Deposition of whole blood platelets on extracellular matrix under flow conditions in preterm infants

N Linder, B Shenkman, E Levin, L SirotA, T H Vishne, I Tamarin, R Dardik, D Lubin, N Savion, D Varon

Background: A previous study showed greater adhesion by platelets of healthy full term infants to subendothelial extracellular matrix (ECM) under flow conditions compared with healthy adult platelets. Aim: To investigate the adhesion and aggregation of platelets from preterm infants on ECM under defined shear conditions.

Methods: In vitro platelet function was investigated in 106 preterm infants, 74 full term infants, and 26 healthy adults. Blood samples were obtained from all infants within 24 hours of birth, and weekly until discharge from preterm infants only. Citrated whole blood was placed in ECM precoated tissue culture plates and subjected to shear stress (1300 s⁻¹) for two minutes using a rotating Teflon cone. Platelet adhesion (surface coverage) and aggregation (average size) to ECM were assessed using an image analyser. Assays for von Willebrand factor (vWF) antigen, ristocetin cofactor, and vWF collagen-binding activity were performed on samples from an additional 70 preterm infants, 23 healthy full term infants, and 24 healthy adults. Preterm infants with hyaline membrane disease (HMD) were analysed separately in both cohorts.

Results: Platelets from preterm infants displayed significantly less platelet adhesion than those from full term infants but similar aggregation and levels of vWF antigen, ristocetin cofactor, and collagen binding activity. Mean surface coverage was 22.0 (8.4)% for preterm infants with HMD, 28.7 (8.0)% for healthy preterm infants, and 35.7 (7.9)% for full term infants. Surface coverage in the preterm infants correlated with gestational age during the first 24 hours only, and did not reach full term levels during 10 weeks of follow up.

Conclusion: Platelet adhesion to ECM is significantly poorer in preterm than in full term infants, and poorer in preterm infants with HMD than in healthy preterm infants. Intrinsic platelet properties rather than the concentration or activity of vWF may be responsible for this difference.

Preterm infants have a higher rate of bleeding diathesis than full term infants and significantly lower levels of most coagulation and anticoagulation factors. A reduced haemostatic functional reserve capacity could contribute to the occurrence of bleeding, especially in the presence of additional risk factors. Adequate platelet function is crucial to the maintenance of normal haemostasis. However, the conventional physiological tests used until recently were limited by the relatively large amounts of blood required and their performance without shear or without the presence of solid phase subendothelial components. Although several sophisticated methods for studying platelet interaction with subendothelial protein ligands under shear conditions have been described, none had the potential for wide clinical use, and they possessed a limited ability to differentiate between the various pathological conditions. The method that we used which requires only small volumes of blood is based on the study of Varon et al, who found that subjection of citrate whole blood to flow on bovine endothelium extracellular matrix (ECM) results in platelet adhesion and aggregation. In our previous study, we used the cone and plate(let) analyser (CPA) to examine platelet adhesion and aggregation on ECM under shear conditions, mimicking most of the complex series of events occurring in vivo during thrombogenesis. We showed that full term infants have increased adhesion and normal aggregation compared with adult controls. This shift in platelet function may provide neonates with a balanced primary haemostasis, compensating for neonatal platelet hyperactivity, which has been shown in vitro without the application of shear stress.

This study was designed to extend the investigation of platelet adhesion and aggregation under flow conditions to preterm infants. The findings may enable us to reach a better understanding of primary haemostasis in this patient population.

MATERIALS AND METHODS

Patients and blood sampling
The study was approved by the local and national ethics committees, and informed consent was obtained for each participant. Two cohorts were defined. The first, in which platelet adhesion and aggregation were measured under defined shear stress, included 106 preterm infants (< 35 weeks gestation), born consecutively between March and August 1997, and 74 healthy full term infants (> 37 weeks gestation, one and five minute Apgar scores 9 and 10 respectively; birth weight > 2500 g) born during April 1997. All infants were born at the Rabin Medical Center, Petah Tiqva, Israel. Twenty six healthy adult volunteers who had not taken medication known to affect platelet function for at least 10 days before blood sampling served as controls for this cohort. The second cohort, in which von Willebrand factor (vWF) antigen assay, ristocetin cofactor activity test, and collagen binding activity assay were performed, included 70 preterm infants born consecutively.

