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Quality Control of Platelets Concentrates; an *In Vitro* Fate Prediction Model System of PCs Transfusion

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Abstract

Introduction: We previously described that metabolic resting preserves old and cold platelets (PLTs) concentrates (PCs). In Badlou et al. 2006 we have established that P-selectin involved as an intermediate in binding, and the combination of PS exposure with (-out) changes in GPIb in phagocytosis of PCs. Using a standard threshold over the interaction between donors PLTs and patient's phagocytes might increase success of transfusion, and decrease side effects, pretransfusion.

The aim of this study is to introduce a novel method, which could be used as an in-vitro prediction model system to envisage PCs fate, posttransfusion.

Materials and Methods: Human PCs were stored for different periods to obtain a wide range of surface expressed P-selectin and-PS exposure. The quantitative change in GPIb was deduced from decreased binding of an anti GPIb- antibody measured by FACS flowcytometry. Correlations established in RT stored-PCs were compared with PCs stored at 0°C, and metabolic suppressed stored at 4°C (MSP4).

Results: Qualitative and quantitative analysis revealed that 'good PCs' which had low P-selectin and PS exposure, and high GPIb expression were not removed by phagopcytes. Comparison studies between C22, C0 and MSP4 showed that at a given amount of PS exposure and GPIb expression, phagocytes immediately removed 'bad PCs'. Threshold- dependent removal might be caused by ageing-dependent lesions. At high GPIb expression was phagocytosis almost 0%, when GPIb expression decreased below certain degree phagocytosis increased, remarkably.

Discussions: Our results signify that these chosen three markers combination measurements are reliable markers for phagocytic (ir-) responsiveness. In the near future, when these thresholds established as standard thresholds then any (Para-)medic can predict on the basis of relatively simple analysis, whether delivered PCs from the blood banks are immunogenic or not. In conclusion, our introduced *in-vitro* model system could be used as the quality control of any randomly selected and prepared PCs, pretransfusion.

Keywords: Transfusion; Platelets; Human; Prediction model system; *In vitro*; Immune response

Introduction

We previously described that metabolic resting preserves better PCs quality and quantity old and cold platelet (PLT) concentrates (PCs) [1]. Moreover, Badlou et al. showed that P-selectin acts as an intermediate marker in binding and the different combinations of PS exposure with (out) conformational changes in GPIbα [2] as intermediates in phagocytosis [3].

Using an in vitro assay for pre-analysis of the interaction between PLTs and macrophages might enhance success of transfusion and decreases side effects significantly. There is little insight in the relative contribution of these regulators of PLT destruction by macrophages in different patients.

There are raising evidences that in the field of PCs transfusion optimal PLT storage in vitro correlates directly with optimal hemostatic effectiveness and survival *in vivo* [4,5].

Moreover, optimal preservation of PLT reactivity results in better PLT functions [2,6,7]. Nevertheless, there is a need for PLT prediction assay that under laboratory conditions is able to predict how PCs behave themselves in the circulating blood of any random patient.

Different markers have been tested as indicators of the so called platelet storage lesion (PSL), such as P-selectin expression (PSE) [7,8], CD40/CD40 ligand [9,10] and glycoprotein (GP) IIbIIIa [11], changes in mean platelet volume (MPV), sphere-to-disc changes [12,13], actin assembly [14,15], serotonin release [16,17], changes in GPIb-V-IX and GPVI expressions [18,19], PS exposure [20,21], loss of swirling [22], osmotic pressure response [23], and changes in PLTs count caused by agglutination [24].

Although each marker provides insight in the activation state of the PLTs, none of them is a perfect predictor of the quality of transfused

PLTs. An additional problem is the lack of comparative studies with data on sensitivity and specificity of the different markers.

Prolonged storage induces a gradual increase in PSE [25-27], PS exposure [3,21], and a decrease in GPIbα expression [28-30]. Leytin et al. described that PSE showed a positive and GPIb expression a negative correlation with phagocytosis of PLTs *in vivo* [8].

Previously we provided evidence that binding of PLTs to macrophages is mediated by surface expressed P-selectin and phagocytosis by exposed PS and changes in GPIb. Changes in GPIb were detected as a decrease in the binding of a PE-labeled Moab against AA1–35on the N-terminal flank and thought to be related to the clustering of GPIb observed after cooling of PLTs [31].

The aim of this study is to introduce a novel *in-vitro* prediction model system to predict PCs fate, posttransfusion. We hypothesis that using changes in these three PLTs markers (P-selectin, PS exposure, and GPIb), would be enough to (dis-)qualify any randomly selected and prepared PCs, pretransfusion.

Materials and Method

This retrospective study of data were prepared and analyzed from 30 different independent human voluntary donors with inform consent as previously described [3]. The quantitative and qualitative binding and phagocytosis assays were done with BD-FACSCalibur flow cytometry, and high confocal (video) microscopic photos acquired under flow, and were measured as described previously [3,30]. The data were analyzed using WinMDI-and Graphpad 6.0 softwares and statistics. The data presented are Mean ± SEM of 30 randomly selected assays with human isolated PCs *in-vitro*.

