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Microparticles in newborn cord blood: Slight elevation after normal delivery

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ABSTRACT

Introduction: Microparticles formed during delivery may add to the well functioning hemostasis, but also to hypercoaguability in the newborn.
We wanted to investigate whether microparticles in newborn cord plasma differ from those in adult plasma in terms of concentration, procoagulant activity, and effect on thrombin generation.
Materials and Methods: Three different techniques were used to analyze microparticles. To enumerate and characterize microparticles, flow cytometry and ELISA, based on the prothrombinase reaction, were used. The effect of microparticles derived tissue factor on thrombin generation was measured indirectly by Calibrated Automated Thrombography in newborn cord and adult platelet free plasma.
Results: The flow cytometric measurements of microparticles showed no significantly increased microparticle concentration in newborn cord compared with adult plasma. By the use of ELISA a significantly increased procoagulant activity of microparticles was found in newborn cord plasma as compared to adult plasma. Initiation of thrombin generation by adding phospholipids alone suggested a higher microparticle activity in newborn cord plasma than in adult plasma.
Conclusions: Our results show a higher impact of microparticles on the hemostatic system in newborn cord plasma than in adult plasma in terms of activity, but not concentration. Calibrated Automated Thrombography and ELISA suggest an increased microparticle activity in newborn cord plasma, but comparable results in microparticle number as determined by flow cytometry argue against strong platelet activation during birth.

Introduction

Despite physiological low levels of some coagulation factors and a poor platelet function in vitro, newborns have an excellent hemostasis and wound healing. Healthy newborns show no easy bruising or bleeding during surgery. In contrast to the poor platelet function in vitro, bleeding times [1,2] and PFA-100 closure times are even shorter in newborns than in adults [3,4]. Increased amounts of ultralarge von Willebrand factor [5,6], increased hematocrit values and higher white blood cell counts in newborn plasma might in part explain these laboratory results and the well functioning primary hemostasis in newborns.

In newborn blood lower concentrations of clotting factors are present, especially of those synthesized in the liver, most of which are vitamin K dependent. This is reflected in a prolonged activated partial thromboplastin time and prothrombin time [7,8]. It was found that the ability to generate thrombin is decreased in healthy newborns [9]. Newborns have a lower endogenous thrombin potential (ETP) and lower peak levels than adults, when continuous thrombin generation (TG) is measured, but lag time is short when low concentrations of tissue factor (TF) are used to trigger the coagulation process [12]. The lower TG can be explained by the low amounts of prothrombin which is together with antithrombin (AT) the most limiting factor for the ETP. Low levels of inhibitors seem to be very important in the newborn hemostasis. Low AT and tissue factor pathway inhibitor (TFPI) allow sufficient TG despite low concentrations of clotting factors [10–13].

Platelets are necessary in primary but also in secondary hemostasis. Many coagulation factors are bound on their surface where the formation of the prothrombinase- and tenase-complex take place, which convert prothrombin into thrombin. These reactions need negatively charged phospholipids as cofactors for complex formation. Platelet activation is crucial, since it involves the flip flop reaction which causes exposure of negatively charged phospholipids on the platelet membrane. Compared with adult controls newborn platelets are hyporeactive to physiological agonists in vitro, resulting in decreased platelet activation and aggregation [14–18]. These data suggest that the hyporeactivity of newborn platelets is at least in part a

Abbreviations: AMC, Amino-methyl-coumarin; AT, Antithrombin; BSA, bovine serum albumin; CAT, Calibrated Automated Thrombography; DMSO, dimethylsulfoxide; ETP, endogenous thrombin potential; FACS, fluorescence-activated cell sorting; FITC, fluorescein isothiocyanate; MPs, microparticles; PFP, platelet free plasma; TG, thrombin generation; TF, tissue factor; TFPI, tissue factor pathway inhibitor.

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relative defect in a shared signal transduction pathway [16,17]. The expression of negatively charged phospholipids is very important for initiation and propagation of TG. Therefore, we recently investigated whether newborn and adult platelet membranes differ in their lipid composition and exposure. However, we did not find a difference between the newborn and adult platelet membrane in their lipid-exposure, composition, and support of TG [19].

Another rich source for phosphoplipids are microparticles (MPs). MPs are microvesicles which are released from the plasma membrane of many eukaryotic cells (leukocytes, endothelial cells, red cells and platelets). They are heterogenous in size and, therefore, many different sizing definitions, from 0.2-2 μm, exist in the literature, but <1 μm is the definition most often used [20–24]. MPs are generated upon cell activation or apoptosis and bear surface antigens reflecting their cells of origin [21,22,25,26]. The distribution of phospholipids is a well regulated process. In resting cells phosphatidylserine and phosphatidylethanolamine are located on the inner membrane layer while phosphatidylcholine and sphingomyelin are located on the external membrane layer [27]. Upon activation of platelets due to increased levels of cytosolic calcium, this lipid asymmetry is lost, resulting in exposure of phosphatidylserine on the outer cell membrane [28]. MP release is an integral part of the membrane flip flop mechanism, and closely associated with the redistribution of phospholipids [29]. MPs have been implicated to play a direct and indirect role in the complex coagulation process [21,22,25]. In particular, elevated numbers of MPs have been described in several diseases associated with thrombembolic complications [30–34]. An inherited defect of lipid scramblase was found in patients with Scott syndrome [35], a rare isolated defect of platelets coagulant activity [36]. These patients show a reduced production of MPs [37] as well as impaired phosphatidylserine expression [38], resulting in severe bleeding. This syndrome underscores the important role of MPs in a normally functioning hemostasis.

