

PROLONGED PLATELET PRESERVATION BY TRANSIENT METABOLIC SUPPRESSION

Bahram Alamdary Badlou

Cover: "Girl in Hibernation" adapted from Google

A drawing of resting of platelets (Platelet Hibernation) and after activation by prolonged storage without recovery made by the Scanning Electron Microscopy (SEM). The middel photo is made by regular Fluorescence Microscopy of the interaction between mepacrine-labeled platelets with THP-1 macrophages.

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PROLONGED PLATELET PRESERVATION BY TRANSIENT METABOLIC SUPPRESSION

HET VERLENGEN VAN DE BEWAARTIJD VAN BLOEDPLAATJES MET BEHULP VAN METABOLE
SUPPRESSIE

ACADEMISCH PROEFSCHRIFT

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door

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It is not important who you are.....

It is important what you want to achieve....

Best future perspective can be yours if you do your best for it...

This is our call ... to change the course in appropriate way. Yes that is what I am trying to do!

Bahram A. Badlou

For my family and my dear father and mother

you all, that I will always love you

wherever you are....

For you dear Majid Mashadi

As I promised you ...

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Abbreviations

AA = amino acids

ACD = (2.5 g tri-sodium citrate, 1.5 g citric acid and 2.0 g D-glucose in 100 mL distilled water)

AEC = adenylate energy charge

AN51 = anti human GPIb, CD42b

Antimycin A = an inhibitor of mitochondrial respiration, inhibits transfer of electrons from cytochrome B to C

BAPTA-AM = a calcium chelator

BC's = buffy coats

$[Ca^{2+}]_i$ = intercellular calcium concentration in platelets

cAMP = cyclic adenosine monophosphate

CD14 = anti human-monocytic and macrophages antigen; GP53-55

CD42b = anti human -GPIb; AN51 clone R7014 unlabeled from Dako cytometry

CD62p = anti human - P-selectin a component of the α -granule membrane in platelets

CD63 = anti human -GPIIb/IIIa

C0 = control group stored on ice

Cyto B = cytochalasin B an inhibitor of actin polymerisation

ER = Energy-reduced platelets induced by glucose deprivation

GPIb = glycoprotein Ib α , a member of GPIb $\alpha\beta$ -V-IX complex receptor at surface of platelets

GlcNAc = N-linked glucosamine, a monosaccharide at GPIb

Glycocalicin = a proteolysis sensitive region of GPIb α receptor, contains vWF binding site

HBSS buffer = (0.3 mM KH_2PO_4 , 13.7 mM NaCl, 417 mM $NaHCO_3$, 31 mM Na_2HPO_4 and 0.5 mM KCl in aqua dest buffer)

Hepes-Tyrode buffer = (137 mM NaCl, 2.68 mM KCl, 0.42 mM NaH_2PO_4 , 1.7 mM $MgCl_2$, and 11.9 mM $NaHCO_3$, pH 7.2)

Mac-1 = monocytes and macrophages CR3b1 receptor; GP170; CD11b

MSP4 = metabolic suppressed platelets prior to storage at 4°C (refrigerator)

PC's = platelet concentrates

PFA = paraformaldehyde 2%

PGI₂ = prostaglandin I₂ used to prevent platelet activation during differential centrifugations

PMA = phorbol 12-myristate 13-acetate

PO₂ = oxygen pressure and tension in the medium

PS = phosphotyrosine

PLT = platelets

PSL = platelet storage lesion

TAPI = N (R)-[2-(hydroxyaminocarbonyl)methyl]-4-methylpentanoyl-L-alanine amine TNF- α protease inhibitor

THP-1 = monocytic cell lines THP-1

TPO = thrombopoietin hormone

TRAP = Thrombin receptor-activating peptide SFLLRN

VWF = von Willebrand Factor

Chapter 1

General introduction

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1.1. Platelet formation, survival

Platelets are anucleated cells that arise in the bone marrow from megakaryocytes under control of thrombopoietin (TPO).¹ After the megakaryocytes mature, proplatelets are formed and the cytoplasm becomes demarcated into platelet fields (Figure 1). Platelets are released into the circulation through a process of megakaryocytic fragmentation.² The minimal platelet age is about 9 days, and the maximum about 19 days.³ Normally, two-third of the platelets released from the bone marrow stay in the peripheral circulation; the remainder is sequestered in the spleen and is freely exchangeable with the circulating platelets.^{1;3} Smith⁴ postulated that in patients with splenomegaly, a larger percentage of the platelets is sequestered in the spleen, and that peripheral thrombocytopenia may develop. There is a direct relationship between the megakaryocyte mass in the bone marrow and the rate at which platelets are released into the circulation.² When bone marrow is maximally stimulated, it can increase platelet production six- fold.⁵ When platelets are rapidly destroyed, increased delivery of platelets to the peripheral blood shows a lag time of approximately 5 days. Even then, thrombocytopenia may continue if the rate of platelet production cannot keep up with the rate of platelet destruction.⁴

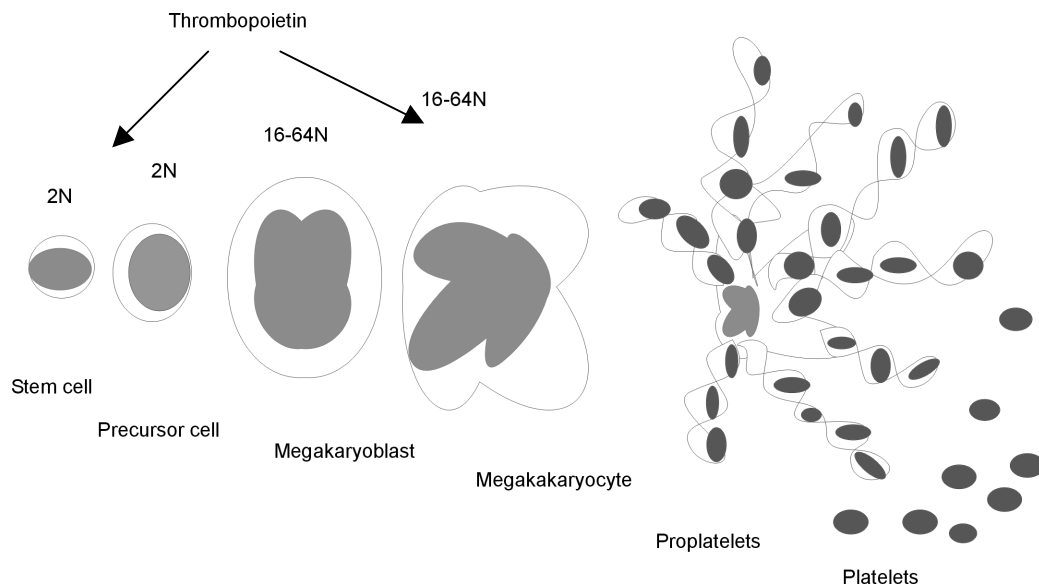


Figure 1. Platelet generation from megakaryocytes

1.2. Platelet functions

In haemostasis, platelets play two major roles:

