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Chapter 8

Increase in glycocalcin levels in PC stored in plasma or synthetic medium for 8 days: comparison with other platelet activation markers

_Vox Sanguinis in press_
INCREASE IN GLYCOCALICIN LEVELS IN PC STORED IN PLASMA AND SYNTHETIC MEDIUM FOR 8 DAYS: COMPARISON WITH OTHER PLATELET ACTIVATION MARKERS


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Abstract

BACKGROUND AND OBJECTIVES: Glycocalcin (GC) is a proteolytic fragment of GpIb and can conveniently be measured in supernatants of platelet concentrates (PCs) by means of a sandwich ELISA. Because of convenience of the assay and easy sample storage, we tested its suitability as a sensitive platelet activation parameter during PC storage.

MATERIAL AND METHODS: Filtered PC in plasma or additive solution were made from 5 pooled buffy coats and were subsequently stored during 8 days at 22 ± 2 °C. Correlation coefficients (r) were calculated after comparison of GC levels with platelet parameters.

RESULTS: A significant increase in GC concentration was found between all subsequent sampling days. PC stored in plasma showed GC levels that correlated well with the soluble P-selectin levels (r = 0.7506), P-selectin (CD62P) expression on the platelet membranes (r = 0.8843), morphology scores according to Kunicki (r = -0.7102), lactate concentrations (r = 0.9216), glucose concentrations (r = -0.8913) and β-thromboglobulin (β-TG) concentrations (r = 0.8913). In PC stored in additive solution, the correlation coefficients with these markers were 0.9209 with soluble P-selectin, 0.7161 with CD62P expression, -0.7474 with morphology score, -0.8908 with glucose concentrations, 0.8923 with lactate concentrations and 0.8908 with β-TG concentrations.

CONCLUSIONS: The GC concentration correlates well with sensitive platelet (activation) parameters, rendering it a sensitive and convenient parameter for platelet activation.
INTRODUCTION

Glycocalicin (GC) is a proteolytic fragment of the α-chain of Gplb. It is the binding site for von Willebrand Factor and can be shed during platelet activation or during platelet destruction. It is present in normal plasma ([3637]) and its function is still unknown. Glycocalicin levels in plasma are dependent on the platelet count and the platelet turnover [27;150]. We used a sandwich ELISA [27] to quantitate GC and correlated the GC levels to other (activation) parameters of platelet concentrates (PCs).

Because platelets become activated during storage of PC, the degree of activation is measured as a parameter of the quality of the platelets. To measure PC quality, various assays are used. In general, the assays now available need to be performed on the day of sampling, which is time- and reagent-consuming, because samples from the different storage days cannot be stored and analysed in one run. We already introduced the soluble P-selectin assay as a convenient and sensitive assay that can be performed after storage of the plasma samples at −80 °C [131]. However, with this assay, during prolonged PC storage, no differences in activation between day 6 and 8 were detected, which can be necessary when performing comparison studies. Although assays for GC applied to PCs during storage have been described before [151-154], these reports did not compare the results with a set of other platelet parameters during storage of PC, so the additional value of this test in PC storage is still unknown.

In the present study, sensitive PC activation assays were compared with the GC assay, and correlation coefficients were calculated to show the relation of GC levels to other parameters. We also tested for the presence of microvesicle-associated GC.