Results

Freshly isolated resting PCs (Figure 1) stored at room temperature for 1 hour showed a single population with high affinity to the GPIb (Figure 2). Retrospective study of interaction data acquired from human PCs and PMA stimulated THP-1 cells under *ex-vivo* condition revealed that less than 5% of fresh PLTs were bound and removed by THP1 phagocytes.

We have chosen this threshold and cut-off limit to build and introduce our novel prediction model system. Moreover, our hypothesis was that it should be possible to assess a reconsider quality control of any random PCs, pretransfusion.

Prolonged storage condition was accompanied by an increase in PLTs binding to and phagocytosis by macrophages in a stepwise manner (Figure 2A and 2B).

In freshly isolated PCs the P-selectin expression and binding were less than 5% but after prolonged storage condition increased binding to macrophages to 25 ± 15% while the removal percentage of PLTs by macrophages was less than <5%.

After 48 hrs storage this fraction decreased to 60% resulting in a step by step increasing of PCs by phagocytes (Figure 2C).

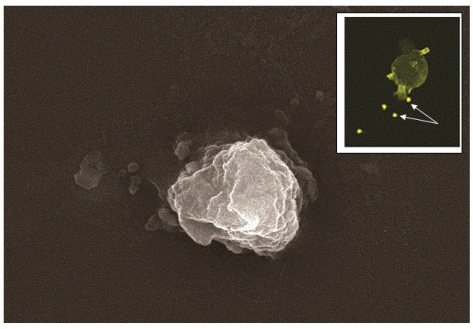

Figure 1: Confocal high resolution microscopy of human platelets in resting state. Insertion. In this video microscopy photo under flow, the PCs labeled with mepacrine (Quinacrine dihydrochloride, Sigma-Aldrisch, Germany) and introduced to the PMA-stimulated THP-1 (un-)differentiated monocytes under *ex-vivo* condition. These photos give an idea about evidences in relation to the resting PCs, which do not bind and phagocyted by phagocytes [3,30].

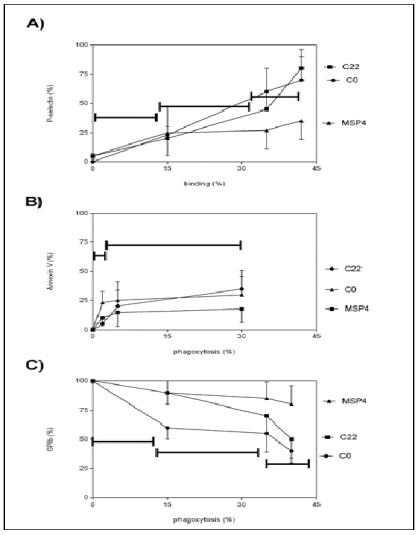

Figure 2: Causal relation between storage temperature, metabolic condition of platelets concentrates versus increasing in phagocytosis. Correlations between A) P-selectin expression vs binding percentage of PCs B) Annexin V binding to Phosphatidyl serine, and C) GPIb expression vs an increase in phagocytosis percentage in platelets from the same donor stored at room temperature (C22), ice 0°C(C0) and in refrigerator 4°C after metabolic suppression (MSP4) after 0, 40 min, 48h and 72 h storage. These figures indicates that the fresh PCs do not bind and phagocyted by macrophages but after prolonged storage step by step, time dependent, and receptors-condition dependently phagocyted by THP-1 macrophages in vitro. Hence, changes in P-selectin expression, PS exposure, and GPIb could be used as a qualification threshold to (dis-) qualify any random PCs prepared by any kind of method.

Fresh PLTs showed a mean ± SEM about 10 ± 8% P-selectin expression in the absence of PMA and 50% in its presence, confirming

the role of PMA as a secretion-inducing agent. Phagocytosis of the same platelets was <5% with and without PMA removal. After 48 hours storage, there were about 50% P-selectin expression without PMA and 60% with PMA. These PLTs showed about 20 and 35% phagocytosis. Our new acquired data are in line with the concept that P-selectin is an intermediate in binding and bringing PCs on phagocytosis.

We next retrospectively studied the correlations between GPIb peak-2 expression versus phagocytosis, as described [30].

Compared to fresh (T0, assumed as 100%) the GPIb expression and conformational change showed a threshold of less than 25% (Figure 2C), below which phagocytosis was less than <6%, but after prolonged storage GPIb expression was decreased and GPIb peak-2 increased in an ageing-dependent manner from 30 up to 90% which correlated linearly with higher phagocytosis (Figure 3).

