KCl, 12 mmol per L trisodium citrate, 10 mmol per L glucose, and 12.5 mmol per L sucrose, 1 µg per mL prostaglandin E_1, pH 6.0 (Buffer B), and resuspended in Buffer A (10 mmol/L *N*-(2-hydroxyethyl)piperazine-*N*′-(2-ethanesulfonic acid) [HEPES], 140 mmol/L NaCl, 3 mmol/L KCl, 0.5 mmol/L $MgCl_2$, 10 mmol/L glucose, and 0.5 mmol/L $NaHCO_3$, pH 7.4) at a concentration of 5×10^8 per mL and were activated by adding 0.1 to 1 U per mL thrombin (Sigma-Aldrich, St. Louis, MO); PRP was mixed with PPP in the ratio 1:1 and it was then activated through the addition of 10 µmol per L adenosine 5′-diphosphate (ADP; Sigma-Aldrich) or 1.5 mg per mL ristocetin (Sigma-Aldrich) for 3 minutes at 37°C under constant stirring (1000 rpm).[6] Buffer A for washed PLTs and PPP for PRP were set as maximum of light transmission.

Microscopy

Ten-millimeter round cover slips (Fisher Scientific, Pittsburgh, PA) were coated with 1 mg per mL poly-L-lysine (Sigma) for 5 minutes, and unbound poly-L-lysine was removed by briefly washing the coverslips with PBS. PLTs were washed once by centrifugation (850 × *g*, 5 min) with 5 vol of Buffer B and added to the center of poly-L-lysine–coated coverslips in Buffer A-bovine serum albumin (BSA) and centrifuged at 200 × *g* for 5 minutes in multiwell plates. PLTs that adhered to the center of poly-L-lysine–coated coverslips were mounted onto microscope slides (Fisher Scientific) with mounting medium (Aqua Poly/Mount, Polysciences, Inc., Warrington, PA) and viewed at RT on a microscope (Axiovert 200, Zeiss, Jena, Germany).

Differential interference contrast objective images were obtained with a 100× lens with a charged coupled device camera and its accompanying software (ORCA-II and Metamorph, respectively, Molecular Devices, Sunnyvale, CA). Images were imported to computer software (Adobe Photoshop, Adobe, San Jose, CA) where image contrast was enhanced equally in test and control samples.

Flow cytometric analysis of glycoprotein and phosphatidylserine PLT surface expression

Human PLTs were analyzed for surface expression of CD42b (GPIbα), CD61 (β3), activation of β3 (PAC-1), and CD62P (P-selectin) by staining with fluorescein isothiocyanate (FITC)-coupled antibodies. Anti-CD42 (clone SZ2) was obtained from Immunotech (Marseilles, France), whereas anti-CD61, anti-CD62P, and PAC-1 were obtained from BD Bioscience PharMingen (San Jose, CA). Surface phosphatidylserine was measured with FITC-coupled annexin V (BD Bioscience PharMingen). Untreated PRP at Day 0 and after 5 and 14 days of storage was diluted with Buffer A to the final concentration of 3×10^5 PLTs per μL. The individual FITC-labeled antibodies were added to a final concentration of 5 μg per mL to untreated PRP or PRP activated with 0.1 U per mL thrombin. Subsequently, the samples were incubated at RT for 30 minutes, and diluted with 2 vol of PLT Buffer A, before the analysis, by flow cytometry on a flow cytometer (FACSCalibur, Becton Dickinson, Mountain View, CA).

Lectin binding to PLTs

The PLT surface β-GlcNAc or galactose content was determined by lectin binding with FITC-conjugated succinylated tritium vulgaris-wheat germ agglutinin (S-WGA; EY Laboratories Inc., San Mateo, CA) or FITC-conjugated ricinus communis I lectin (RCA I; EY Laboratories Inc.), respectively. Lectin analysis of PLTs was performed at Day 0 or after 5 or 14 days of RT or refrigerated storage. PLTs were isolated from PRP by centrifugation.[4] PLT counts were determined with a hemocytometer (Bright Line, Hausser Scientific, Horsham, PA) under a phase-contrast microscope at 400× magnification; the PLT count was adjusted to 0.3×10^7 per 100 μL of Buffer A and incubated with 0.1 μg per mL FITC-conjugated S-WGA or 0.1 μg per mL FITC-conjugated RCA I for 40 minutes at RT. PLT samples were immediately analyzed by flow cytometry. For each sample, 10,000 events were acquired.