MATERIALS AND METHODS

Preparation of PCs

The study was performed in 4 experiments, each with 8 PCs, thus in total 32 PCs were tested. For each experiment, 40 whole blood units (500 ± 50 ml, blood group A positive) were collected from normal blood donors in PVC-DEHP bottom-and-top containers with 70 ml of citrate-phosphate-dextrose (CPD) (T&B, Biopack, Composphex, NPBI, Emmer-Compascuüm, The Netherlands). After collection, whole blood was stored overnight at 20°C on butane-diol cooling plates [30] until the next day, when PCs were prepared from pooled buffy coats (BCs). Whole-blood units were centrifuged (8 minutes, 2817 x g, brake 3, start-up time 70 s, slow-down time about 270 s, 20 °C; Hettich Roto Silenta R/P, Dépex, De Bilt, The Netherlands). After centrifugation, BCs were collected by means of an automated device for component preparation, the Compomat [84](version used, G4) with Compomaster software (NPBI). Five BCs were pooled to prepare one PC. Plasma
or GAC (gluconate, acetate, citrate: 90 mM NaCl, 23 mM sodium gluconate, 27 mM sodium acetate, 5 mM KCl, 3 mM MgCl$_2$, 0.32% (w/v) trisodium citrate, 294 mosm, pH 7.4) was added to the BC pool up to a gross weight of 500 g. One medium type was used for each experiment. BC-pools in plasma (n = 16) were centrifuged at 704 x g for 5 minutes (brake 3, 20°C). BC-pools in GAC (n = 16) were centrifuged at 313 x g for 5 minutes (brake 3, 20°C). The platelet-rich supernatants were pressed and transferred to a satellite container. PCs were filtered within 10 minutes after preparation over Sepacell PL-10A filters (Asahi, Japan) and were stored in UPX80 platelet storage containers (JMS1000, JMS, Hiroshima, Japan) or PO containers (Compoflex, NPBI) (each type of container 8 plasma PCs and 8 GAC PCs). Volumes of the pooled PCs were calculated by dividing the net weight of the PCs by the specific gravity of plasma (1.026 g/cm$^3$) or GAC with less than 10% remaining plasma (1.002 g/cm$^3$).

**PC storage and sampling**

Concentrates were stored in a platelet incubator (22 ± 2°C) on a horizontal flatbed shaker (1 cycle/s)(Helmer labs Inc, Noblesville, IN, USA). Samples were taken aseptically via a sampling site coupler (NPBI) on day 0 (directly after preparation and filtration), days 1, 3, 6 and 8. For preparation of supernatants, 1 ml of PC was centrifuged at 12,000 x g for 5 min. The supernatants were stored at -80°C until analysis.

**Platelet and leukocyte counting**

Platelets and leukocytes were counted with an electronic counter (AcT10, Coulter Electronics, Mijdrecht, The Netherlands). For this purpose, PCs were diluted (1:4) in PBS. Leukocytes were counted after filtration in 5 times diluted PCs with acridine orange (2.5 mg/L PBS) in a Nageotte bright-line counting chamber (Superior, Bad Mergentheim, Germany) by fluorescence microscopy (Leitz, Wetzlar, Germany).

**Blood gas parameters**

Within 5 minutes after sampling, PCs were used for blood gas measurements (pH, pCO$_2$, pO$_2$) in a blood gas analyser (CBI Corning 238 pH/Blood Gas Analyzer, Chiron Diagnostics NV, Houten, The Netherlands).

**Glycocalicin assay**

The GC concentration (in arbitrary unites (A.U.)/ml) in the supernatants of PCs was determined with a sandwich enzyme-linked immuno-sorbent assay.
Glycocalicin in PCs stored in plasma and synthetic medium

(ELISA) with two non-competitive monoclonal antibodies, as previously described [27].

**Soluble P-selectin assay**

The soluble P-selectin concentration was quantified by a sandwich-type enzyme-linked immuno-sorbent assay (ELISA) [131] in the supernatants of the PC samples. 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.

**FACS analysis of CD62P**

To measure CD62P expression on platelets, the monoclonal antibody CLB-Thromb/6 (CLB, Amsterdam, The Netherlands) against P-selectin (CD62P antigen) was used. In short, flowcytometric analysis was performed, and the percentage of platelets that reacted with the monoclonal antibody CLB-Thromb/6 was calculated. IgG1 against an irrelevant murine control antigen was used as a negative control [129].

**Glucose and lactate concentrations**

Glucose and lactate concentrations were measured in the supernatants of PCs, as previously described [130].

**β-thromboglobulin release**

The amount of β-TG in supernatants was measured with a radio-immuno assay (RIA) (Kodak Clinical Diagnostics Ltd, Amersham, UK).