Abbreviations: CPA, cone and plate(let) analyser; ECM, extracellular matrix; GP, glycoprotein; HMD, hyaline membrane disease; PBS, phosphate buffered saline; vWF, von Willebrand factor
between April and December 1999, and 23 full term infants born during April 1999. Twenty four healthy adult volunteers served as controls for the second cohort.

**Sample collection**

Venous blood samples were obtained during the first 4 hours of life from all infants. In the first cohort, additional samples were collected weekly from the preterm infants until discharge. All blood samples were drawn into plastic syringes and immediately transferred to plastic tubes containing trisodium citrate (0.129 M).

**Procedure for the cone and plate(let) analyser (CPA)**

The method has been described in detail elsewhere. Briefly, 0.2 ml samples of clotted blood were placed in tissue culture wells precoated with subendothelial ECM derived from bovine corneal endothelium. Blood was subjected to flow (1300 s⁻¹) for two minutes using a rotating Teflon cone. The wells were washed with phosphate buffered saline (PBS) stained with May-Grünwald stain and analysed with an image analysis system (Galai, Migdal Haemek Israel).

Two parameters of platelet function were evaluated: platelet adhesion to ECM, defined as the percentage of total area covered by platelets (surface coverage), and platelet aggregation on the ECM, defined as the average size of the ECM bound objects.

**Immunoturbidimetric assay of vWF antigen**

vWF antigen was tested with the immunoturbidimetric assay, which measures the change in light transmission by a suspension of microlatex particles precoated with antibodies against vWF, in the presence of tested plasma. The STA-Liatest vWF kit (Stago, France) was used.

**Assay of ristocetin cofactor**

Ristocetin makes conformational changes in the vWF structure, allowing it to bind to glycoprotein Ib (GPIb) on the platelet membrane. The rate of ristocetin induced platelet agglutination is related to vWF activity. The ristocetin cofactor assay kit (Helena Laboratories, Beaumont, Texas, USA) measures the agglutination of formalin fixed normal platelets by ristocetin in the presence of test plasma. The percentage of vWF activity is obtained from aggregometer tracings.

**vWF collagen binding activity**

This assay was performed according to a previously described method with minor modifications. Plasma samples diluted 1:100 with PBS containing 0.2% bovine serum albumin were placed on multwell plates precoated with collagen type III (5 μg/ml) and blocked with 1% bovine serum albumin in PBS for 15 minutes. After incubation for two hours, the bound vWF was incubated for one hour with a solution of peroxidase conjugated antibody to vWF (1:4000). After each step the microtitre wells were washed three times with PBS. The chromogenic reaction was performed by addition of phenylenediamide (0.5 mg/ml) containing 0.5 μl/ml 30% H₂O₂. The reaction was terminated by the addition of 1.6% H₂SO₄, and the absorbance was read at 2932 nm on an ELISA reader.

**Statistical analysis**

Statistical analysis was performed with BMDP Statistical Software, 1990. One way and two way analysis of variance and covariance with repeated measures, Fisher’s exact test, Pearson’s χ² test, paired t test, and logistic regression analysis were used, as appropriate. Correlations were derived by Student’s linear regression analysis.

**RESULTS**

**First cohort**

The mean (SD) birth weights of the 106 preterm infants and 74 full term infants were 1412.2 (482.8) g and 3184.5 (540.7) g respectively; mean (SD) gestational ages were 30.5 (3.2) weeks and 39.6 (1.5) weeks respectively. A healthy preterm group of 52 infants was formed after the exclusion of 54 infants with one or more major pathologies including: hyaline membrane disease (HMD; n = 26), hyperbilirubinaemia (serum bilirubin > 150 mg/l; n = 9), sepsis (either positive blood or cerebrospinal fluid culture any time during their stay in the neonatal unit; n = 16), intraventricular haemorrhage (n = 4), thrombocytopenia, or coagulation disorder (n = 8).

