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The platelet hyporeactivity of extremely low birth weight neonates is age-dependent

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ABSTRACT

Introduction: We have previously demonstrated that, as compared to adults, the platelets of extremely low birth weight (ELBW) neonates are markedly hyporeactive on day 0 – 1 of life. The purpose of this study was to examine the age dependency of this hyporeactivity.
Materials and Methods: On days 0 – 1, 3 – 4, and 10 – 14, peripheral blood was collected from 14 stable ELBW neonates and compared to peripheral blood from normal adults run in parallel. Whole blood flow cytometry was used to examine the activation-dependent increase in platelet surface P-selectin (reflecting degranulation) and platelet surface binding of factor V/Va (reflecting platelet surface procoagulant activity) and decrease in platelet surface glycoprotein (GP) Ib (the von Willebrand factor receptor).
Results: In the physiologic milieu of whole blood, ELBW neonatal platelets on days 0 – 1 and 3 – 4 were markedly less reactive than adult platelets. However, by day 10 – 14, the platelet function of ELBW neonates improved significantly, although not completely to adult levels.
Conclusions: The age-dependent platelet hyporeactivity of ELBW neonates demonstrated in this study may be a contributing factor to the similar age-dependent propensity of ELBW neonates to intraventricular hemorrhage.

The development of the hemostatic system is incomplete at the time of birth. Delayed maturation has been documented for all the major hemostatic components: platelets [1], the coagulation system [2], and the fibrinolytic system [3]. Platelet reactivity has been shown to be relatively decreased in term and preterm neonates [1,4–6]. Extremely low birth weight (ELBW) neonates are a group of particular interest in this regard because, unlike term neonates, they have a propensity for serious intraventricular hemorrhage [7]. We [5,6] and others [8] have previously demonstrated that, as compared to adults, the platelets of ELBW neonatal are markedly hyporeactive on the first day of life. The mechanism of this platelet hyporeactivity appears to be, at least in part, the result of an impaired receptor-mediated signal transduction defect [1]. However, the age-dependency of this phenomenon in ELBW neonates is unknown. In this study, we therefore examined, in individual ELBW neonates, the relationship between age and platelet reactivity.

Materials and methods

Study population

The study was approved by the Human Subjects Committee of the University of Massachusetts Medical School. Parents of the neonates and the adult control volunteers (n = 12) gave written informed consent for their participation in the study. Clinically stable ELBW neonates (n = 14) were defined as: birth weight <1 kg, gestation <30 weeks, Apgar score >4 at 5 minutes, and no known congenital or acquired disorder. No mother or baby had been given aspirin or any other anti-inflammatory agents (including indomethacin). Neonates were excluded from analysis if their mothers had any of the following: diabetes, hypertension, preeclampsia, eclampsia, HIV seropositivity, hemostatic disorder, active infection, drug or alcohol abuse. No neonate had a platelet count outside the normal range (150 – 450 × 10^9/L). Cranial ultrasounds performed on days 0 – 1, 3 – 4, and 10 – 14 were normal in all neonates. Adult normal volunteers were excluded from participation if, within the previous 10 days, they had ingested medications known to affect platelet function. No adult volunteer had a history suggestive of abnormal hemostasis or medical conditions such as coronary artery disease, diabetes, or renal disease.

Blood sampling

On days 0 – 1, 3 – 4, and 10 – 14, peripheral venous blood was drawn by venipuncture through a 21 gauge needle from ELBW neonates. The

Abbreviations: ELBW, extremely low birth weight; FITC, fluorescein isothiocyanate; GP, glycoprotein; PE, phycoerythrin.

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blood was collected at each of the 3 age intervals for all 14 ELBW neonates (*i.e.*, the same neonate was sampled 3 times). Normal adult donor blood was drawn by venipuncture through a 21 gauge needle from the same adult donor in parallel with each of the 3 blood samples from ELBW neonates. The normal adult donors were randomly selected and assigned, and were not a relative of the neonate. In both neonates and adults, the first 0.5 mL of collected blood was discarded before the collection of 0.5 mL of blood directly into 3.8% sodium citrate.

