Optimising quality of platelet transfusions
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Chapter 7

Soluble P-selectin as a parameter for platelet activation during storage

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Chapter 7

SOLUBLE P-SELECTIN AS A PARAMETER FOR PLATELET ACTIVATION DURING STORAGE

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Abstract

Platelet concentrates stored at room temperature deteriorate. The so-called storage lesion is characterised by morphological changes and a loss of functionality.

To develop an assay for early platelet activation in platelet concentrates, the morphological score, β-TG release and P-selectin expression were determined and compared with the amount of soluble P-selectin. An ELISA was used to quantify soluble P-selectin in the storage medium.

We found a significant correlation between the amount of soluble P-selectin and the percentage of P-selectin-positive platelets (flowcytometric analysis) \( r = 0.7449; \ p < 0.0001 \) or the amount of β-TG release \( r = 0.6837; \ p < 0.0001 \). The morphological score also correlated significantly (negative) with the amount of soluble P-selectin \( r = -0.7669; \ p = 0.0002 \). From day 0 to day 8, the amount of soluble P-selectin increased constantly from \( 219 \pm 49.2 \) ng/ml to \( 556 \pm 102.3 \) ng/ml.

The detection of soluble P-selectin can be used to quantify activation of platelets during storage. The immuno-assay for soluble P-selectin is more sensitive than the flowcytometric analysis of the percentage of P-selectin-positive cells and allows earlier detection of platelet activation.
INTRODUCTION

Platelets stored as concentrates gradually deteriorate, with morphological changes and loss of functionality. This is the so-called 'platelet storage lesion' [136]. With tests in which the morphology score, the expression of activation antigens or the release of β-thromboglobulin (β-TG) is determined, the quality of stored platelets can be (semi-)quantified [107;129].

The CD62P antigen (P-selectin, GMP-140 or PADGEM), a platelet α-granule protein (Mr 140 kD), is re-distributed to the plasma membrane during platelet activation and degranulation [137;138]. Surface-expressed P-selectin mediates the binding of activated platelets to neutrophils and monocytes [139-142] involved in early inflammatory reactions.

Due to alternative splicing there are two different isoforms of P-selectin in platelets: one with and one without a transmembrane part [142;143]. Upon activation of platelets, P-selectin without a transmembrane part is released from the α-granules, whereas membrane-integrated P-selectin can be partially released by proteolysis. Both types of soluble P-selectin in the medium can be quantified with a sandwich immuno-assay in which two monoclonal antibodies directed against different epitopes of the P-selectin molecule are used [144]. In this study the amount of soluble P-selectin is compared with three other activation parameters of activation: morphology, percentage of cells expressing P-selectin and the amount of released β-TG.

MATERIALS AND METHODS

Blood collection and platelet concentrate preparation

Platelet concentrates (PC) were prepared in two different ways, i.e. the platelet-rich plasma (PRP) method [104] and the buffy-coat (BC) method [87].

500 ± 50 ml blood were collected in 70-ml of citrate-phosphate-dextrose (CPD) in PVC/DEHP quadruple systems (NPBI, Emmer-Compascuum, The Netherlands). After storage at 20 ± 2°C for 12-16 hours [30], the blood was processed to PRP-PC or BC-PC. For PRP-PC, the blood was first separated into red cells and PRP by centrifugation for 7 min at 1,000 g at 20°C. The PRP was pressed under reduced flow into an empty satellite bag by an automated device for component preparation, the Compomat with Compomaster software [84](NPBI). The PRP was subsequently centrifuged for 6 min at 3,000 g at 20°C. The Compomat was then used with a different programme to remove the plasma until about 70 ml of plasma was left. The platelet pellet with the 70 ml of plasma was left undisturbed for 90 min at room temperature before manual resuspension. Subsequently, the PRP-PC were stored overnight in 600-ml PVC/DEHP bags, in a platelet incubator (22 ± 1°C) on a horizontal flatbed shaker (1 cycle/s ) (Helmer labs Inc, Noblesville, IN, USA) before pooling.
Five PRP-PC of the same ABO blood group were pooled with the use of a sterile connecting device (SCD 312, Haemonatics, Braintree, MA, USA). The individual PRP-PC were selected by platelet count so that the final platelet concentration was similar in all 8 pools used in this study.

The blood for BC-PC was processed to PC from single donations [87] with the use of a Compomat according to standard methods as described before [106].

**Filtration**

Only the pools of PRP-PC were filtered through the PL50HF leukocyte depletion filter (Pall Biomed. Products CO., Glen Cove, N.Y., USA), according to the instructions of the manufacturer. Because of their low initial leukocyte numbers (<0.1*10^6/ml), BC-PCs were not filtered.