- 1) Platelets arrest bleeding from severed blood vessels.⁶ When platelets encounter a disturbance in the endothelial surface, platelets adhere, aggregate and prevent excessive blood loss.
- 2) They provide phospholipids that act as the catalytic surface for the coagulation cascade^{7:8}

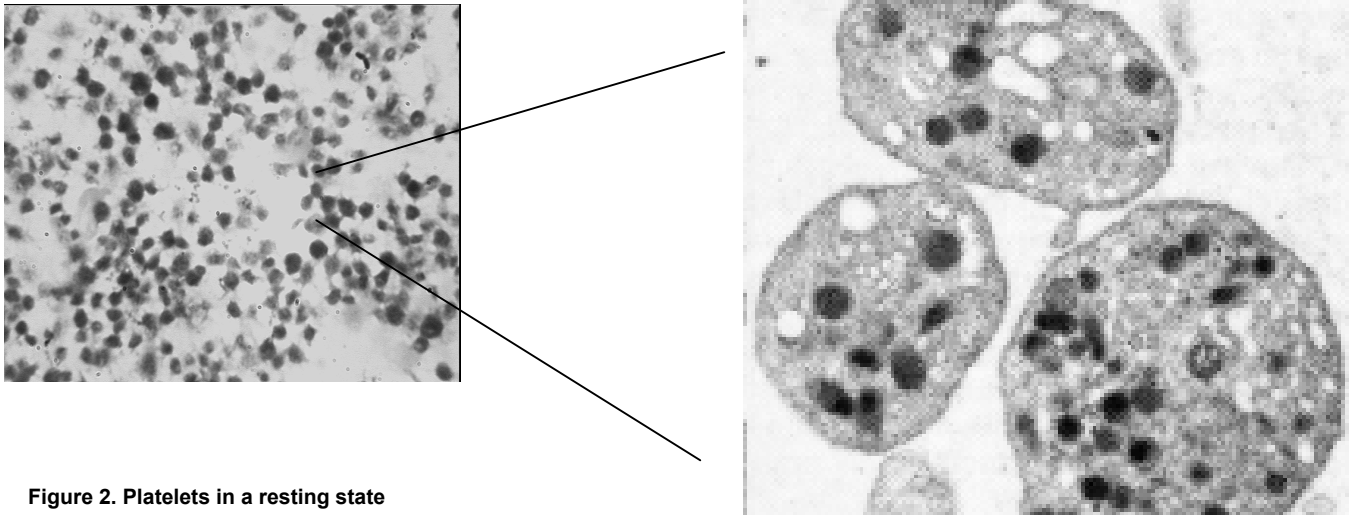


Figure 2. Platelets in a resting state

Platelets remain in a resting condition through release of NO and PGI₂ from the endothelium (Figure 2). This keeps platelets in a resting state by inducing formation of cyclic GMP (cGMP) and AMP (cAMP), which are major inhibitory second messengers.⁹ Other activators of a cAMP increase are prostaglandin E₁ (PGE₁), PGE₂, PGD₂, PGI₂ and adenosine.⁹⁻¹¹ Upon activation by stimulating agents, platelets undergo a rapid transition in shape from a discoid morphology to spheres with pseudopods.¹² Intima injury associated with endothelial denudation and plaque rupture expose subendothelial collagen and von Willebrand factor (vWF), which support prompt platelet adhesion and activation.¹³ The platelet is extremely sensitive to changes in the environment. A variety of stimuli such as physiological activators, artificial surfaces, mechanical stress, low temperature, and drugs induce a disc-to-sphere transformation.^{12;14;15} Platelet responses start with binding of the glycoprotein (GP) Ib-IX-V complex to vWF in the injured vessel wall. Rolling platelets slow down by adhesion to exposed vWF and undergo shape change (Figure 3).¹⁶

Platelet-specific adhesion receptors mediate these interactions. Other membrane glycoproteins, such as the collagen receptor GP VI, trigger platelet activation. Engagement of GPIb-IX-V or GP VI ultimately leads to platelet aggregation mediated by the integrin, αIIbβ₃ (GPIIb-IIIa).¹⁶⁻¹⁸ After spreading, platelets start releasing ADP and TXA₂.¹⁷ Subsequently, the generation of thrombin starts, which is very strong thrombogenic factor. Platelets form stable aggregates and seal the damaged vessel wall.¹⁹ Following clot retraction, fibrinolysis starts that dissolves the fibrin network and makes room for wound healing processes.^{19;20}

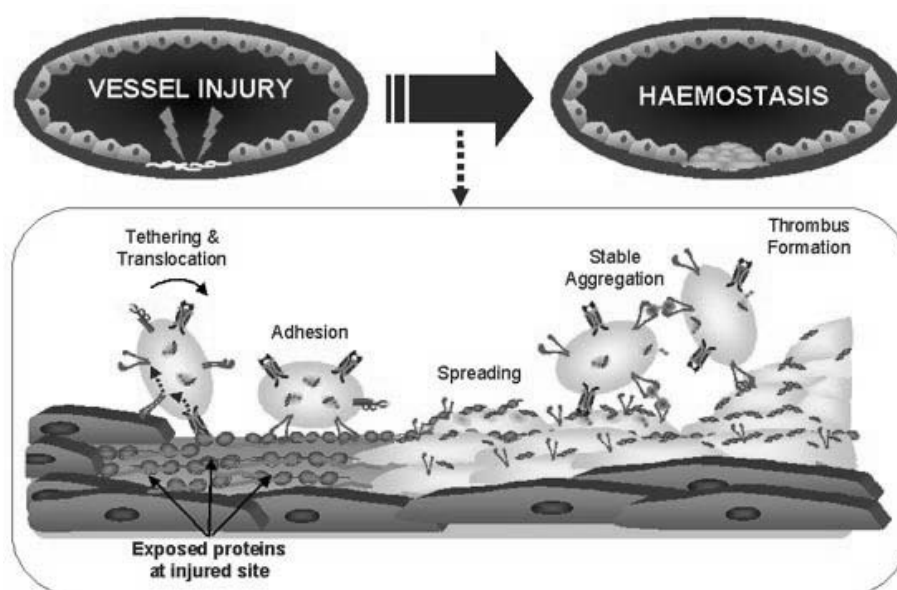


Figure 3. Platelets function at the vasculature

1.3. Platelet cytoskeleton and organelles

Platelets contain two major skeletons: 1) the membrane skeleton and 2) the cytoskeleton. Contractile proteins consist of actin (both as globular and as filamentous actin), and myosin². Filamentous actin (F-actin) is especially found close to the plasma membrane. Myosin is mostly found in the cytosol of platelets. In resting platelets 40% of the actin exists in the form of F-actin, which is not highly cross-linked.^{21;22}