In vitro PLT phagocytosis assay with stimulated THP-1 cells

Differentiated THP-1 phagocytic cells (1×10^6 cells/mL) were activated by the addition of 150 ng per mL phorbol 12-myristate 13-acetate for 15 minutes at 37°C, plated onto human albumin (1 mg/mL)-coated 24-well plates (1×10^6 cells/well), and allowed to adhere for 45 minutes at 37°C in RPMI.[7] Before incubation with THP-1 cells, PLTs were labeled with 1.8 μmol per L dye (CellTracker Orange CMTMR, Invitrogen, Carlsbad, CA), washed by centrifugation, and resuspended in Buffer A as described.[6] Labeled chilled-rewarmed or RT PLTs (5×10^8 cells/mL) were added to each well containing 10^6 differentiated THP-1 cells and allowed to incubate in Ca^{2+}- and Mg^{2+}-containing Hanks' balanced salt solution for 30 minutes at 37°C under gentle agitation in a horizontal shaker (Classic Series C24KC shaker, New Brunswick). The majority of surface associated PLTs were removed through digestion with 0.05 percent trypsin-ethylenediaminetetraacetate (Invitrogen), followed by the addition of trypsin inhibitors for 5 minutes. THP-1 cells were detached from the wells and incubated with a FITC-anti-CD61 monoclonal antibody, which recognizes the integrin β3 on the PLT surface, to exclude the surface-attached PLTs. PLT ingestion was determined and quantified by flow cytometry (FACSCalibur, Becton Dickinson). The percentage of phagocytes positive for CM-Orange fluorescence, when incubated with 22°C PLTs, was set to 1 to calculate the ratio of the phagocytic ingestion of the chilled PLTs.

PLT counts and pH measurement in PCs

PLT count was determined with a hemocytometer (Bright Line, Hausser Scientific) under a phase-contrast microscope at 400× magnification by two different observers, in duplicate. Furthermore, the PLT counts were verified with a flow cytometry with 5.5 μmol per L calibration fluorescent rainbow beads (Spherotech Inc., Libertyville, Illinois).[9] The pH of RT or refrigerated PCs was determined with pH indicator strips (colorpHast, pH 6.5-10.00, EMD Chemicals Inc., Gibbstown, NJ).

Flow chamber studies

PCs from whole blood were obtained following a routine standard preparation from the Rhode Island Blood Center. PCs were stored at 4°C for a period of 14 days. To test PLT function, 3 mL of PRP was removed from each PC, rewarmed for 1 hour at 37°C, and washed with modified Tyrode-HEPES buffer (137 mmol/L NaCl, 0.3 mmol/L Na_2HPO_4, 2 mmol/L KCl, 12 mmol/L $NaHCO_3$, 5 mmol/L HEPES, 5 mmol/L glucose, pH 7.3) containing 0.35 percent BSA and prostaglandin I_2 (Sigma-Aldrich). Washed PLTs were labeled with 2.5 μg per mL calcein (Invitrogen) and added to PLT-poor whole blood immediately before perfusion in a parallel-plate flow chamber system (calcein-labeled PLTs accounted for 70%-80% of all PLTs in the whole-blood sample). A sili-

cone gasket with a flow path height of 127 μm was placed between a flat perfusion chamber (Glycotech, Rockville, MD) and a 35-mm tissue culture dish coated with 200 μg per mL collagen Horm (Nycomed, Munich, Germany) for 1 hour at RT. Perfusion was carried out at a wall shear rate of 1000 per second for 4 minutes. PLT adhesion was visualized with an inverted microscope (Axiovert 135, Carl Zeiss MicroImaging Inc., Thornwood, NY) equipped with a 100-W HBO fluorescent lamp source (Optiquip, Highland Mills, NY) and a silicon-intensified tube camera (C 2400, Hamamatsu, Middlesex, NJ) connected to an S-VHS video recorder (AG-6730, Panasonic, Matsushita Electric, Tokyo, Japan). Images were analyzed with software (NIH Image 1.61, National Institutes of Health, Bethesda, MD).

RESULTS

Stable enzymatic galactosylation of refrigerated PCs

At least one form of β-1,4-galactosyltransferase is present on PLTs.[8] Thus, enzymatic galactosylation of PLT glycoproteins can be achieved, by adding the nucleotide-substrate donor UDP-galactose to washed PLTs. Under laboratory conditions, the addition of 200 μmol per L UDP-galactose to washed PLTs or freshly prepared PRP and incubation for 30 minutes at 37°C was sufficient to achieve an approximately twofold increase in RCA I binding.[8] In this study, we investigated whether galactosylation of PLTs is possible in PCs prepared for patient transfusion. Sterile-filtered UDP-galactose was injected directly into PCs, and galactosylation was monitored with the galactose-specific lectin (RCA I) and the β-GlcNAc-specific lectin (S-WGA). Enzymatic galactosylation of PCs was achieved after the addition of 800 μM UDP-galactose and incubation at 37°C for 40 minutes, as demonstrated by an approximately 1.6-fold increase in RCA I binding. Galactosylation is stable for more than 14 days as determined by the elevated RCA I lectin-profile of galactosylated PCs compared to control PLTs. In contrast, the RCA I binding profile of RT-stored PCs decreases slightly, suggesting a slow loss of surface galactose residues during storage (Fig. 1A). PLTs kept in a physiologic buffer and refrigerated for short periods (hours) have significantly elevated S-WGA binding profiles.[8] PCs require prolonged refrigeration (≥24 h, not shown) to develop elevated S-WGA binding profiles consistent with increased exposure and/or clustering of exposed β-GlcNAc residues on the PLT surface (Fig. 1B). Consistent with our previous results, PLTs treated with UDP-galactose before refrigeration fail to have increased S-WGA binding profiles after prolonged refrigeration, suggesting stable galactosylation (Fig. 1B). Furthermore, binding of the S-WGA lectin to RT-stored PLTs decreases slightly, suggesting a decrease in exposure of β-GlcNAc on the PLT surface. These results suggest that the enzymatic galactosylation of PCs remains stable following prolonged refrigeration.