Twenty three preterm infants who were found to have HMD, without sepsis, serum bilirubin of > 150 mg/l, or coagulation disorders were separated into a distinct subgroup for comparison. All were diagnosed by a qualified radiologist, and all required supplementary oxygen with either mechanical ventilation or continuous positive airway pressure support. Table 1 gives the characteristics of the healthy preterm infants, the preterm infants with HMD, the healthy full term infants, and healthy adult controls of the first cohort.

The healthy preterm infants were older (p < 0.001), had higher birth weights (p < 0.001), and higher Apgar scores at one and five minutes (p < 0.001) than the preterm infants with HMD. Significantly more preterm (whole group) than full term infants were born by caesarean section (76/106, 72% v 6/74, 8%, p < 0.01). However, within the healthy preterm subgroup, there were no significant differences in gestational age or birth weight between infants delivered by caesarean section and those delivered vaginally.

Table 1 presents platelet adhesion (surface coverage) and aggregation (average size) during the first 24 hours of life for all the infant groups studied. Surface coverage was lower in the total preterm than the full term population (24.2 (9.3) % v 35.7 (7.9)%; p < 0.001), and lowest in the preterm infants with HMD (22.0 (8.4%) compared with the healthy preterm infants (28.7 (8.0%); p < 0.01) and the full term infants (35.7 (7.9%); p < 0.0001). On multivariate analysis, HMD was a significant factor for low surface coverage (p < 0.05), independent of weight, gestational age, or Apgar score. Aggregation was similar in the healthy preterm, HMD preterm, and full term infants.

During the first 24 hours of life, surface coverage correlated with gestational age in all infants (fig 1) (R² = 0.30, p < 0.05).

| Table 1 First cohort: clinical characteristics and platelet function in infants and adult controls |
|-------------------------------------------------|-------------------------------------------------|-------------------------------------------------|-------------------------------------------------|-------------------------------------------------|
| Characteristics                               | Preterm                                        | HMD                                           | Healthy                                        | Adult                                           |
| Gestational age (weeks)                        | Healthy (n=52)                                 | HMD (n=23)                                    | Healthy (n=52)                                 | Adult (n=26)                                    |
| Birth weight (g)                               | 32.1 (2.2)                                     | 28.6 (3.0)                                    | 39.6 (1.5)                                     | -                                               |
| Birth weight (g)                               | 1611.0 (429.9)                                 | 1190.5 (457.1)                                | 3184.5 (540.7)                                 | -                                               |
| Average age (adhesion) (%)                     | 28.7 (8.0)                                     | 22.0 (8.4)                                    | 35.7 (7.9)                                     | 19.4 (4.6)                                      |
| Surface coverage (adhesion) (%)                | 44.0 (12.7)                                    | 43.9 (16.8)                                   | 44.7 (4.9)                                     | 47.32 (15.2)                                    |
| Platelet count (cells x 10⁶/ml)                | 268.7 (92.5)                                   | 217.4 (115.8)                                 | 242.0 (61.0)                                   | 208.0 (48.0)                                    |

Note: all values are mean (SD).

*p<0.01 for preterm v full term and healthy preterm v HMD preterm, p<0.001 for full term v HMD preterm.

Downloaded from http://fn.bmj.com on November 6, 2017 - Published by group.bmj.com
Figure 1  Surface coverage during the first 48 hours of life correlated with gestational age.

However, during follow up (weeks 1–10 of life), the preterm infants showed no increase in surface coverage or correlation with corrected postconceptional age.

Platelet count correlated with both surface coverage and average size, but packed cell volume, white cell count, and absolute neutrophil count did not. No correlations were found between mode of delivery and surface coverage or average size.