Platelet activation alters the structure of the membrane skeleton through Ca^{2+} -dependent proteolysis²² of actin-binding protein.²¹⁻²³ Disruption of the membrane skeleton causes a decreased stability in the lipid bilayer and an increased mobility of membrane glycoproteins.²³ This permits the disc-to-sphere change.^{23;24} Upon stimulation of platelets the cross-linking increases and polymerization of globular actin (G-actin) takes place.^{14;21} In addition to the cytoplasmic actin filaments, platelets contain a membrane skeleton, which is composed of short actin filaments cross-linked by actin-binding protein.²⁴ This membrane skeleton supports the plasma membrane and is linked to the plasma membrane mainly by the GPIb-V-IX complex.^{14;21;24} Phosphorylation of myosin light chains causes the myosin molecule to associate with actin filaments.²³ In addition, platelets contain the microtubules, which consist of tubulin. It is present at the periphery of the platelet, just beneath the membrane skeleton.^{25;26} When the microtubule is disrupted by an increase in $[\text{Ca}^{2+}]_i$ or exposure to cold the platelets change in shape.^{14;27} Shape-change occurs at small increases in Ca^{2+} , while aggregation and secretion of granules take place at higher Ca^{2+} levels.²⁸ This indicates that the microtubules are also important for the maintenance of the shape of platelets.

1.4. Platelets contain three different types of secretion granules

Platelets contain three major secretion granules: 1) α - granules 2) dense granules and 3) lysosomal granules. The contents of the α - granule can be divided into platelet-specific and non-platelet specific proteins. These proteins are involved in the promotion of haemostasis and tissue repair.²⁹ The internal side of the α - granules membrane contains the GPIIb/IIIa complex and P-selectin. Both proteins are exposed on the platelet membrane upon secretion.³⁰⁻³¹ P-selectin is a good marker for platelet activation because in the resting state it is not surface-expressed. Its surface expression correlates with the release of α - granule content. P-selectin can not be re-internalized following secretion.^{32;33} The second important granule is the dense- granule, which contains mainly Ca^{2+} , inorganic pyrophosphate, ATP, ADP, serotonin and catecholamine.^{34;35} ADP and serotonin play an important role in amplifying the platelet response. The significance of the dense granules is illustrated in hereditary and acquired storage pool deficiency.^{36;37} Both diseases are characterized by a depletion of the content of the dense granules and this deficiency leads to defect in the secondary phase of aggregation and to a bleeding tendency.³⁸ The lysosomal granules are common cell organelles, which play a role in the cellular degradation system as well as in autolytic processes.

1.5. Platelet mitochondria

Platelet mitochondria are an important subcellular compartment and are responsible for oxidative phosphorylation. Platelet mitochondria consist of an outer membrane, an intermembrane space, an innermembrane, and the matrix. The energy production is a result of electron transport from the matrix to the innermembrane space leading to phosphorylation of ADP to ATP. In this process oxygen serves as the electron acceptor. Oxidative ATP synthesis is coupled to transmembrane proton fluxes.

The flow of electrons from NADH or FADH_2 to O_2 through protein complexes located in the inner membrane leads to the pumping of protons out of the mitochondrial matrix. Metabolic energy is generated by the conversion of fatty acids to CO_2 . Although the rate of ATP resynthesis varies among different donors the actual availability of energy is present in the steady-state levels of metabolic ATP (ATPm) and metabolic ADP (ADPm). A better reflection of the energy status in the cell is the adenylate energy charge ratio, $\text{AEC} = (\text{ATP} + \frac{1}{2} \text{ADP}) / (\text{ATP} + \text{ADP} + \text{AMP})$. This concept was developed by Atkinson.^{39;40} who described the energy stored in the reactants of the adenylate kinase reaction (Figure 4). The AEC is a very sensitive marker of the cell's energy status. Normal platelets have an AEC of 0.92 to 0.94.⁴¹ When this parameter is lowered by gradual inhibition of ATP resynthesis, platelet responses fall. For instance, a fall to 0.8 is accompanied with 50% inhibition of acid hydrolase secretion.³⁷ For a similar inhibition of dense and α -granule secretion, the AEC ratio must fall to 0.65, whereas for 50% inhibition of aggregation an AEC of less than 0.55 is required.^{37;42}

Thus, the different platelet responses show a different energy requirement both with respect to the metabolic ATP level and the AEC ratio. Akkerman et al. described that the relative importance of AEC and ATPm can be studied over a wide range by incubating platelets with inhibitors of mitochondrial respiration and oxidative phosphorylation in a medium depleted of glucose.⁴³ Suboptimal O_2 supply also reduced the levels of ATPm and ADPm, and enhanced lactate production leading to a fall in extracellular pH (Figure 5).⁴⁴ Cytochromes B and C play a pivotal role as electron carriers in the

respiration chain of platelets during rest and activation. Antimycin A blocks electron transport from cytochrome B to C (Figure 4).

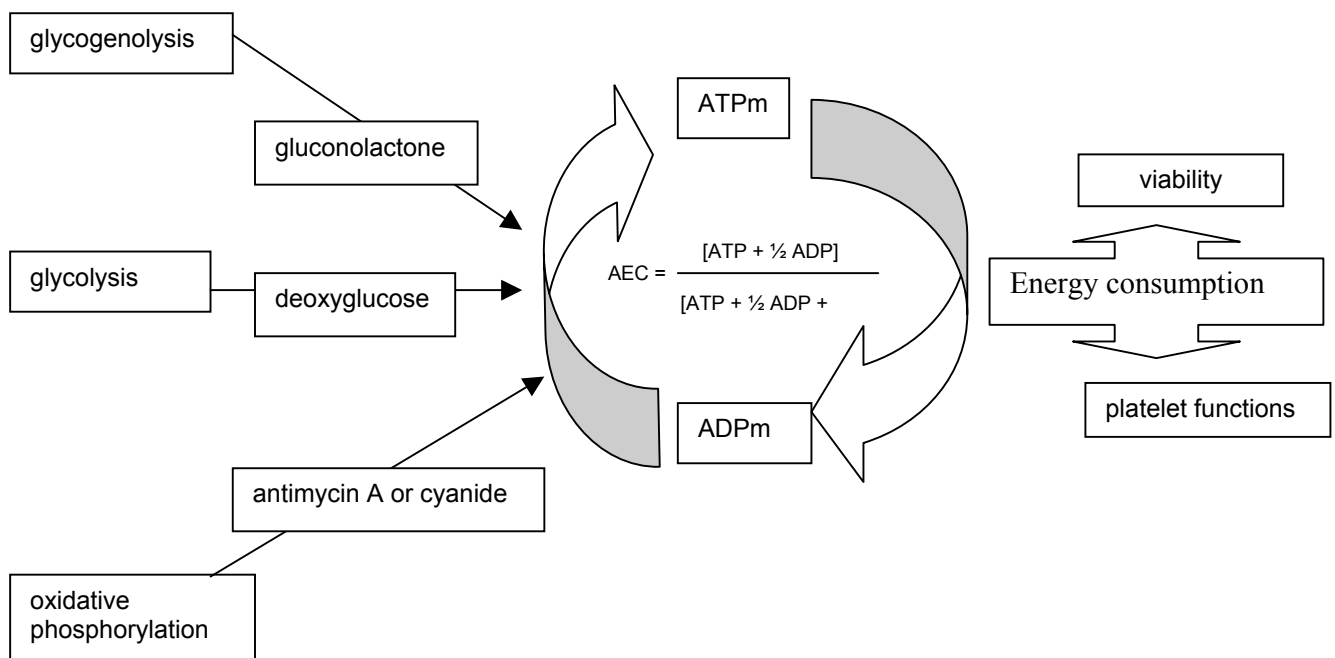


Figure 4. Metabolic suppression by different inhibitors, adjusted from Akkerman et al. 1985. Adenylate energy charge (AEC)