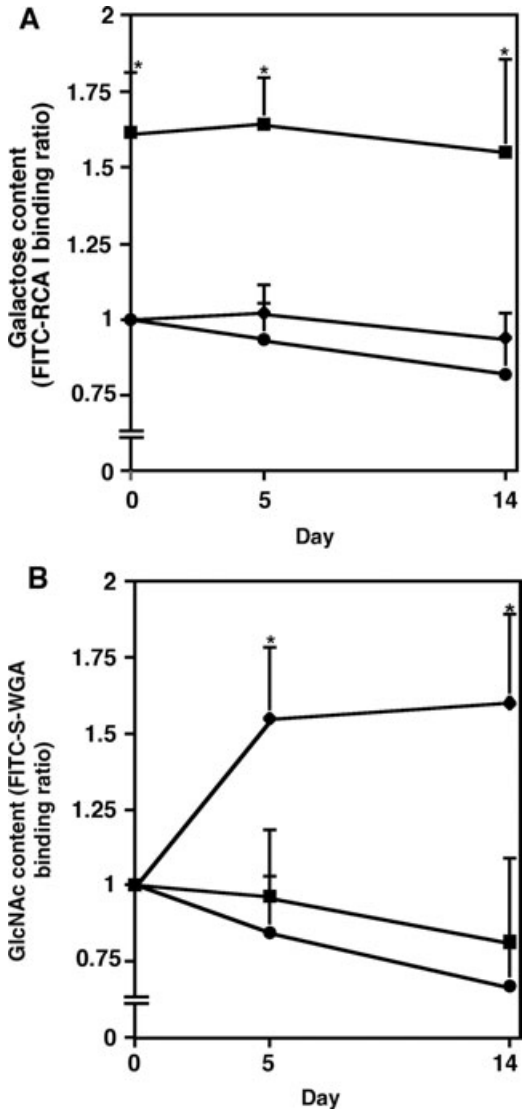

Fig. 1. Lectin binding to galactosylated PLTs in PCs. (A) RCA I binding; (B) S-WGA binding. Results represent the ratio of lectin binding to untreated or galactosylated PLTs at Days 0, 5, and 14. Values are mean ± SEM of four experiments. Statistical analysis of RCA I binding was performed with ANOVA followed by Tukey-Kramer multiple comparisons test. For all three time points, an asterisk indicates p values of less than 0.01 for the difference between refrigerated galactosylated PLTs (RGP) and either refrigerated PLTs (RP) or RT-stored PLTs (RTP). Statistical analysis of S-WGA binding was performed with Kruskal-Wallis test followed by Dunn's multiple comparisons test. In B, an asterisk indicates p values of less than 0.05 for the difference between RTP and RP. (■) RGP; (▲) RP; (●) RTP.

Galactosylation inhibits phagocytosis of refrigerated human PLTs by THP-1 macrophages

Refrigerated murine PLTs are cleared in vivo in the liver most likely through an interaction between GPIbα on PLTs surface with the lectin domain of integrin αMβ2 on macrophages. Galactosylation of transfused murine PLTs refrigerated for short periods (hours) prevents their clearance from the circulation.[7] Differentiated human monocytic THP-1 cells can be used as an in vitro model for refrigerated PLT clearance.[6] We therefore used differentiated human monocytic THP-1 cells to determine whether galactosylation of human PCs refrigerated for extended periods of time can prevent their phagocytosis. Prolonged RT storage of PCs leads to increased phagocytosis (Fig. 2). Phagocytosis of untreated refrigerated PLTs by THP-1 cells increases by approximately twofold after not more than 5 days of refrigeration and continues to increase by fourfold after 14 days of refrigeration when compared to the rate of phagocytosis of PLTs measured on the day PC was received from the blood bank (Fig. 2). In contrast, the phagocytic ratio of galactosylated PLT remains unchanged after 5 days of refrigeration, and although it increases by approximately twofold after 14 days of refrigeration (Fig. 2), it remains significantly lower than that of untreated refrigerated PLTs. These results indicate that galactosylation of refrigerated PLTs is stable and antagonizes PLT recognition and phagocytosis by THP-1 macrophages in vitro.