Second cohort
The mean birth weights of the 70 preterm and 23 full term infants were 1417.3 (642.1) g and 3250.4 (530.7) g respectively; mean gestational ages were 29.9 (3.3) weeks and 39.1 (1.3) weeks respectively. Using the same criteria as for the first cohort, six preterm infants with intraventricular haemorrhage, sepsis, hyperbilirubinaemia, and coagulation disorders were excluded. Two preterm subgroups were defined: healthy preterm infants (n = 38) and preterm infants with HMD (n = 26). Table 2 shows the results for the preterm infant subgroups, full term infants, and adults. There were no significant differences between the whole preterm group and full term infants with respect to vWF antigen (165.3 (33.5)% v 166.5 (33.5)%), ristocetin cofactor 215.9 (73.3)% v 253.3 (88.2)%), and collagen binding activity (0.88 (0.27) v 1.11 (0.12) units). Adult values were significantly lower than all infant values (p < 0.01). No correlation was found between the levels in any of the assays and gestational age.

DISCUSSION
The CPA test has been found suitable for diagnosis and monitoring of treatment in various platelet disorders. Blood samples from patients with severe von Willebrand disease show very low adhesion (surface coverage) and aggregation (average size) compared with samples from healthy volunteers, mainly under a relatively high shear rate (1300 s⁻¹). However, during follow up (weeks 1–10 of life), the preterm infants showed no increase in surface coverage or correlation with corrected postconceptional age.

Platelet count correlated with both surface coverage and average size, but packed cell volume, white cell count, and absolute neutrophil count did not. No correlations were found between mode of delivery and surface coverage or average size.

Second cohort
The mean birth weights of the 70 preterm and 23 full term infants were 1417.3 (642.1) g and 3250.4 (530.7) g respectively; mean gestational ages were 29.9 (3.3) weeks and 39.1 (1.3) weeks respectively. Using the same criteria as for the first cohort, six preterm infants with intraventricular haemorrhage, sepsis, hyperbilirubinaemia, and coagulation disorders were excluded. Two preterm subgroups were defined: healthy preterm infants (n = 38) and preterm infants with HMD (n = 26). Table 2 shows the results for the preterm infant subgroups, full term infants, and adults. There were no significant differences between the whole preterm group and full term infants with respect to vWF antigen (165.3 (33.5)% v 166.5 (33.5)%), ristocetin cofactor 215.9 (73.3)% v 253.3 (88.2)%), and collagen binding activity (0.88 (0.27) v 1.11 (0.12) units). Adult values were significantly lower than all infant values (p < 0.01). No correlation was found between the levels in any of the assays and gestational age.

Table 2  Second cohort: clinical characteristics of infants and von Willebrand factor (vWF) function

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Preterm</th>
<th>HMD (n=26)</th>
<th>Full term (n=23)</th>
<th>Adult (n=24)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gestational age (weeks)</td>
<td>Healthy (n=38)</td>
<td>32.2 (3.0)</td>
<td>28.5 (2.7)</td>
<td>39.1 (1.3)</td>
</tr>
<tr>
<td></td>
<td>(n=20)</td>
<td>161.3 (693.3)*</td>
<td>1192.3 (479.9)*</td>
<td>3250.4 (530.7)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>165.1 (27.4)</td>
<td>162.8 (39.7)</td>
<td>166.5 (33.5)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Birth weight (g)</td>
<td>Healthy (n=38)</td>
<td>193.8 (73.3)</td>
<td>228.6 (74.7)</td>
<td>253.3 (88.2)</td>
</tr>
<tr>
<td></td>
<td>(n=20)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>vWF antigen (%)</td>
<td>Healthy (n=38)</td>
<td>32.2 (3.0)</td>
<td>28.5 (2.7)</td>
<td>39.1 (1.3)</td>
</tr>
<tr>
<td></td>
<td>(n=20)</td>
<td>161.3 (693.3)*</td>
<td>1192.3 (479.9)*</td>
<td>3250.4 (530.7)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>165.1 (27.4)</td>
<td>162.8 (39.7)</td>
<td>166.5 (33.5)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>vWF collagen binding (arb. units)</td>
<td>Healthy (n=38)</td>
<td>0.89 (0.26)</td>
<td>0.77 (0.30)</td>
<td>1.11 (0.12)</td>
</tr>
<tr>
<td></td>
<td>(n=20)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ristocetin cofactor (%)</td>
<td>Healthy (n=38)</td>
<td>193.8 (73.3)</td>
<td>228.6 (74.7)</td>
<td>253.3 (88.2)</td>
</tr>
<tr>
<td></td>
<td>(n=20)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

All values are mean (SD). Adult values significantly lower than all infant values for each characteristic, p<0.01.