Compared with other cell types, unstimulated platelets have a high rate of energy production and consumption. This is exceptional for a cell that lacks a number of energy consuming processes that are common in other cells, e.g. protein synthesis, biosynthesis of complex carbohydrates, etc. This has led to hypothesis that resting platelets build up a store of energy that is liberated during aggregation and secretion.^{45;46;37} In this respect the role of actin is of special importance since it undergoes a rapid polymerization-depolymerization cycle.^{46;47}

Platelets resynthesize metabolic ATP in glycogenolysis, glycolysis, and oxidative phosphorylation and consume the energy stored in metabolic ATP in the various energy utilizing processes present in unstimulated platelets (basal energy consumption). During shape change, aggregation, and secretion, the energy consumption increases (incremental energy consumption). The balance between energy production and consumption is depicted by the AEC. The energy in ATP and ADP is expressed as ATP equivalents (ATP_{eq}) in which 1 ATP_{eq} represents the energy liberated in the conversion of 1 mole of ATP to 1 mole of ADP.

Rapid abolishment of ATP resynthesis is achieved with metabolic inhibitors, each specific for a certain pathway (gluconolactone, deoxyglucose, antimycin A). This causes a rapid fall in metabolic ATP and, later, in metabolic ADP. An important feature of platelets with reduced energy production and consumption is that the cells lose their responsiveness to activating agents. Apparently, under conditions of limited energy availability priorities are set to preserve cell integrity at the expense of cell

functions. Thus, it is possible to prevent platelet activation by transient metabolic suppression and to restore functionality prior to infusion by restoring energy supply.

Platelets preserve their rapidly accessible energy in the form of metabolic ATP and ADP (ATPm and ADPm) as opposed to storage ATP and ADP in the dense granules, which is not accessible to energy demanding functions.⁴⁵ With sufficient glucose and O₂ in plasma the combined glycolytic and mitochondrial activity suffices to maintain stable levels of ATPm and ADPm and an optimal adenylate energy charge AEC.^{41;48}

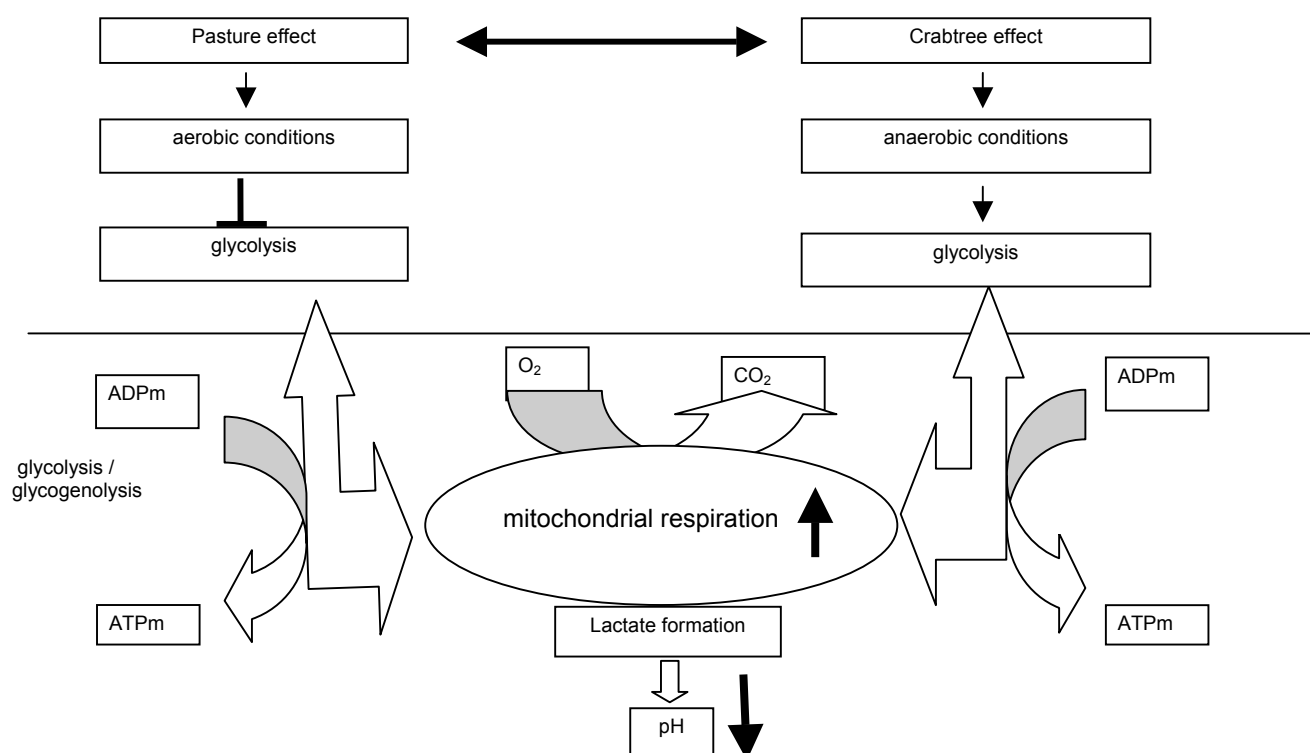


Figure 5. Platelet metabolic energy production. Rapidly accessible metabolic energy is metabolic ATP (ATPm) and ADP (ADPm).