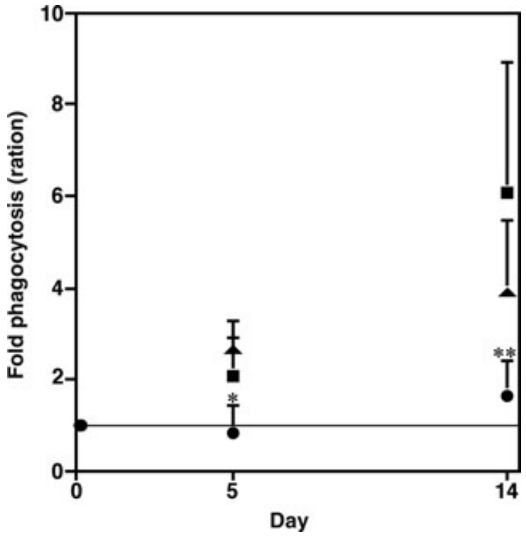

Fig. 2. Effect of galactosylation on the ingestion of PLTs by THP-1 macrophages. The results are normalized for the mean number of CM-Orange–positive THP-1 macrophages after incubation with fresh PLTs. Data are mean ± SEM of four experiments. Statistical analysis for Day 5 was performed with ANOVA followed by Tukey-Kramer multiple comparisons test. *A p value of less than 0.01 for difference between RP and RGP and a p value of less than 0.05 for difference between RTP and RGP. Statistical analysis for Day 14 was performed with Kruskal-Wallis test followed by Dunn's multiple comparisons test. **A p value of 0.01 for difference between RTP and RGP. (■) RTP; (▲) RP; (●) RGP.

PLT counts and pH remain stable in refrigerated PCs

A decrease in PLT count, or alterations in pH values below 6.2 or above 7.6, have been shown to significantly diminish PLT transfusion efficiency. We determined PLT counts and the pH of PCs stored not more than 14 days at RT or the counts and pH of galactosylated and untreated PCs maintained at 4°C. Independent of the storage condition, PLT counts, as well as the pH, remain unchanged after 5 days. PLT numbers significantly decrease after 14 days of storage under all tested condtions (Fig. 3A). However, the decline in PLT count is most prominent in RT PLTs. Refrigeration maintains pH within a PLT bag for 14 days (Fig. 3B). Finally, galactosylation does not have a significant effect on either PLT count or pH.

Refrigeration protects PLT agonist response in vitro

To evaluate the effect of RT, refrigeration, and galactosylation and refrigeration on in vitro function of stored human PLTs, we assessed the ability of PLTs from each concentrate to aggregate in the presence of thrombin or ADP and to agglutinate in the presence of ristocetin. Independent of the storage conditions, PLTs activation responses decrease after more than 5 days of storage. In all cases, refrigerated PLTs maintain agglutination and aggregation responses better than RT-stored PCs, particularly after the stimulation with thrombin and ristocetin (Fig. 4). In addition, PLT galactosylation significantly improves the agglutination induced by ristocetin when compared with untreated refrigerated PLTs, RT PLTs at Day 5, or after 2 weeks of storage (Fig. 4C). PLTs stored at RT for 2 weeks aggregated or agglutinated to the agonists poorly.

The ability of PLTs to change shape is preserved by refrigeration

From the standpoint of survival and hemostatic function, the ability of activated PLTs to change shape may be of greater importance than the preservation of discoid shape at rest. At sites of vessel injury, PLTs undergo a complex shape change that includes an initial rounding of the disc to a sphere, followed within seconds by the extension of filopodia and circumferential spreading, suited to cover

