No differences in support of thrombin generation by neonatal or adult platelets

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Summary
Newborns have, despite low clotting factors and poor in vitro platelet function, a well functioning hemostasis. The reason for this is still not completely clear. The aim of our study was to investigate whether phospholipids in neonatal platelets differ from those in adult platelets in their total amount, in their exposure on the platelet surface, and their effect on thrombin generation (TG). Methods: Clotting times of newborn and adult platelet-rich plasma were measured. Effect of newborn and adult platelets on TG was measured by means of CAT (calibrated automated thrombography). In addition, the effect of newborn and adult platelets with or without stimulation by ionophor on TG was measured in a purified prothrombinase complex. Phosphatidylycerine-exposure (PS) of newborn and adult platelets was measured by flow cytometry of annexin V binding. The amount of phospholipids (PL) was determined by means of mass spectrometry. Results: Clotting times of platelet-rich plasma (PRP) of newborns stimulated with ionophor showed a significant lower reduction of clotting time than in adult PRP. No differences in the support of TG between neonatal and adult platelets were found in neonatal or adult plasma by means of CAT. In the purified system TG was increased by adding ionophor stimulated platelets but no difference was evident between stimulated newborn and adult platelets. Flow cytometric analysis showed no difference in annexin V binding between adult and newborn platelets. The results of mass spectrometry showed a very similar amount and pattern of PL of adult and newborn platelets. Conclusion: Our results do not provide any evidence that a different PL content or expression of neonatal platelets may alter TG in neonates.

Schlüsselwörter
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Zusammenfassung
Newborns have low levels of many clotting factors, especially of the prothrombin complex and the contact factors. This would predispose adults to easy bruising, but newborns have no such easy bruising and excellent wound healing. The reason for the well functioning clotting in newborns seems to be the low levels of inhibitors.

In vitro aggregation of neonatal platelets is impaired in response to most agonists. Expression of pseudopods, glycogen deposits and visible microtubular structures are lower after stimulation with thrombin. Platelet aggregation with platelet-rich plasma is markedly lower in neonates when platelets are stimulated with ADP, epinephrine, collagen, or thrombin, while aggregation with whole blood shows no differences between neonates and their mothers when ADP and collagen is used as the stimulating agents. Primary hemostasis in newborn is not impaired (1). Bleeding time in healthy neonates is shorter than in older children and adults (2), and PFA-100 closure times are shorter than with adult blood (3, 4). This might be explained by the increased von-willebrand-factor (VWF) plasma concentration and VWF-collagen binding activity in newborns (5).

Platelets are not only important in primary haemostasis but are also essential in initiation and propagation of the coagulation process. Platelet adherence with expression of negatively charged phospholipids is important for initiation and propagation of thrombin generation.

Consistent with the hypothesis, it was reported that neonatal platelets have a relative impairment in their ability to mobilize calcium, an important mediator of many platelet functions (6). Therefore, the so-called flip-flop mechanism leading to exteriorisation of phospholipids also might be impaired.

Therefore, we investigated in this study, whether a difference exists between the phospholipid (PL) composition and expression of the platelet membrane of newborns to that of adults by analyzing the following parameters:

- phospholipid-composition by mass spectrometry,
- phosphatidylserine (PS)-exposure by annexin V binding using flow cytometry,
- support of phospholipid-exposure on the thrombin generation by means of calibrated automated thrombography using a fluorogenic assay and a purified system using a chromogenic substrate.

### Materials and methods

#### Blood sampling

Approved by the local ethics committee, blood was collected from umbilical cord of neonates (gestational age 38–40 weeks). Additionally, blood was taken from peripheral veins from healthy adult volunteers who were not taking any medication influencing coagulation.

#### Devices

Clotting times after recalcification were measured in platelet-rich plasma (PRP) to which A23187, a calcium-ionophor or Buffer, was added.

#### Thrombin generation

The fluorogenic assay via Calibrated Automated Assays were performed by means of Fluroskan Ascent plate reader (Thermo Lab-systems, Helsinki, Finnnland) and Thrombinoscope software (Thrombinoscope BV, Maastricht, The Netherlands) as described by the manufacturer.

For the chromogenic method the thrombin generation was measured by Athos microplate reader 2001, from Labtec instruments GmbH (Salzburg, Austria).

#### Flow cytometry

For analyzing we used FACScan flow cytometer equipped with LysysTM II (Becton Dickinson, Franklin Lakes, USA) software.

#### PS-amount analysis

Mass spectrometric analysis was performed with a triple quadrupole instrument model TSQ Quantum Ultra coupled to an Accele UPL system (Thermo, San Jose, USA).