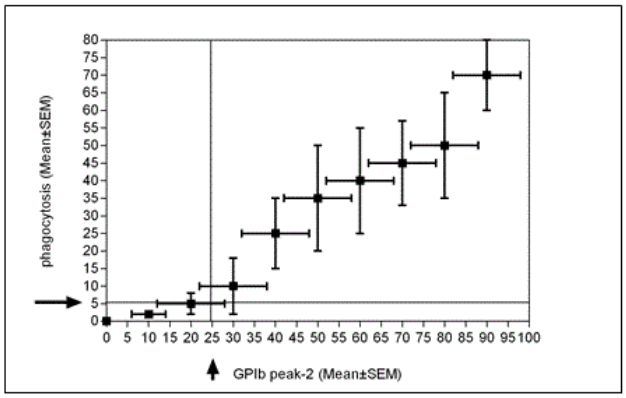

Figure 3: GPIb clustering-like appearance (GPIb peak 2) versus aggravation of phagocytosis of C22 platelets after 0, 40 min, 48h and 72 h storage. The data presented are the Mean ± SEM of 20 different experiments *in vitro*. This figure illustrates the PCs based on quantitative GPIb-decay (-dependent) phagocyted by macrophages with a threshold of 25% (n=30). Hence, GPIb degradation and changes could be used as biomarker of the recipient's immune response.

Correlations between either P-selectin expression versus binding percentage of PCs; and Annexin V binding to phosphatidyl serine (PS), or GPIb expression versus an increase in phagocytosis percentage indicated that the fresh PCs do not bind and phagocyted by macrophages. Therefore, immune response should be minuscule.

After prolonged storage and ageing-related membrane lesions step by step, time dependent, and receptors-condition dependently the PCs removal increased. Consequently, any significant changes in P-selectin expression, PS exposure, and GPIb could be used as a qualification threshold to (dis-) qualify any random PCs prepared by any kind of method, pretransfusion.

Discussion

Different extra- and intracellular PLTs markers have been tested as indicators for PCs quality and quantity. PLTs in rest were not removed by phagocytes, which their external and internal proteins are suppressed to remain in their places. In this retrospective study we observed that a slight degree of PS exposure (<5%) and GPIb change (<20%) do not induce phagocytosis, remarkably. There appears to be a threshold that primes phagocytes to bind and remove PLTs. Moreover only when shape and surface changes exceed this threshold level, PLTs become 'stepwise' targets for destruction and phagocytosis. It is noteworthy that phagocytosis of a fraction of PLTs does not mean whole population would be destroyed subsequently. Any PLT that remain intact and kept all ex- and internal proteins in their position will not be phagocyte as we observed after recovery of metabolic suppressed PLTs (MSP4/48 hrs) [3,30]. Beside we observed that fresh PLTs show a slight degree of P-selectin expression, PS exposure and GPIb change but they were not phagocytized [3,30]. We found a marginal threshold for PSE, and even in fresh PCs a slight increase in PSE induced binding to macrophages but finally did not turn over to removal of fresh PCs, however. In old PCs only metabolic recovered PCs has lower phagocytosis which indicates GPIb changes and PS exposure are not irreversible processes and could be repaired metabolically pretransfusion [3,30,32-35]. Interestingly, metabolic suppression prior to cold storage greatly suppressed the expression of phagocytic signals. The relation between PS exposure and GPIb change *versus* phagocytosis appears to follow the correlation established with RT stored but the low PS expression and GPIb change hamper reliable calculations.

The finding that metabolic suppression also reduces the change in GPIb receptor during prolonged storage is still unexpected phenomenon. GPIb is the receptor that mediates platelet adhesion to VWF [6]. The cooling of PLTs is thought to trigger irreversible GPIb clustering which provides a signal for rapid destruction by phagocytes, confirmed Hoffmeister et al. [35-37] In contrary, also storage at RT (18-24°C) shows also old PCs more prone to phagocytosis, suggesting that the same mechanism might control the removal of RT-stored PCs, eventually.

Taken together, an slight increase (<0.5%) in the PSE, PS exposure and GPIb changes induced more binding to phagocytes but did not further result in clearance of PCs. In contrast, higher than 30% PS exposure and GPIb change showed direct stepwise increase in phagocytosis with a certain threshold. If the regulation of PLT binding and phagocytosis by macrophages are restricted to surface expression of P-selectin, PS, and changes on GPIb, One might expect that correlations established here might valid for any random PCs stored under different conditions.

It is clear that the present correlations between surface markers for PCs destruction by macrophages are based on an in vitro system with matured THP-1 cells. At present it is uncertain to which extent this set up is an accurate reflection of the destruction of PCs under *in-vivo* conditions. If such a correlation could be established in future studies, the sensitivity of transfused PCs to be destructed by macrophages could be predicted on the basis a few, rather simple quantitative and qualitative assay(s)-analysis. In the near future, if by any company, pharmaceutical drugs developed and introduced, which can prevent PCs damages and/or restore stored PCs lesion pretransfusion, all can be qualitatively controlled by such simple combination assay.

In conclusion, our results signify that the chosen three markers combination measurement are representative for phagocytic (ir-) responsiveness. These parameters measured *in-vitro* could be used as the quality control markers of any randomly selected and prepared PCs, pretransfusion. In the near future, when these thresholds established as standard thresholds then any (Para-)medic can predict

on the basis of relatively simple analysis, whether delivered PCs from the blood banks are immunogenic or not.

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