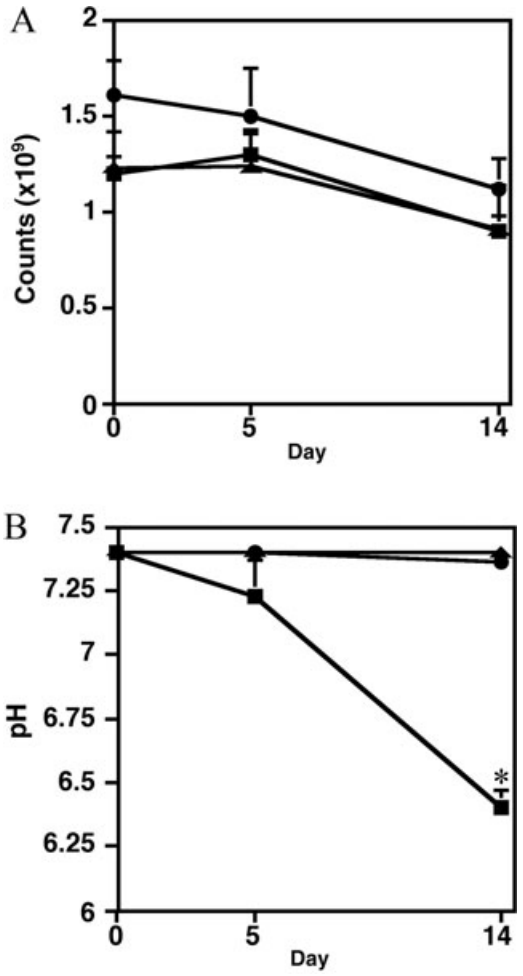

Fig. 3. Impact of storage conditions on PLT count and pH. (A) PLT count; (B) pH of PCs. Data shown are mean ± SEM from four to six experiments. Statistical analysis of data was performed with Kruskal-Wallis test followed by Dunn's multiple comparisons test. *A p value of less than 0.01 for difference between RTP and RP and for the difference between RTP and RGP. (■) RTP; (▲) RP; (●) RGP.

maximum surface area on damaged vessel surfaces. Galactosylated, or untreated refrigerated, PCs retained their abilities to extend lamellae and filopodia as observed by phase contrast microscopy after 14 days of storage (Figs. 5F, 5G, respectively). PLTs stored for 5 days at RT

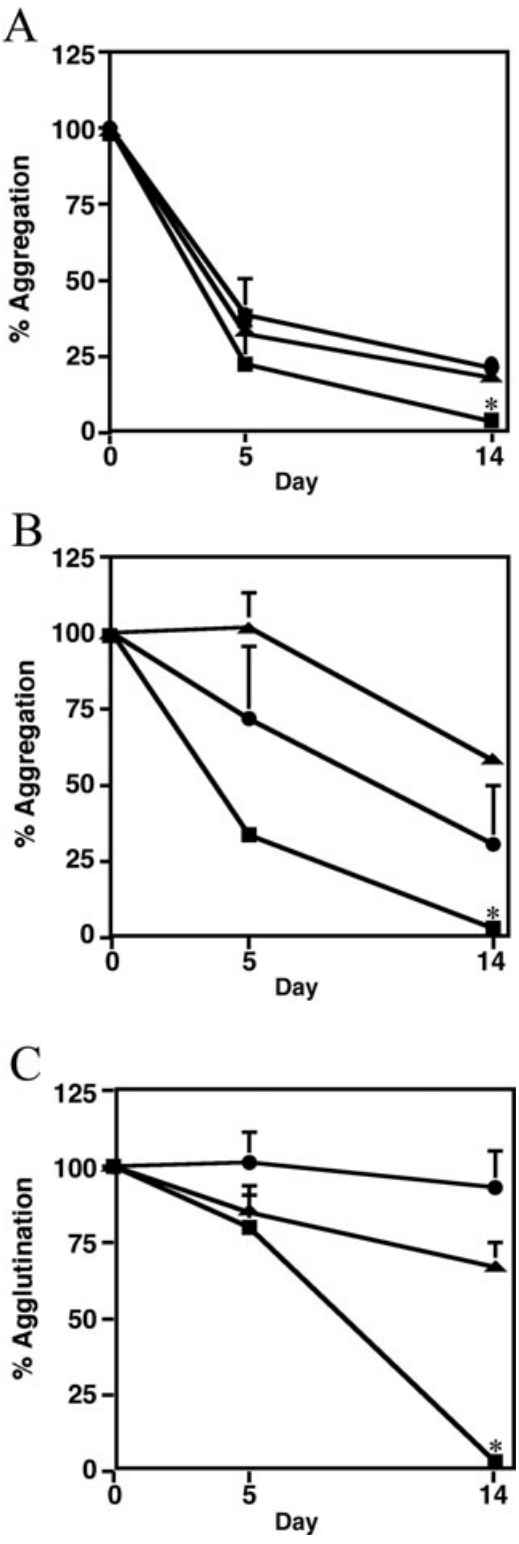

Fig. 4. Effect of PLT storage conditions on the aggregation and agglutination of PLTs. The PLTs were stimulated with 10 µmol per L ADP (A), 0.1 U per mL thrombin (B), or 1.5 mg ristocetin (C) at 37°C. The data are the mean ± SEM of four experiments. Statistical analysis of the response to each agonist was performed with Kruskal-Wallis test followed by Dunn's multiple comparisons test. In A, an asterisk indicates a p value of less than 0.05 for the difference between RTP and RP. In B, an asterisk indicates a p value of less than 0.05 for the difference between RTP and RP. In C, an asterisk represents a p value of less than 0.05 for the difference between RTP and RGP and a p value of less than 0.01 for the difference between RTP and RGP. (■) RTP; (▲) RP; (●) RGP.