The absence of glucose compromises optimal energy parameters and impairs platelet functions.⁴⁹ Under prolonged hypoxic conditions, platelet mitochondria and caspases are directly involved in platelet apoptosis.⁴⁸⁻⁵¹

In O₂-rich conditions glycolysis is suppressed (Pasteur effect) conversely, in O₂-poor conditions glycolysis is not suppressed and lactate production increases (Crabtree effect) (Figure 5). Genova et al. described that mitochondria are strong producers of reactive oxygen species (ROS), and at the same time, vulnerable to the oxidative damage produced by their action on lipids and proteins.^{52;53} In particular, damage to mitochondrial DNA induces alterations to the polypeptides encoded by this DNA in the respiratory complexes, resulting in a decrease of electron transfer and a further production of ROS.^{52;53} This deficiency in mitochondrial energetic capacity is considered a cause of cell ageing. Complex I would be the enzyme most affected by ROS, since it contains seven of the 13 subunits encoded by mitochondrial DNA.⁵²

Leytin et al proposed the view that platelet mitochondria may be an important apoptotic target during isolation and prolonged storage.⁵⁴ In addition, it has been suggested that depletion of growth factors or treatment with apoptotic stimuli artificially induces anucleated cells to undergo apoptotic features that are indistinguishable from those of their parent nucleated cell during apoptosis.^{54;55} Therefore, the collective evidence so far suggests that in platelets apoptosis may be induced by mitochondria.⁵⁶ After chronic exposure to various harmful stimuli platelet mitochondrial function reduces⁵³, for instance after: prolonged exposure to cold ($< 15^{\circ}\text{C}$);⁵⁷⁻⁵⁹ exposure to high concentrations of mitochondrial inhibitors e.g. cyanide and antimycin A;⁵¹ or by an increase in lactate production during a prolonged anaerobic condition.^{60,52;53}

1.6. Platelet receptors involved in platelet function and survival

Platelets contain different receptors and phospholipids involved in platelet function. The major membrane receptors are: GPIb-V-IX complex (vWF receptor), GPIIb/IIIa (fibrinogen receptor), GPVI (collagen receptor). The major phospholipid involved in procoagulant activity of platelets is phosphatidyl serine (PS), which in the resting state is kept in the inner leaflet of the plasma membrane by energy and Ca^{2+} -dependent processes. The major adhesive molecule expressed from α -granule to the surface of platelets after activation is P-selectin. The GPIb-V-IX complex is present at the surface of platelets and composed of 4 transmembrane subunits: GPIb α (135 kD, 610 residues), GPIb β (25kD, 181 residues), GPV (82 kDa, 544 residues) and GPIX (22kD, 160 residues) in the ratio of 2:2:1:2.^{61,62} The full complex is formed in the endoplasmic reticulum and transported to the Golgi for further modifications. The GPIb-V-IX complex is highly glycosylated (Figure 6). GPIb represents the major sialoglycoprotein on the plasma membrane of platelets and the different N- and O-linked oligosaccharides which are ligand binding domains are primarily localized in the α -subunit of GPIb α .⁶¹

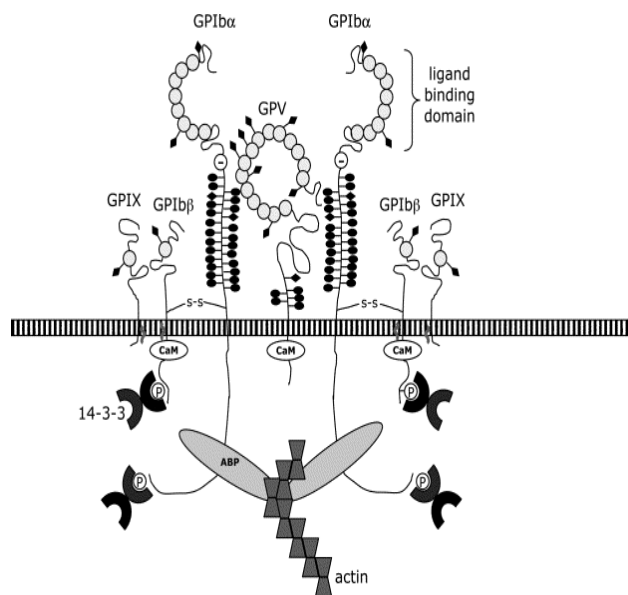


Figure 6. Model of the GPIb-V-IX complex adapted from Canobbio et al. 2004. The cytoplasmic tail of GPIb α and GPIb β are constitutively phosphorylated. Calmodulin (CaM), adapter protein 14-3-3 (14-3-3) that has a phosphorylation site (P), actin binding protein (ABP), N-linked monosaccharides (○), N-acetyl glucosamine (GlcNAc), O-linked monosaccharides (●)

The C-terminal serine of GPIb α is constitutively phosphorylated and is under control of cAMP-dependent protein kinase A. Canobbio et al⁶¹ described that the cytoplasmic domains of the single subunits of the GPIb α interact with the cytoskeletal actin-binding protein and 14-3-3 ξ adapter proteins. The β -subunits of GPIb contain N-linked oligosaccharides which are covalently linked to GPIb α and non-covalently to GPIX. The cytoplasmic domain of GPIb β interacts with calmodulin and adapter protein 14-3-3 ξ .¹⁷ GPV is composed of an extracellular domain with 15 Leucine-rich-repeats (LRRs), a single transmembrane domain, and a cytoplasmic tail of 16 amino acids.^{63;64} The cytoplasmic tail of GPV associates with calmodulin and 14-3-3 ξ protein as well.¹⁷ GPV binds via ligand binding domains and the different N- and O-linked oligosaccharides to GPIb α . GPIb on platelets may also mediate platelet-endothelium or platelet-leukocyte adhesion by recognition of P-selectin or Mac-1, respectively.¹⁷

P-selectin (also known as CD62p, GMP-140, and PADGEM) is a component of the α granule membrane that becomes surface-expressed after activating agents induce exocytosis of secretion granules.^{65;66} It mediates the tethering of platelets to leukocytes *in vitro* and *in vivo*.⁶⁶⁻⁶⁸ Another important feature of platelet activation is the membrane appearance of negatively charged phospholipids such as PS.^{69;70} PS is also exposed on cells going into apoptosis.^{50;54;71-73} PS exposure may form recognition sites for destruction of senescent platelets and a similar mechanism might remove stored platelets from the circulation.^{71;72;74;75}

1.7. Prolonged preservation of platelet viability and function

The storage of platelet concentrates at room temperature facilitates bacterial multiplication⁷⁶ and introduces changes in platelets indicative for activation and initiation of apoptosis.^{58;77} Improvements have been sought in lowering the storage temperature. Platelet metabolic energy production shows a decrease approximately by factor 2 per 10°C, lowering in storage temperature.⁷⁸

Attempts to preserve platelet membrane integrity have been based on addition of glycine, trehalose, and DMSO during chilling and freezing of isolated platelet concentrates.^{59;79} Eventually, platelets become activated and form aggregates that make them vulnerable for rapid clearance by phagocytes *in vivo*.^{15;57-59;80} Despite all these problems, the anticipated advantages of cold storage are so powerful that several laboratories continue to investigate possible alternatives to oppose the disadvantages of the chilling process.

Under current investigation is the addition of N-linked monosaccharide to preserve GPIb-V-IX complex against clustering during chilling and rewarming of PC's.⁸⁰ Other investigations focus on addition of second messenger regulators to increase cAMP, cGMP levels to prevent platelet activation during storage.⁷⁶ Other laboratories investigate which fraction of platelet poor plasma (PPP) (> 10 to 30%) in combination with a platelet additive solution optimally preserves platelet function.⁸¹⁻⁸³

Figure 7. Platelet concentrates isolated from human whole blood in the Blood banks by the PRP isolation method and stored in polyolefin plastic bags (600ml), which are permeable for gas exchange.