*p<0.01 compared with full term.
short bleeding time shown in full term newborns may result from raised levels of circulating vWF and/or increases in high molecular mass vWF multimers. On the basis of these data, the second part of the study (cohort 2) was designed to investigate the levels and activity of circulating vWF in preterm and full term infants. Unexpectedly, neither the concentration of vWF nor its activity, as determined by the level of vWF antigen in plasma, ristocetin cofactor, and assay of collagen binding activity, differed significantly between the two groups.

The reason for the lower adhesion of preterm platelets may be an intrinsic platelet hypoactivity. Platelets from preterm infants have been shown to be less reactive than those from full term infants and adults to physiological agonists. Furthermore, preterm platelets have reduced thromboxane A2 productivity. Decreased platelet function has not been related to altered binding characteristics of the thromboxane A2 receptor, but may lie in the transduction pathway after the receptor.

As described in our previous study, unlike surface coverage, average size did not differ between full term neonates and adults. This was also true in the present study for preterm infants. This finding suggests that vWF functions under high shear conditions to maintain platelet adhesion to the ECM surface. Average size is an important variable in solid phase platelet aggregation, depending mainly on the GPIIb-IIIa receptor, which is present at similar levels in adults and neonates. The decrease in average size to that of a single platelet in patients with Glanzmann’s thrombasthenia is confirmed in patients receiving treatment with GPIIb-IIIa antagonist.

In conclusion, platelet deposition on ECM under flow conditions may be an intrinsic platelet hypoactivity. Platelets from preterm infants and adults to physiological agonists. Platelet adhesion conditions may be an accurate and specific representation of the state of platelet function in preterm infants. Platelet adhesion to ECM is significantly poorer in preterm than full term infants. Intrinsic platelet properties, rather than concentration or activity of vWF, may be responsible for this difference.

ACKNOWLEDGEMENTS

We thank Dr. E. Fallen-Gozanti for editorial assistance, B. German and B. Raviv for technical assistance, the staff of the Neonatology Department of Schneider Children’s Medical Center of Israel for help in collecting blood samples, and Gloria Gin zach and Marian Propp for editorial and secretarial assistance.

Authors’ affiliations

N Linder, L Sirota, T H Vishne, Department of Neonatology, Schneider Children’s Medical Center of Israel, Petah Tikva, Israel
E Levin, D Lubin, Department of Neonatology, Chaim Sheba Medical Center, Tel Hashomer, Israel
B Shenkman, I Tamarin, R Darkin, D Varon, Institute of Thrombosis and Hemostasis, Chaim Sheba Medical Center
N Savion, Eye Research Institute and Sackler Faculty of Medicine, Tel Aviv University, Tel Aviv, Israel

REFERENCES


www.archdischild.com
Deposition of whole blood platelets on extracellular matrix under flow conditions in preterm infants

N Linder, B Shenkman, E Levin, L Sirotka, T H Vishne, I Tamarin, R Dardik, D Lubin, N Savion and D Varon

Arch Dis Child Fetal Neonatal Ed 2002 86: F127-F130
doi: 10.1136/fn.86.2.F127

Updated information and services can be found at:
http://fn.bmj.com/content/86/2/F127

These include:

References
This article cites 22 articles, 6 of which you can access for free at:
http://fn.bmj.com/content/86/2/F127#BIBL

Email alerting service
Receive free email alerts when new articles cite this article. Sign up in the box at the top right corner of the online article.

Topic Collections
Articles on similar topics can be found in the following collections
Child health (1515)
Infant health (857)
Neonatal health (928)

Notes

To request permissions go to:
http://group.bmj.com/group/rights-licensing/permissions

To order reprints go to:
http://journals.bmj.com/cgi/reprintform

To subscribe to BMJ go to:
http://group.bmj.com/subscribe/