MPs may be found in increased numbers after birth. Platelets may be activated during the stress of delivery but so far no evidence for platelet activation is published. Newborns seem to be hypercoaguable after delivery, both thrombin-antithrombin complexes and D-dimer are significantly elevated in newborns after birth suggesting activation of the coagulation system in newborns [39]. Among children newborns are the largest group developing thrombembolic events [40].

Several studies on MPs in newborns have been published over the last decade. Flow cytometry was used to evaluate number and activity of MPs. Methods range from platelet activation with calcium ionophores following flow cytometric measurements for MPs in whole blood [41] to comparison of platelet derived MPs and platelet counts in preterm newborns [42]. However, none of these publications include MP sensitive functional methods to evaluate the impact on prothrombinase reaction and resulting thrombin generation.

In this study we investigated if newborn cord plasma compared with adult plasma contains higher levels of MPs which add to the hypercoaguable state but also may contribute to the well functioning hemostasis in newborns. We used three different methods to analyze MPs: The MP concentration was measured by the use of flow cytometry, MPs procoagulant activity by means of their catalytic surface was measured by the use of ELISA, and the dependency of TG on MPs derived TF was determined by means of Calibrated Automated Thrombography (CAT).

Materials and Methods

Reagents

For flow cytometry measurements fluorescein isothiocyanate (FITC) labeled Annexin V and Annexin V Binding Buffer 10 X concentrate, containing 0.1 M HEPES (pH 7.4), 1.4 M NaCl, and 25 mM $CaCl_2$ solution, were both purchased from BD Biosciences (San Jose, CA, USA). Syringe filters with 0,8 μm Versapor® membrane were purchased from Pall (East Hills, NY, USA). Trucount™ Tubes were obtained from BD Biosciences (San Jose, CA, USA). Microparticle size standard, 1 μm, was purchased from Fluka (Sigma-Aldrich, Buchs, Switzerland).

The ZYMUPHEN MP-Activity kit was purchased from HYPHEN BioMed (ZAC Neuville Universite, France). Measurements of TG were performed by use of a Thrombin Calibrator, platelet poor plasma (PPP) reagent with a content of 5 pM TF and 4 μM phospholipids as well as TF free MP reagent with a phospholipid content of 4 μM phospholipids, which were all purchased from Thrombinoscope BV (Maastricht, The Netherlands). The fluorogenic substrate Z-Gly-Gly-Arg-amino-methyl-coumarin (AMC), purchased from Bachem (Bubendorf, Switzerland) was solubilized in pure dimethylsulfoxide (DMSO) purchased from Sigma. Fluobuffer consisted of 60 mg/ml bovine serum albumin (BSA) and 20 mM HEPES, which were both obtained from Sigma. Calcium chloride was purchased from Merck (Darmstadt, Germany).

Blood collection and preparation of platelet free plasma (PFP)

Blood of 28 adult volunteers (female: 15; male: 13) was drawn with a 21 gauge needle from the antecubital vein, without applying venostasis, into precitrated S-Monovette® premarked tubes (3 ml) from Sarstedt, containing 0.30 ml 0.106 M trisodium citrate solution. Approved by the local ethics committee, cord blood was obtained from 31 term (gestational age 38–40 weeks) newborns (female: 17; male: 14) immediately following uncomplicated delivery into the above mentioned citrate tube and processed within one hour. Blood counts were measured before and after preparing PFP, by using a Sysmex KX 21 cell counter. The second blood count measurement was performed to exclude platelet contamination of the samples. To prepare PFP, blood was centrifuged at room temperature for 20 minutes at $1550 \times g$ without brake. The supernatant plasma was carefully removed, aliquotted (1 ml), snap- frozen and stored at −80 °C for further analysis.

Preparation of microparticles for flow cytometry

The aliquots were thawed at 37 °C and centrifuged at $18000 \times g$ for 30 min at room temperature to pellet MPs in PFP. Afterwards 970 μl of the supernatant was removed and the MPs were resuspended in 970 μl phosphate buffered saline, pH 7.4, which was previously filtered through a syringe filter with 0.8 μm Versapor® membrane. After a second centrifugation step with the same parameters, 97% of the supernatant was again removed. The MP pellet was resuspended in 200 μl Annexin Binding Buffer (diluted 1:10) to provide a basis for calcium dependent Annexin V- binding.

Flow cytometric detection of microparticles

100 μl of the MP suspension were transferred into Trucount™ Tubes containing a known number of fluorescent beads to enumerate MPs. FITC labeled Annexin V was used to identify MPs by binding phosphatidylserine. Annexin V was titrated in preliminary experimentes to determine the optimal labelling volume (5 μl). MPs were then incubated in the dark with 5 μl Annexin V for 20 minutes at room temperature. MPs were defined as particles 1 μm in diameter or smaller in size, removable by ultracentrifugation, and positive for binding Annexin V. Before starting the measurement, samples were diluted with Annexin binding buffer to a final volume of 705 μl. The samples were analyzed within one hour by using a BD™ LSR II, 4 laser, flow cytometer with BD FACSDiva™ 6.0 software. Forward- and side-scatter were set in logarithmic scale. A MP size standard was used to set the microparticle gate (MP gate), using forward- and side-scatter (Fig. 1A). MPs were differentiated from signal noise by threshold settings of 4000 forward scatter and 200 side scatter. Out of all events falling into the predefined MP gate, only Annexin V positive events were defined and enumerated as MPs (Fig. 1B). The acquisition was terminated after 1500 counts were reached in the counting bead gate