Whole blood flow cytometry

Neonatal and adult samples were drawn and processed at the same time, and this was consistent between age samples. Anticoagulated blood was processed between 20 and 30 minutes after the blood draw – a differential time frame that does not alter platelet surface P-selectin, platelet surface GPIb, or platelet surface factor V/Va binding. Agonists were selected based on our previous studies [5,6], because the specific goal of the present study was to determine whether the previously-demonstrated [5,6] platelet hyporeactivity of low birth weight neonates is age-dependent.

Platelet surface P-selectin and GPIb

The method has been described previously [5]. One hundred μL of citrated whole blood was incubated with 100 μL of the peptide gly-pro-arg-pro (GPRP) (Calbiochem, San Diego, CA) (final concentration 2 mM) to inhibit fibrin polymerization and platelet aggregation [9,10]. The platelets in 20 μL of this diluted whole blood were incubated undisturbed (to prevent platelet aggregation) for 15 min at 22 °C with 20 μL of either the stable thromboxane A_2 analog U44619 10 μM (Cayman Chemical, Ann Arbor, MI), the combination of ADP 20 μM (BioData, Hatboro, PA) and epinephrine 20 μM (Sigma, St. Louis, MO), or modified Tyrode's buffer only. Activation was stopped by fixation in 1% formaldehyde (22 °C, 30 min). The samples were then diluted 10-fold in modified Tyrode's buffer and stored at 4 °C (for a maximum of 18 h) before labeling with monoclonal antibodies. The samples (20 μL) were incubated (22 °C, 15 min) with 5 μL of a mixture of a saturating concentration of either biotinylated P-selectin-specific monoclonal antibody S12 (Centocor, Malvern, PA) or biotinylated GPIb-specific monoclonal antibody 6D1 (provided by Dr. Barry S. Coller, Rockefeller University, New York, NY) and of a near saturating concentration of fluorescein isothiocyanate (FITC)-conjugated GPIIIa-specific monoclonal antibody Y2/51 (DAKO, Carpinteria, CA), followed by an incubation (22 °C, 15 min) with phycoerythrin (PE)-streptavidin (3.5 μL) (Jackson ImmunoResearch, West Grove, PA). Samples were diluted 10-fold with modified Tyrode's buffer and analyzed within 24 h in a Coulter EPICS XL flow cytometer (Beckman Coulter, Miami, FL). The flow cytometer was equipped with a 500 mW argon laser (Cyonics, San Jose, CA) operated at 15 mW and a wavelength of 488 nm. After identification of platelets by gating on both FITC positivity and log forward and side light scatter, binding of the biotinylated antibody was determined by analyzing 5,000 individual platelets for PE fluorescence. As previously described [11,12], platelet surface GPIb was expressed as mean fluorescence intensity (MFI) and background binding, obtained from parallel samples with purified biotinylated mouse IgG (Calbiochem), was subtracted from each test sample. As previously described [11,12], platelet surface P-selectin was analyzed as percent positive platelets, without subtraction of the isotype control. As previously described [13,14], the above methods of fixation result in no significant differences in fluorescence intensity between samples analyzed immediately and samples analyzed within 24 h.