#### Statistical analysis

Statistical analysis was performed with help of SPSS Version 15.0 (SPSS Inc., Chicago, USA)

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**Fig. 1**

Thrombin generation (TG) curves of platelet poor plasma (PPP) of

- a) newborns
- b) adults

Newborn platelets or adult platelets were added.

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USA). Students’ t-Tests were calculated between results of newborn and adult platelets and p-values less than 0.05 were considered to be significant.

Results

Clotting times

Stimulation of platelets shortened recalcification time with newborn platelets as well as with adult platelets. Newborn plasma stimulated with the ionophor had a reduction of clotting time of $10 \pm 12.2\%$; adult plasma had a reduction of $27 \pm 18.3\%$ (mean values; $p < 0.001$).

Thrombin generation by newborn platelets

We examined 12 pairs of newborn and adult platelet suspensions. Newborn platelets were added to newborn or adult platelet-poor plasma (PPP) and adult platelets were added to newborn plasma as described and thrombin generation was continuously measured.

Newborn platelets supported thrombin generation in the same way as adult platelets (Fig. 1a, b). Thrombin generation (TG) was higher in adult plasma but no difference was seen whether newborn or adult platelets were added to the adult PPP. Also, TG was lower in newborn plasma regardless of adult or newborn platelets had been added. No significant difference was found between newborn or adult platelets in the effect on TG whether they were added to adult or newborn plasma.

Thrombin generation with 15 pairs of adult and newborn platelets with or without stimulation by ionophor in a purified prothrombinase complex was measured. The thrombin generation was increased by adding the ionophor with newborn as well as with adult platelets and there was no difference between the effects of stimulated newborn and adult platelets (Fig. 2).

PS-exposure

PS-exposure of five pairs of newborn and adult platelets by annexin V binding was measured. The binding of annexin V before and after stimulation of the platelets with ionophor or thrombin was identical in newborns and adults ($p < 0.05$) (Fig. 3).

PL-amount

The relative distribution of fatty acid composition of PS of 15 newborns and 12 adults was acquired with mass spectrometry. Analysis of data resulted in a similar pattern for platelet membrane PS in adults and newborns, in respect to overall fatty acid chain length and degree of unsaturation (Fig. 4). Furthermore, the overall distribution of phosphatidylcholine (PC), PS and phosphatidylethanolamine (PE) was calculated, showing PC as the major PL class in platelet membranes (Fig. 5). Again, no difference between newborn and adult PL distribution could be found.

Discussion

The phospholipid surface of cells, especially of platelets, is very important for the coagulation process. Tissue factor bearing cells and platelets are two critical coagulation components where large amounts of thrombin are generated on their surfaces (7). Cell activation, apoptosis, and cell stress can induce redistribution of phospholipids and phosphatidylserine becomes rapidly exposed in the outer leaflet of the plasma membrane where the prothrombinase- and the tenase-complex are assembled (8). The phospholipid content of newborn and adult platelets may show differences. Existing differences of the...
membrane polarity might explain the good thrombin generation in neonatal platelets, if more negatively charged phospholipids were expressed on newborn platelets.

Results of our investigation do not show significant differences in the total amount of PS between newborn and adult platelets. Furthermore, flow cytometry analysis with annexin V, which binds to PS in the presence of calcium, does not show differences in the expression of phosphatidylserine in the outer leaflet of the plasma membrane after addition of ionophor as well as of thrombin. Our study shows that differences in phospholipid content are not prominent between newborn and adult platelets.

Ionophor is the strongest trigger for the exteriorisation of phospholipids, but it is not physiologic. Neonatal platelets have been shown to show a poor response to thrombin (9, 10), but we do not show any differences in the expression of PS even after stimulation with thrombin, suggesting that the flip-flop mechanism for exteriorisation of phospholipids works well despite the fact neonatal platelets have an impairment in their ability to mobilize calcium.

In contrast to our experiments on clotting time using a purified system shortening after ionophor stimulation in adult PRP is more pronounced than in newborn plasma. This indicates that the exposure of PS alone on phospholipid surfaces is not enough in itself to affect the clotting of native whole blood, or to induce clotting in recalcified plasma depleted of platelets (11). Another discovery which supports additional mechanisms is the description of patients with a platelet defect with spontaneous PS exposure named "Stor-morken syndrome". These patients have a bleeding tendency and no increase in thrombin formation (12, 13).

However, our data show that the thrombin generation is increased by adding the ionophor with newborn as well as with adult platelets and there is no difference between stimulated newborn and adult platelets in a purified system.

Conclusion

Newborn platelets are not different to adult platelets in their phosphatidylserine content and the flip-flop mechanism leading to the exteriorisation of phospholipids seems to work. Therefore, our results do not suggest that a different phospholipid content or expression on the platelet membrane of newborns compared to adults has an influence on the thrombin generation.

Conflict of interest

All authors declare that there is no conflict of interest.

References