**Platelet morphology**

For platelet morphology, 50 μl of PC were fixed in 250 μl of glutardialdehyde (0.5% in PBS, v/v) and stored at 4°C. Samples for morphology were stored for a maximum of 4 days and scored according to a slight modification of the method of Kunicki [107] by light microscopy (Leitz, Wetzlar, Germany) with oil immersion (1000x). A total of 100 platelets was scored: for calculations the number of perfect discs and almost flat platelets (with dendrites) was multiplied by 4, filled discs with dendrites by 2 and spheres by 1.
Ultracentrifugation of PC supernatants

To check whether GC was microvesicle-associated in the form of Gplb, PC supernatants, prepared as described above, were subjected to centrifugation for 15 min at 100,000 x g (Airfuge, Beckman Instruments, Palo Alto, CA, USA) [155]. GC assays were performed in the pellet and in the supernatant obtained after this high-speed centrifugation step.

Statistical analysis

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

RESULTS

Composition of PC

PC contained 301 ± 70.5 x 10^6 platelets in a volume of 344 ± 30.9 ml. The total leukocyte numbers were below 7.7 x 10^5, resulting in a depletion of more than 95.5%. The PC stored in GAC contained 7 ± 1.3% plasma (n = 16) (further referred to as GAC).

Increase in glycocalicin levels during storage of PC

During 8 days of storage, the GC levels increased in plasma PC as well as in GAC PC (fig. 8.1). For the sake of clarity, only data for UPX80 containers are shown (data from PO bags showed a similar pattern). The increase in GC concentration was significant between all subsequent PC sampling days (p < 0.05, n = 8, plasma; p < 0.003, n = 8, GAC). In a group of healthy volunteers, the mean GC concentration in plasma was 294 ± 75 AU/ml (n = 95).

Fig. 8.1 – The increase in GC concentration during storage of PC stored in plasma (solid line) or GAC (broken line).
Other platelet activation markers during storage

The changes in the different (activation) parameters in PC during storage for 8 days in PO as well as in UPX80 storage containers were comparable with previous studies [106;133]. Figure 8.2a-f shows the levels of the different parameters during storage in UPX80 containers, which were comparable with those in PO containers. The pH levels decreased slightly during storage but were still above 6.8 at day 6 and above 6.7 at day 8. pO₂ recovered to stable values (90-110 mmHg) during storage after an initial decrease between day 0 and 1, and pCO₂ slightly decreased (initial value 75 and 22 mmHg, respectively, for plasma or GAC) followed by stabilisation during the storage period, indicating good gas exchange in combination with metabolic activity.

![Graphs showing changes in different platelet parameters during storage](image)

**Fig. 8.2a-f - Changes in platelet (activation) parameters during storage of PC in GAC and plasma.** Concentrations or scores of different platelet (activation) parameters during storage of PC in plasma (solid lines) or GAC (broken lines); soluble P-selectin (a), CD62P expression (b), morphology score (c), glucose concentration (d), lactate concentration (e) and β-TG release (f).
Correlation of glycocalcin levels with other activation markers

The correlation coefficients of GC levels with the other (activation) parameters of PC are shown in table 8.1. For this comparison, all data of PO and UPX80 containers for day 1, 3, 6 and 8 were used, resulting in a considerable number of observations for this analysis. The correlation coefficients were highly significant (p < 0.0001).

Table 8.1: Correlation coefficients for comparisons of GC with other parameters

<table>
<thead>
<tr>
<th>GC correlated with:</th>
<th>Plasma</th>
<th>GAC</th>
</tr>
</thead>
<tbody>
<tr>
<td>sP-selectin conc.</td>
<td>0.7506</td>
<td>0.9209</td>
</tr>
<tr>
<td>CD62P (P-sel. expr.)</td>
<td>0.8843</td>
<td>0.7161</td>
</tr>
<tr>
<td>morphology</td>
<td>-0.7102</td>
<td>-0.7474</td>
</tr>
<tr>
<td>glucose</td>
<td>-0.8913</td>
<td>-0.8908</td>
</tr>
<tr>
<td>lactate</td>
<td>0.9216</td>
<td>0.8923</td>
</tr>
<tr>
<td>ß-TG</td>
<td>0.8622</td>
<td>0.8921</td>
</tr>
</tbody>
</table>

Data from day 1, 3, 6 and 8 for PC stored in plasma (n = 63) and GAC (n = 64) were used for calculations of correlation coefficients.