Currently, the isolated platelet concentrates from pooled buffy coats are stored in sterile containers made of special, gas permeable plastics (nominal volume between 1 and 1½ L). Whole blood is collected into systems that consist of multiple bags. Whole blood is collected in a container with a citrate-based anticoagulant. In the currently used bottom-and-top systems, the collection bag is connected by tubing to a 'top' bag intended for plasma, and a 'bottom' bag for the red cells. The latter bag is connected by tubing to a leuko-reduction filter, that is connected to a container with a red cell storage solution. After the collection of blood, the unit is centrifuged and subsequently placed in a semi-automated machine for transfer of plasma and red cells into the two satellite bags.⁸⁴ During processing the collection bag is squeezed and plasma and red cells are transferred simultaneously from the two outlets into the two bags. The buffy coat remains in the collection container and can be used for production of platelet concentrates. Currently, five buffy coats and one unit of plasma from one of the donors are pooled in a buffy coat pooling system. This system consists of a pooling bag with 6 leads intended for the buffy coats and plasma. The pooling bag is further connected to a leuko-reduction filter that in its turn is attached to a platelet storage bag. After soft spin centrifugation, the platelet rich plasma is expressed on a semi-automated machine through the filter to the platelet storage bag. These pooled units can be stored for up to 7 days after blood collection. Two different plastic bag systems are available for preparation of blood components from a single unit of whole blood.^{84;85} Most experience has been gained in The Netherlands with the quadruple-bag system, which consists of a collection bag with three integrated satellite bags; one of the bags contains preservation fluid for the red cells. After the collection of blood, the unit is centrifuged and subsequently placed in a semi-automated machine for transfer of plasma and RBC's into the two satellite bags.⁸⁴ During processing the collection bag is squeezed and plasma and red cells are transferred simultaneously from the two outlets into the two bags.

1.8. Platelet isolation, storage and transfusion procedures in the blood bank

In the past thirty years the use of blood components for treatment of thrombocytopenic patients has increased significantly. Specific devices (Figure 7) have been developed to prepare separate fractions from a unit of whole blood for transfusion: plasma, buffy coat and red blood cells (RBC). For PC's there are three methods to isolate platelets from whole blood in use in blood banks: 1) PRP method, 2) apheresis, and 3) buffy coat method. The PRP method carried out by direct isolation of PRP and RBC from whole blood, this method is mainly used in USA (Figure 7). On demand five or six single donor units of platelets are pooled for preparation of the PC's containing a fraction of plasma about 10 to 30%.^{83;86-88} The buffy coat method is used in European blood banks, where from whole blood direct platelet poor plasma, buffy coat and RBC are isolated and prepared for transfusion (Figure 8).⁸⁹ PC's produced by pooling of buffy coats (4-6) and a unit of PAS or PPP. It has been demonstrated that white cell can be significantly reduced by special filters either in the blood bank or at the bedside.⁹⁰⁻⁹² At present PC's, independent of their production are stored at room temperature, continuously are laying on flat bed shakers in gas permeable bags to maintain O₂ tension and to lose CO₂.

1.9. Current additive solutions for platelet storage and quality control of PC's

Replacing plasma with synthetic media for storage has the advantage of reducing the allergic reactions posttransfusion, and reduces the need for plasma.^{93,94} There is an extensive body of literature about the use of synthetic media for storage of platelets.^{82;83;95;96} The semiautomated devices prepare buffy coats on a large scale, and 4 to 20 buffy coats can be pooled immediately after preparation and resuspended in specific synthetic media with 10 to 30% plasma.^{83;96} Platelets resuspended in additive solutions show a gradual increase in P-selectin expression and lactate production during prolonged storage. Hence, a minimum of 30% plasma remains is necessary to preserve premature activation.^{81;97} Major progress has been made by the introduction of acetate (PAS-II).^{86,98} Eggen et al. showed that addition of potassium, gluconate and magnesium to additive solution, better preserves use of PC's.^{83;87;99} Similar results were seen with platelets prepared by buffy coats and apheresis methods, despite differences in equipment, the preparation technique and the final platelet counts. Gullikson et al. described that storage of platelets in PAS-IIIM (containing potassium, magnesium and acetate) improves maintenance of platelet function and allows a plasma reduction to 20%.^{96;97}

Methods to evaluate the effect of additive solutions on PC's are based on P-selectin expression,^{100;101} and PS exposure (PS flip-flop).^{50;54;77} Morphological score, hypotonic shock response, and shape change are also used by a number of Blood banks (Table 1).^{59;101;102} Today it would be valuable to develop a so-called golden standard for *in vitro* methods for evaluation of platelet function, which can be used to predict platelet haemostatic effectiveness and survival *in vivo*.

Currently, different quantitative measurements of P-selectin expression and annexin V binding to exposed PS^{56;68;79;103-107} carried out by FACS flowcytometry in combination with aggregation studies that offer valuable information about platelet function, while for survival there is still no good tool yet.

2.0. Attempts to improve platelet survival posttransfusion

After about 9 days leukocytes bind to old platelets and remove them from the circulation by various phagocytic cells in spleen, liver, and kidney.^{65;73;80;108} There is little insight in the mechanisms that regulate stored platelet binding and phagocytosis by macrophages after transfusion.

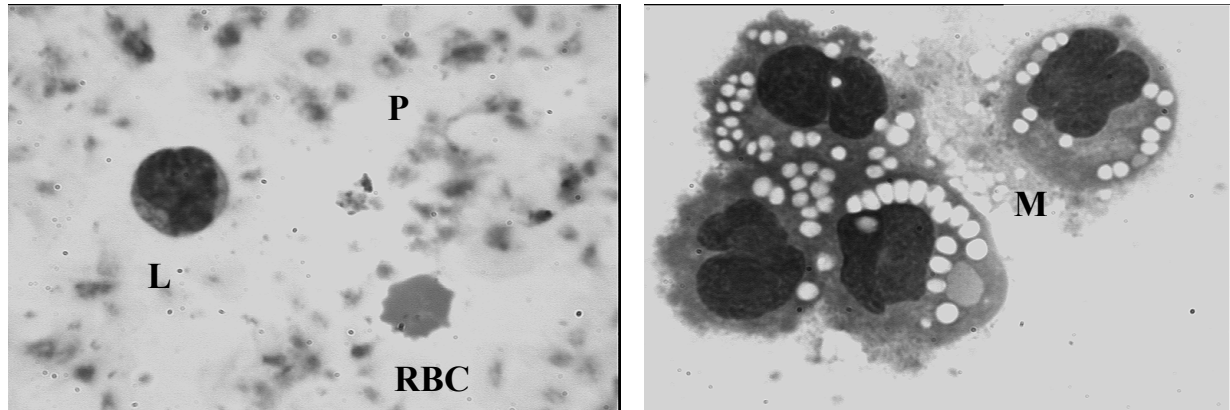


Figure 8. Platelet rich plasma (PRP) isolated from whole blood mainly contains platelets (P), lymphocytes (L), monocytes (M) and sometimes red blood cells (RBC).