**Morphology**

For morphological evaluation, 50 μl of a platelet sample were fixed with 250 μl of 0.5% glutaraldehyde in phosphate-buffered saline (room temperature) and stored for future evaluation at 4°C. Morphology was judged by a modification of the Kunicki score [107] by light microscopy (Leitz, Wetzlar, Germany) with oil immersion (1000x). The number of discs per 100 cells was multiplied by 4, the number of dendrites by 2 and the number of spheres by 1.

**β-thromboglobulin release**

The release of β-TG in the supernatant of platelet concentrates was measured after centrifugation of a sample (5 min at 12,000 x g). The supernatants were stored at -80°C. β-TG was measured with a RIA (Kodak Clinical Diagnostics Ltd, Amersham, UK).

**Membrane-expressed P-selectin**

The expression of P-selectin on platelets was measured with the CLB-Thromb/6 monoclonal antibody against P-selectin. In short, flowcytometric analysis was performed and the percentage of platelets that reacted with the monoclonal antibody CLB-Thromb/6 was calculated. IgG1 against the cat-antigen was used as a negative control [129].

**Soluble P-selectin concentration**

To quantify soluble P-selectin, a sandwich-type ELISA was set up; two non-competitive monoclonal antibodies were used, one of which was biotinylated. P-selectin purified from plasma by immuno-absorption was used as a standard. The
inter-assay and intra-assay variation for P-selectin was 8 and 7%, respectively (n = 8).

In 37 control samples of plasma from freshly drawn blood, the concentration of soluble P-selectin was 155 ± 24 ng/ml (mean ± SD).

Statistical analysis
Statistical comparisons and correlation coefficient calculations were made with the computer programme Instat2 (GraphPad Software, San Diego, CA, USA). P-values < 0.05 were considered significant.

RESULTS

Changes between collection and component preparation
The amount of soluble P-selectin in plasma was measured within a half-hour and 17 hours after blood collection and was found to increase from 167 ± 49.9 ng/ml to 219 ± 49.2 ng/ml (p < 0.0001) (fig. 7.1). The blood was stored as whole blood on butane-diol cooling plates [30]. Plasma was prepared by centrifugation (5 min at 12,000 g). During processing of the blood to prepare PRP-PC or BC-PC and storage for 1 day, the soluble P-selectin concentration significantly increased in the platelet concentrates prepared by the PRP method (277 ± 42.6 ng/ml; p < 0.05) but not in those prepared by the BC method (260 ± 72.8 ng/ml). On day 1, a significantly higher percentage of P-selectin-positive platelets was found in PRP-PC (17.6 ± 3.1%) compared with platelets in concentrates prepared with the BC method (12.4 ± 5.6%; p < 0.05).
Fig. 7.1 - Soluble P-selectin concentrations (mean ± SD; open bars) in plasma were measured directly after blood collection (WB(0)), after overnight storage as whole blood (WB(17)) and one day after preparation of BC PC (BC(41)) or PRP-PC (PRP(41)). WB is whole blood; number in parentheses is time in hours since blood collection. For comparison the percentage of P-selectin-positive cells for BC-PC and PRP-PC is also shown (mean ± SD; shaded bars).

Storage of filtered PRP-PC

During 8 days of storage, a constant increase was seen of soluble P-selectin and of the percentage of P-selectin-positive cells in filtered pools of PRP-PC (r = 0.7449; p < 0.0001) (fig. 7.2). These two parameters correlated significantly. An increase in the concentration of soluble P-selectin occurred between day 1 and 3 and between day 3 and 6 (p < 0.0001). The concentration did not differ between day 6 and 8. The percentage of P-selectin-positive cells differed significantly between day 3 and 6 as well as between day 6 and 8 (p < 0.0005).
Soluble P-selectin as a parameter for early platelet activation

Fig. 7.2 - Soluble P-selectin (mean ± SD; open bars) and percentage of P-selectin-positive cells (mean ± SD; shaded bars) were measured during storage for 8 days of filtered PRP-PC.

There was a significant correlation between the amount of soluble P-selectin and β-TG release ($r = 0.6837; p < 0.0001$). The β-TG concentration increased and showed a pattern identical to the soluble P-selectin concentration between day 1 and 3 and between day 3 and 6 ($p < 0.005$). There was no difference between day 6 and 8 (fig. 7.3).

Fig. 7.3 - Soluble P-selectin (mean ± SD; open bars) and β-TG concentration (mean ± SD; shaded bars) were measured during storage for 8 days of filtered PRP-PC.
The morphological score showed a negative correlation with the amount of soluble P-selectin (fig. 7.4), a decline in morphology went together with an increase in soluble P-selectin concentration ($r = -0.7669; p < 0.0001$). A decrease in the morphology score occurred during storage between day 1 and 3, day 3 and 6 and between day 6 and 8 ($p < 0.0001$).