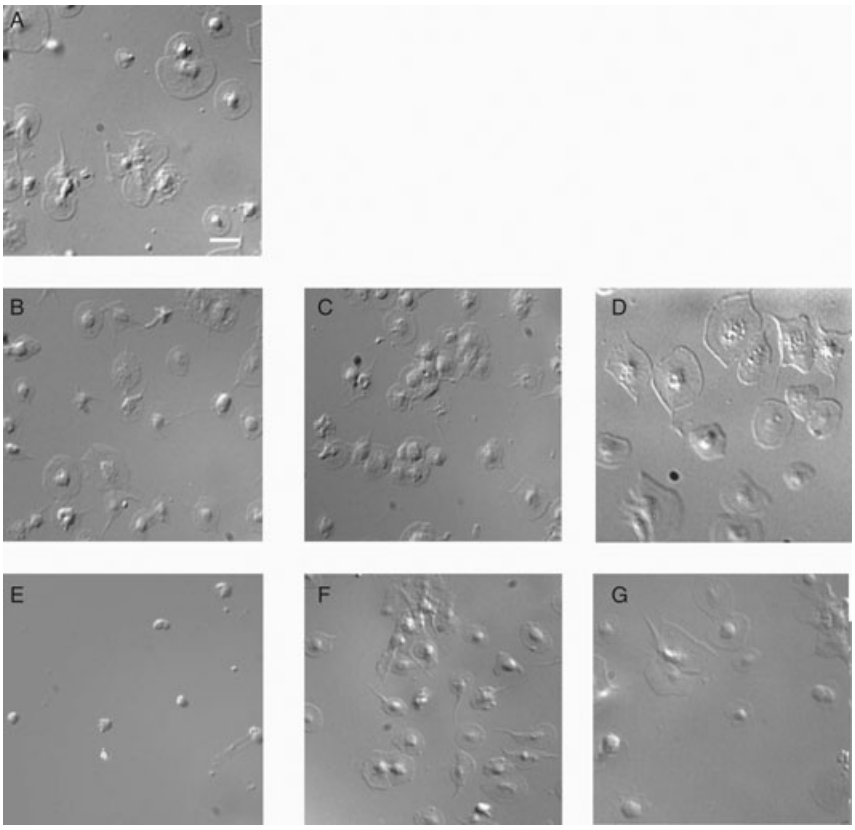

Fig. 5. Morphology of stored PLTs after adhesion and incubation on a glass surface. (A) Fresh PLTs (Day 0, 22°C); (B) 5-day RTP (Day 5, 22°C); (C) 5-day RP (Day 5, 4°C); (D) 5-day RGP (Day 5, 4°C plus UDP-galactose); (E) 14-day RTP (Day 14, 22°C); (F) 14-day RP (Day 14, 4°C); (G) 14-day RGP (Day 14, 4°C plus UDP-galactose). Bar, 10 μm.

also retain their ability to spread on glass (Fig. 5B), but after 2 weeks of storage, fail to spread (Fig. 5E). In addition, galactosylated and refrigerated or untreated refrigerated PLTs do not form increased microaggregates after long-term storage when compared to the RT control (not shown).

Prolonged refrigeration of PLTs does not impair their ability to adhere to collagen under flow

Thrombus formation on collagen surfaces is a multistep process that requires ligand binding to PLT receptors such as GPIbα, GPVI, and integrin αIIbβ3.[10] We evaluated the ability of PLTs derived from PC to form thrombi in vitro during perfusion over collagen-coated surfaces. PLTs, in PC released by blood banks and analyzed immediately on arrival in the laboratory, adhered approximately 50 percent less well to the collagen-coated surface when compared to freshly drawn PLTs prepared under laboratory conditions (Fig. 6, Day 0). Refrigeration of PCs for 5 or 14 days, however, did not impair further the ability of the PLTs to adhere to collagen surfaces, indicating that extended PLT storage in the cold does not grossly diminish their hemostatic function (Fig. 6).