Current concepts predict that their clearance occurs via the same mechanism as the destruction of senescent cells.^{54;73;77;109;110} An additional problem is that platelets form complexes with monocytes (PMC) *in vivo*. Different studies show that platelets and leukocytes also form complexes after isolation of donor blood.^{13;111;112} It is still unclear which cell is first activated to form PMC's.

Holvoet et al.¹¹³, described that platelets interact with leukocytes but not with phagocytes. They show that activated platelets mediate the homing of leukocytes by interaction with the subendothelial matrix under shear stresses.¹¹³ This indicates that the binding and complex formation of platelets with different cells is a selective process.

According to the literature, there are different possibilities as to how platelets are removed from the circulation. Wagner et al.⁶⁶ described that platelet binding is regulated by P-selectin and PSGL-1 interaction but P-selectin positive platelets were not phagocytosed implicating that phagocytosis is not always a next step after platelet binding to leukocytes. Obviously, other signals are needed to ingest activated and bound platelets by phagocytes. Hoffman et al. described that binding signals differs from phagocytosis signals and that PS exposure is a prerequisite signal for ingestion of cells.

Fadok et al.⁷⁵ described that apoptotic processes are involved in the clearance of PS exposed cells and platelets. Leytin et al.⁵⁵ described that platelet P-selectin is involved in the rapid phase and GPIb in the delayed phase of platelet clearance after transfusion. Hoffmeister et al.⁸⁰ described that rapid binding and phagocytosis of chilled-rewarmed platelets is exclusively regulated by GPIb clustering and exposure of N-linked monosaccharide N-acetyl glucosamine that trigger hepatic macrophages Mac-1 to bind and remove these platelets.^{80;114}

Furthermore, Josefsson EC et al.¹¹⁴ have specified that the macrophage $\alpha\text{M}\beta_2$ integrin αM lectin domain mediates the phagocytosis of chilled-rewarmed platelets. Unfortunately, they did not show the exact mechanism that underlies the GPIb clustering of cold and room temperature stored platelets.

	Quality control of platelet function for platelet concentrates	
	Resting platelet	Activated platelets
< 1960	morphology , MPV, count	aggregation, adhesion tests
> 1970	morphology , MPV, count	aggregation, adhesion tests
> 1980	morphology , MPV, count , shape changes, Δ pH	aggregation, osmotic shock, adhesion tests
> 1990	morphology score , MPV, count, shape changes, Δ pH	aggregation, adhesion, P-selectin expression, osmotic and hypotonic shock response, glyocalicin release
> 2005	morphology score , MPV, count, GPIb, P-selectin expression and changes in the GPIb-V-IX receptor, bacterial screening tests, and pathogen inactivation technologies, proteomics and genomics	aggregation, adhesion, P-selectin expression, HSR, Δ pH, changes in the GPIb-V-IX receptor, PS exposure, gas exchange of plastic bags, functional proteomics

Table 1. Changes in platelet tests and criteria for platelets quality during last 50 years. Mean platelet volume (MPV), hypotonic shock response (HSR), changes in pH levels (Δ pH).

It is also unclear which signals function as ‘bind me signals’ in the chilled platelets and whether the same signals function, as “eat me signals”. Tait et al. 1999, and Hoffmann et al. 2001 postulated that binding, engulfment and ingestion of cells by phagocytes is a step-by-step process and each step needs a specific signal to proceed.^{68,72} Ingestion of bound cells would not be carried out without exposure of PS and binding to the PS-receptor at the surface of phagocytes (Figure 9).

Taken together all abovementioned findings illustrate the complexity of platelet removal from the circulation. These studies were performed with human PC’s isolated and stored at different conditions, and then reinjected to mice and rabbits for survival studies. Therefore, the results cannot comply be extrapolated the human situation.

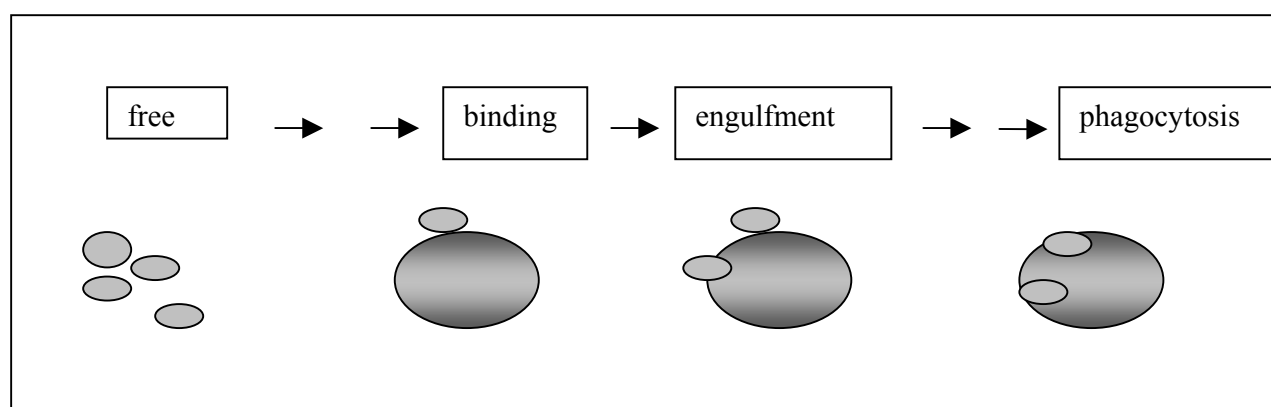


Figure 9. Platelet phagocyte interaction occurs in different steps

2.1. Optimal storage conditions for platelet transfusion

The storage of platelet concentrates at room temperature is accompanied by an increase in bacterial multiplication and changes in platelets, indicative for early activation, apoptosis and membrane damage. The risk of microbial multiplication is the reason that the FDA limits the storage time of platelet concentrates to 5 days.

New technologies developed to improve and prolong shelf life of PC's by pathogen inactivation and bacterial screening prior to prolonged storage, which have promising potentials.^{60;81;115} Recently, bacterial screening is mandatory in the USA. Attempts to preserve platelet function by decreasing the storage temperature primes platelets for rapid clearance. PC's are optimally preserved when their haemostatic effectiveness is kept intact and their survival is normal.

While the platelet storage lesion (PSL) is accompanied by swelling due to loss of membrane control of ion movement, the apoptotic and morphological changes are associated with membrane shrinkage. Both phenomena, are associated with the appearance of PS on the cell surface.¹¹⁰

Rinder et al¹⁰⁸ postulated that elucidation of the reversible aspects of the PSL may result in improved function and survival of transfused platelets. Plasma rescue to an optimal pH improved morphology scores, stabilized osmotic recovery, and completely restored platelet secretory responses, as measured by aggregation, glycoprotein IIb/IIIa up-regulation, and α -granule release.¹⁰⁸ In a limited number of studies, plasma rescue was accompanied by preserved *in vivo* platelet recovery and survival after autologous transfusion after 5 days of storage.