![Graph showing soluble P-selectin (mean ± SD; open bars) and morphology score according to Kunicki (mean ± SD; shaded bars) measured during storage for 8 days of filtered PRP-PC.](image)

**Fig. 7.4** - Soluble P-selectin (mean ± SD; open bars) and morphology score according to Kunicki (mean ± SD; shaded bars) were measured during storage for 8 days of filtered PRP-PC.

**Analysis of the time dependency of the correlation**

When combining the data of percentage of P-selectin-positive cells and the amount of soluble P-selectin obtained in either PRP-PC (pooled, filtered) or BC-PC (single, low leukocyte count), a significant correlation between these two parameters was found over the whole storage period of 8 days (table 7.1). However, comparing the data at different storage intervals, it was found that the correlation coefficient was higher during the first days than during the final days of storage (table 7.1), ending up with a non-significant correlation for the data obtained at day 6 and 8 of storage.
Soluble P-selectin as a parameter for early platelet activation

Table 7.1: Time dependency of the correlation between soluble P-selectin and the percentage of P-selectin-positive cells

<table>
<thead>
<tr>
<th>Storage days</th>
<th>Correlation coefficient</th>
<th>n</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>1, 3, 6, 8</td>
<td>0.7423</td>
<td>32 PRP, 22 BC</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>1, 3, 6</td>
<td>0.7196</td>
<td>24 PRP, 22 BC</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>3, 6, 8</td>
<td>0.4351</td>
<td>24 PRP, 11 BC</td>
<td>&lt;0.005</td>
</tr>
<tr>
<td>6, 8</td>
<td>0.1912</td>
<td>16 PRP, 11 BC</td>
<td>0.1697</td>
</tr>
</tbody>
</table>

The correlation coefficient between the concentration of soluble P-selectin and the percentage of P-selectin-positive cells was calculated with all available data for PRP-PC and BC-PC during a specified storage period.

DISCUSSION

We consider the amount of soluble P-selectin a valid and sensitive parameter for detection of platelet activation in vitro.

The marked change in the amount of soluble P-selectin correlated with changes in the other tested parameters, commonly known as activation markers [32;106;129;145]. Also, the increase in the amount of soluble P-selectin in concentrates prepared by the PRP method, in which platelets are pelleted, was significantly greater than in concentrates prepared by the BC method. This is in accordance with the increase in the percentage of P-selectin-positive cells in a previous study [32]. Thus, the amount of soluble P-selectin is a valid parameter for platelet activation, as also found by Divers et al. [146].

We found that already during storage as whole blood on cooling plates the concentration of soluble P-selectin increases significantly. In an earlier study, we found that the percentage of P-selectin-positive platelets was not significantly different immediately after blood collection and after overnight storage and preparation of the platelet concentrates [129]. A further increase in the amount of soluble P-selectin was found during storage of platelet concentrates from day 1 to 6, whereas the increase in the percentage of P-selectin-positive cells was only significant between day 3 and 6. Thus, the amount of soluble P-selectin is a more
sensitive marker for platelet activation than the percentage of P-selectin-positive cells, especially during the first days of storage.

In the BC-PC, stored as single concentrate with a low leukocyte content, there was a significant correlation between the percentage of P-selectin-positive cells and the soluble P-selectin concentration during storage for 6 days. In general, the correlation coefficient between these parameters decreased during storage. This is due to the fact that the number of P-selectin-expressing cells still increased during the final days of storage, when the amount of soluble P-selectin remained nearly constant. This might be due to proteolysis of soluble P-selectin in this phase.

At no time did we find a correlation between the percentage of P-selectin-positive cells and the soluble P-selectin concentration in samples from non-filtered PRP-PC (leukocyte contamination 70 x 10⁶/pool). This might be explained by binding of P-selectin to leukocytes [147-149]. This is being further investigated and should be considered as a limitation of the concentration of soluble P-selectin as a parameter for the quality of platelets.

The quantitative assay for soluble P-selectin, based on an ELISA, has a logistic advantage for routine use in the laboratory, because plasma samples can be stored at -80°C. To determine the percentage of P-selectin-expressing platelets, concentrates must be fixed in glutardialdehyde and analysed by flowcytometry within one day.

The strong correlation of known parameters of platelet activation with the soluble P-selectin concentration in combination with the sensitivity and easy handling of the assay makes soluble P-selectin quantification a useful tool for monitoring (early) platelet activation.