Refrigeration of PCs induces P-selectin exposure but diminishes the loss of GPIbα

We evaluated PLT activation, measured by flow cytometric analysis of P-selectin expression and PAC-1 binding, as well as the effect of storage on GPIbα, αIIbβ3, and phosphatidylserine expression levels after refrigeration or RT storage. Approximately 50 percent of PLTs in freshly received PCs have an activated integrin αIIbβ3 as judged by PAC-1 binding (Table 1). In addition, approximately 3.5 percent of freshly received PLTs bind annexin V (Table 1). After 1 U per mL thrombin activation, fresh PCs became approximately 92 percent positive for PAC-1. PCs stored at RT for 5 or 14 days are approximately 58 or approximately 80 percent positive for PAC-1, refrigerated PCs are approximately 65 or approximately 70 percent positive for PAC-1 after 5 or 14 days of storage, respectively. PC RT storage for 14 days diminishes PAC-1 binding by PLTs to approximately 75 percent after 1 U per mL thrombin activation, whereas activated PLTs from PCs refrigerated for 2 weeks are approximately 85 percent for PAC-1. The percentage of PLTs in PCs that are positive for P-selectin staining increases from approximately 22.4 percent at Day 0 to approximately 33 percent after 5 days of storage and increases to approximately 65.1 percent after 2 weeks of RT storage. The percentage of P-selectin-positive PLTs from PCs refrigerated for 5 or 14 days is approximately 65.7 or 75.3 percent, respectively, independent of the galactosylation (Table 1). After storage for 5 days and independent of the storage conditions, we detected no significant loss of β3 from the PLT surfaces. Prolonged PLT storage at RT did, however, lead to significantly lower surface expression of GPIbα compared to refrigerated PLTs. Phosphatidylserine surface expression increased in all PCs after 5 days, but was slightly higher in refrigerated PCs, approximately 11.4 ± 2.9 to 12.0 ± 5.3 percent versus 7.4 ± 2.4 percent from RT-stored PLTs. This trend reversed after 14 days of storage as PLTs from RT PCs bound approximately twofold more annexin V (53.7 ± 14.6%) than refrigerated PCs (27.2 ± 5.1%-28.9 ± 11.5%).

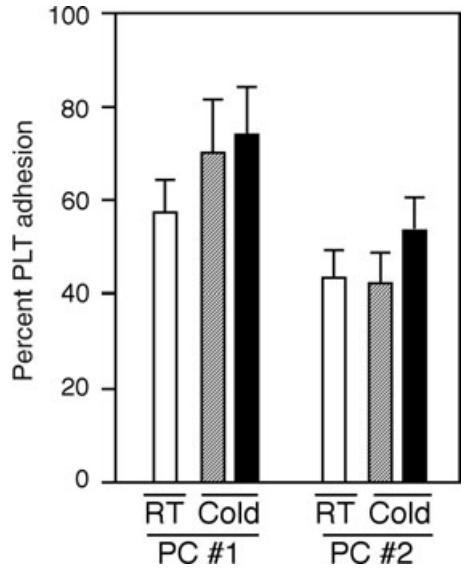

Fig. 6. Effect of storage temperature on the adhesion of PLTs to collagen-coated surfaces under flow. Data are expressed as fraction of fresh adherent PLTs relative to adherent PLTs from PCs. Values are mean ± SEM of three experiments for each PC. (□) Values of Day 0 PCs, (▨) values on PCs refrigerated for 5 days, and (■) values on PCs refrigerated for 14 days.

DISCUSSION

We have previously described a method for galactosylation of murine and human PLTs prepared under laboratory conditions[6] that inhibits phagocytosis of murine PLTs chilled for short periods (<4 hr) and prolongs their circulation in vivo. Here, we evaluated if PLT galactosylation was possible in PCs procured under standard blood banking conditions. The level and stability of galactosylation was assessed with lectins with known carbohydrate specificities. Binding of RCA I, a lectin that binds to terminal β-galactose residues was increased by approximately 1.6-fold after galactosylation with 800 µmol per L UDP-galactose, a concentration 4-fold higher than that required to achieve galactosylation of washed human PLTs. The extent of galactosylation of human PLTs in concentrates was sufficient to inhibit their phagocytosis in vitro after chilling for 14 days and to prevent the increase of S-WGA lectin binding, a surrogate marker for the αMβ2 lectin domain.

What changes affect PLT circulation after transfusion? Refrigeration causes PLTs to round up and rapidly lose their discoid shape. Others and we have previously demonstrated[6,8,11,12] that discoid shape per se is a poor predictor of PLT circulation. Here we describe that P-selectin level on the PLT surface increased with prolonged storage in the cold independent of galactosylation (Table 1).

TABLE 1. Flow cytometric analysis of surface expression of PLT glycoproteins (CD42b), CD61, activation markers (PAC-1 and P-selectin), and phosphatidylserine exposure with annexin V*