Platelet surface binding of factor V/Va

The method has been described previously [6]. An aliquot of 300 μL of citrate anticoagulated blood was microcentrifuged (13,000 g, 2 min) to prepare autologous plasma. Twenty-four μL of the citrated whole blood sample was diluted with 72 μL of GPRP (final concentration 2.5 mM) (to inhibit fibrin polymerization and platelet aggregation [9,10]) and 48 μL of autologous plasma (to provide adequate concentrations of coagulation factors). The samples were then incubated (37 °C, 10 min) in HEPES-Tyrode's buffer (137 mM NaCl, 2.8 mM KCl, 1 mM $MgCl_2$, 12 mM $NaHCO_3$, 0.4 mM Na_2HPO_4, 0.35% bovine serum albumin, 10 mM HEPES, 5.5 mM glucose, pH 7.4) with either calcium ionophore A23187 80 μM (Calbiochem) and $CaCl_2$ 3 mM or HEPES-Tyrode's buffer alone. The samples were then incubated (22 °C, 15 min) with a near saturating concentration of PE-conjugated CD41-specific monoclonal antibody (DAKO) together with either a saturating concentration of the FITC-conjugated [10] factor V/Va-specific [15] IgG_1 monoclonal antibody V237 (American Diagnostica, Greenwich, CT) or FITC-conjugated mouse IgG_1 isotypic control (Boehringer Mannheim, Indianapolis, IN). Samples were then fixed for 20 min at 22 °C with ultrapure EM grade formaldehyde (Polysciences, Warrington, PA) 1% final concentration, and then diluted 27-fold in HEPES-Tyrode's buffer. All samples were analyzed within 2 h in the Coulter EPICS XL flow cytometer described above. Platelets were identified by PE-CD41-positivity and log forward and side light scatter. Procoagulant activity was determined by the binding of the FITC-conjugated, factor V/Va-specific monoclonal antibody V237. The FITC and PE fluorescence was detected using 525 nm and 575 nm band pass filters, respectively. In each assay, 10,000 individual platelets (and/or platelet-derived microparticles) were analyzed. Background binding, obtained from the samples with FITC-conjugated mouse IgG_1, was subtracted from each test sample.

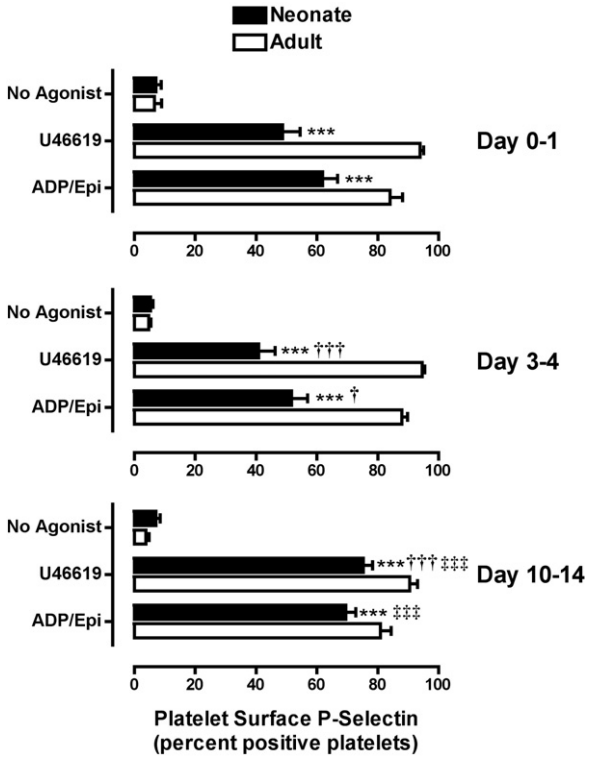

Fig. 1. Platelet surface P-selectin in ELBW neonates *vs.* adults on days 0 – 1, 3 – 4, and 10 – 14. In a whole blood flow cytometric assay, platelet surface P-selectin was determined in the presence of U46619 10 μM (a stable thromboxane A_2 analog), the combination of ADP 20 μM and epinephrine 20 μM, or no agonist. Citrated whole blood was obtained from 14 ELBW neonates (solid bars) and 12 adult controls (open bars). Data are expressed as percent of platelets that were P-selectin-positive (mean ± SEM). Data were analyzed using repeated measures ANOVA with Newman-Keuls multiple comparison post-tests. *** indicates <0.001 for ELBW neonates *vs.* adult controls under same conditions. † and ††† indicate $p<0.05$ and <0.001, respectively, for results at day 3 – 4 or day 10 – 14 vs. day 0 – 1. ‡‡‡ indicates $p<0.001$ for results at day 10 – 14 vs. day 3 – 4.