When the data for day 8 were excluded (because no glucose was left in the GAC PC after day 6), similar results were obtained (not shown).

Soluble and microvesicle-bound GC

To ensure that GC found in the PC supernatants was soluble GC released from platelets and not bound (in the form of Gplb) to microvesicles that might be formed during storage [156; 157], PC supernatants were ultracentrifuged. After this centrifugation, more than 80% of the GC was recovered in the supernatant, indicating that, indeed, the GC had been released from platelets and was not bound to a high degree to microvesicles (table 8.2).
**DISCUSSION**

During storage of PC, a continuous increase in glycocalcin (GC) is found, released from GpIb on the platelet membranes by proteolytic splicing. The increase in GC levels is significant between all subsequent storage days for PC stored in plasma or GAC. The fact that already on day 1, the GC level in GAC-stored PC is 100 AU/ml means that about 80 AU/ml are shed between blood collection and day 1 of storage (starting level of GC concentration in GAC is caused by the 7% of normal plasma GC value (294 AU/ml), *i.e.* 20 AU/ml). Compared to the plasma concentration of GC in healthy donors, no significant increase in GC concentration between blood collection and day 1 of storage for PC in plasma is observed. Early platelet activation between blood collection and day 1 of PC storage [32;131] can explain early shedding of GC.

The average increase in GC per day in plasma stored PC is 75 AU/ml and in GAC 45 AU/ml. So, in contrast to a study of Kunishima et al. [158] of PC in a 15% plasma + Seto solution, we found differences (*p* < 0.0001) in kinetics of GC increase by platelets stored in synthetic medium (GAC) or plasma. This slower increase in GAC in our study can probably be explained by the very low plasma concentrations, resulting in low amounts of plasma proteases (*i.e.* calpain, plasmin, trypsin and elastase) [159;160] These proteases cleave GpIbα, generating soluble GC in the supernatant. This stabilising effect of GAC was not seen with the other (activation) parameters, which are not influenced by proteases. Therefore, the amount of plasma should be taken into account if media are compared by means of GC measurements.
Because GC concentrations in PC supernatants after ultracentrifugation were similar to those before ultracentrifugation, we conclude that the GC we measured is for the most part soluble and not associated with platelet-derived microvesicles [155] in the form of Gplb. To quantify microvesicles, it is desirable to use a platelet antigen that is not sensitive to proteolytic cleavage. Preliminary data obtained in our laboratory suggest that GpIV (or CD36 antigen) might be suitable for this purpose.

In a previous study [131], in which soluble P-selectin levels were measured, no significant differences in sP-selectin between storage day 6 and 8 were observed. This means that the assay is not longer discriminating at that time. However, the GC assay showed significant differences in GC levels between day 6 and 8; thus, this assay is still discriminating for changes after prolonged storage.

Compared to other platelet storage parameters, such as P-selectin expression, morphology score, β-TG concentration, lactate concentration and glucose concentration, highly significant correlations are found with the GC concentration data over the whole storage period.

To check whether GC is a suitable parameter for platelet storage lesion, we set up an additional limited study (n = 2) with paired PCs stored under normal and under unfavourable conditions (limited gas exchange, in containers covered with plastic labels). The limited gas exchange resulted in PCs with low morphology scores, low swirling, low pH values and high CD62P expression compared to PCs stored under normal conditions. In these PCs, the release of GC was at the third, sixth and eighth day of storage 103, 41 and 31% higher, respectively, compared to those in PCs stored under normal conditions. The supernatants of PCs stored under limited gas exchange contained at day 8 almost the total amount of GC.

In conclusion, we consider the GC sandwich ELISA as a valuable parameter for platelet storage studies for the following reasons
- (1) the GC sandwich ELISA is a convenient assay, because samples can be stored at −80 °C pending analysis, with results correlating well with other commonly used parameters;
- (2) besides the β-TG assay it is the only platelet activation parameter that can be measured after storage of the samples for longer than a week and still showing differences in activation between days 6 and 8. In contrast to the β-TG assay, no radioactive material is needed.

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