	Day 0			Day 5			Day 14		
Surface phenotype	RT	4°C plus UDP-galactose	4°C	RT	4°C plus UDP-galactose	4°C	RT	4°C plus UDP-galactose	4°C
GPIbα (CD42b) (MFI)	163.8 ± 18.4	165.7 ± 10.1	154.3 ± 20.4	174.9 ± 17.1	124.6 ± 9.6	129.5 ± 24.7	40.4 ± 7.0*	77.1 ± 10.5	77.2 ± 5.3
CD62P (%)	22.4 ± 8.0	28.1 ± 5.7	25.9 ± 6.4	33 ± 8.7	60.8 ± 4.6	65.7 ± 5.9	65.1 ± 6.4	76.3 ± 2.1	75.3 ± 3.7
β3 CD61 (%)	99.5 ± 0.1	99.2 ± 0.4	99.7 ± 0.01	99.1 ± 0.2	99.5 ± 0.1	99.5 ± 0.1	98.1 ± 1.3	98.7 ± 0.3	97.9 ± 1.5
PAC-1 no thrombin (%)	53.4 ± 13.7	51.6 ± 7.0	52.6 ± 14.2	58.6 ± 4.6	64.3 ± 5.9	64.3 ± 7.7	82.3 ± 6.0	72.2 ± 6.8	71.7 ± 6.6
PAC-1 plus thrombin (%)	92.9 ± 3.5	91.2 ± 2.9	91.5 ± 6.4	89.9 ± 5.9	95.7 ± 0.8	94.3 ± 3.4	75.1 ± 8.2	83.8 ± 2.2	86.4 ± 1.5
Annexin V (%)	3.14 ± 1.5	3.7 ± 1.2	3.7 ± 1.6	7.4 ± 2.4	11.4 ± 2.9	12 ± 5.3	53.7 ± 14.6	27.2 ± 5.1	28.9 ± 11.5

* Data for CD42b expression are presented as the mean fluorescence intensity (MFI) of PLTs labeled with specific antibodies and compared with the appropriate immunoglobulin G control. Data for CD61, PAC-1, CD62P, and annexin V expression are presented as percentage of positive cells compared with the appropriate controls. PAC-1 no thrombin = measurement of intensity of PAC-1 staining without added thrombin. PAC-1 + thrombin = measurement of intensity of PAC-1 staining in the presence of 0.1 U per mL thrombin. Values given as mean ± SEM of four to five experiments. Statistical analysis of CD42b expression was performed with ANOVA followed by Tukey-Kramer multiple comparisons test.

* $p < 0.05$ for the difference between RT PCs (RT) and refrigerated PCs (4°C) or refrigerated glycosylated PCs (4°C plus UDP-galactose).

Increase in P-selectin expression in refrigerated PLTs or refrigerated galactosylated PLTs during storage, however, did not result in increased phagocytosis by differentiated THP-1 cells. This is not unexpected because thrombin activation of PLTs, a treatment leading to PLT shape change and P-selectin expression, does not impair PLT survival.[12,13] PLTs stored at RT storage also progressively increase phosphatidylserine exposure,[14,15] a parameter that in several studies has been correlated with PLT clearance in vivo.[16,17] Induction of PLT apoptosis by carbonyl cyanide *m*-chlorophenylhydrazone, however, led to PLT clearance without significant induction of phosphatidylserine exposure.[18] We found that in PCs, refrigerated or maintained at RT for 5 days, phosphatidylserine expression increased by approximately 3.0- or by 2.3-fold, respectively. After 14 days of RT PLT storage, phosphatidylserine exposure increased by approximately 17-fold. The refrigeration of PCs offered some protection against phosphatidylserine exposure as it increased by approximately 7-fold. The ratio of phagocytosis of 5-day-old RT-maintained PLTs increases by approximately 2-fold, a fact that could be attributed to the increased phosphatidylserine levels. A similar elevated phosphatidylserine exposure in 5-day refrigerated and galactosylated PLTs, however, did not induce in vitro phagocytosis (Fig. 2). After long-term storage, PLTs are phagocytized more efficiently, which correlates with phosphatidylserine values.

The evaluation of the PLT function in vivo is difficult because in vitro assays do not reliably predict in vivo PLT circulation or hemostatic function. Refrigerated, or galactosylated and refrigerated, PLTs in concentrates, despite losing their discoid shape, retain their ability to extend lamellae and filopodia and to adhere on collagen-coated surfaces under blood flow conditions. Our in vitro functional analysis of PLTs shows that simple refrigeration, with or without prior galactosylation, best preserves PLT responses to agonists in vitro. Galactosylation and refrigeration has an additional protective effect on PLT function: maximally preserving GPIbα surface expression and optimizing agglutination in response to ristocetin, compared with nongalactosylated refrigerated PLTs. In contrast, RT-stored PLTs lose their ability to spread, to aggregate, and to agglutinate after 14 days of storage. Therefore, these PLTs would be expected to function less efficiently once transfused.

The clinical relevance of our findings reported here remains to be established. Although we have demonstrated that galactosylation can preserve the circulation of PLTs stored for short periods under refrigeration, refrigeration of PLTs in PLT-rich plasma does not result in accelerated clearance until at least 8 hours of storage time.[19] It is therefore possible that a different clearance mechanism might operate after longer-term storage in plasma.

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