Nevertheless, "storage lesion-injured" platelets restore hemostasis and show good corrected count increments (CCIs) after transfusion into thrombocytopenic animals. These results suggest that at least some aspects of impaired platelet function caused by *in vitro*-storage are readily reversible *in vivo*.¹⁰⁸

A clearer understanding of the different aspects of platelet injury could lead to the development of improved storage conditions for PC's, which might allow to prolong the period of platelet storage and to improve post transfusion function.

2.2. Scope of this thesis

Platelet transfusion therapy is associated with several problems, including refractoriness and transmission of infectious agents and transfusion reactions.¹¹⁶⁻¹¹⁹ In addition, there is a significant decrease in platelet responsiveness to activating agents⁷⁹ and appearance of phagocytosis markers during prolonged storage resulting in decreased haemostatic effectiveness and rapid clearance from the circulation after transfusion.¹⁰⁵ Improvements have been sought in lowering the storage temperature from 22 to 0 – 4 °C and replacing plasma by synthetic media to prolong preservation time and suppress allergic reactions. However, platelets become activated by a sharp decrease in temperature and this so-called cold-induced platelet lesion has long been a major argument against platelet storage at low temperature.

Previous work has shown that suppression of energy generation reduces the capacity of platelets to respond to activating agents. Platelets could even sustain a short period of blocked energy generation without losing their functional properties.

The present study was undertaken to investigate whether metabolic arrest would protect platelets against cold-induced platelet disturbances and would make it possible to store platelets in the cold for a prolonged period of time. Specific questions that were addressed included:

1. Does transient metabolic arrest followed by storage at 4°C better preserve platelet functions than conventional storage (Chapter 2)?
2. Does transient metabolic arrest followed by storage at 4°C suppress the surface expression of phagocytotic signals that would lead to enhanced platelet destruction after transfusion (Chapter 3)?
3. If phagocytosis of cold-stored platelets is mediated via clusters of the Von Willebrand Factor receptor GPIIb α , is it possible to design a method for quantitative analysis of GPIIb α clustering (Chapter 4)?
4. What are the quantitative relations between the surface markers on platelets for platelet-macrophage interaction and binding/ phagocytosis by macrophages (Chapter 5)?
5. Originally, transient metabolic arrest was induced by incubating platelets in glucose-free, antimycin A-containing medium, conditions that are incompatible with platelet transfusion. Can metabolic arrest be induced without antimycin A under conditions that are suitable for platelet transfusion (Chapter 6).
6. Finally, the results of these studies were compared with current literature in order to design strategies for better storage conditions in the near future (Chapter 7).

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Chapter 2

Prolonged platelet preservation by transient metabolic suppression

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Abstract

Background: In this study whether metabolic suppression can be used to preserve platelet (PLT) function during prolonged storage was investigated.

Study design and Methods: Washed human platelets were incubated without glucose and with antimycin A to block energy generation. Metabolic suppressed platelets (MSP) were stored for 72 hrs at different temperatures to find the optimal storage temperature. Controls were incubated with 5 mM glucose and stored at 22°C and 4°C.

Results: Following metabolic recovery with glucose, MSP stored at 37, 22, and 4°C showed (i) an increase in basal P-selectin expression (PSE) reaching > 40% after about 2, 20 and 48 hrs, (ii) a decrease in TRAP-induced PSE inversely related to the increase in basal PSE, (iii) a decrease in TRAP induced aggregation reaching < 30% after about 4, 24 and >72 hrs. When compared with control suspensions, MSP stored at 4°C better preserved a low basal PSE and in addition showed a better adhesion to surface coated-von Willebrand Factor and fibrinogen in a flow chamber.

Conclusions: Metabolic suppression prior to storage at 4°C contributes to better preservation of platelet function.

Keywords: platelet preparation and storage, platelet function, energy, P-selectin, aggregation, adhesion.

Introduction

Modern blood banking procedures aim to provide platelet concentrates (PC's) with optimal haemostatic effectiveness^{1;2} and minimal bacterial contamination.^{2;3} Unfortunately, current procedures for storage of PC's are accompanied by a gradual activation of the platelets as illustrated by surface-expression of the α -granule marker P-selectin and release of granule contents.^{4;5} In addition there is an exponential increase in bacterial growth.^{3;6} These factors limit the storage time to 5 - 7 days and longer storage leads to decrease of platelet viability and increases the chance for febrile reactions.^{7,8} The changes in platelets inflicted during storage are an inevitable consequence of the idea that platelets are best preserved under optimal metabolic conditions at 22°C in bags made permeable to O₂ and CO₂.^{1;9;10} Consequently, current PC's contain platelets that after transfusion fail to survive for more than about 4 days.¹¹⁻¹⁴

The cause for the removal from the circulation of stored platelets is not entirely clear. P-selectin (also known as CD62p, GMP-140, and PADGEM) is a component of the α granule membrane that becomes surface-expressed after activating agents induce exocytosis of secretion granules.¹⁵ It mediates the tethering of platelets to leukocytes *in vitro* and *in vivo*¹⁵⁻¹⁸ and might mediate the clearance of platelets from the circulation,¹⁵ although this has been denied. Attempts to improve the quality of PC's include the application of lower storage temperatures by chilling and freezing of PC's and the use of different cryoprotective agents e.g. trehalose and DMSO.^{13;19;20} An important drawback of storage at low temperature is the risk of spontaneous platelet activation, so called cold-induced activation^{21;22} and the chance that upon transfusion platelets are rapidly phagocytosed by liver macrophages.

Earlier studies have shown that platelets sustain a short period of metabolic suppression without losing their capacity to aggregate and secrete their granule contents.^{13;23;24} Metabolic suppression was induced by a glucose-free medium thereby preventing anaerobic energy generation and the presence of antimycin A, an inhibitor of mitochondrial ATP resynthesis.²⁵ The result was a rapid fall in the adenylate energy charge (AEC), a sensitive reflection of the rapidly accessible metabolic energy in the cell, from a normal level of 9.2 to values as low as 0.2-0.3.^{25;26} At this stage platelets were unresponsive to aggregation- and secretion-inducing stimuli but when energy production was restored by addition of glucose platelet functions recovered.

In this study, we investigated whether metabolic suppression can be used to keep platelets in an unresponsive state during platelet storage while preventing irreversible damage of the capacity to expose P-selectin, aggregate and to bind to adhesive surfaces under flow.

The results show that the successive induction of metabolic suppression, storage at 4°C and recovery with glucose at 37°C better preserve platelet functions than conditions that sustain energy metabolism during storage or are based on cold preservation without prior induction of a low energy turnover.

Materials and methods

We obtained: antimycin A from Sigma Chemicals (Mannheim, FRG), monoclonal antibodies CD42b-FITC, CD42b-PE (R7014) and CD62p-PE (R7200) from Dako A/S (Glusdorp, Denmark), Bovine Serum Albumin (BSA) and Tween-20 from Organon Technika, (Eppelheim, FRG), paraformaldehyde from Sigma-Aldrich, (Mannheim, FRG), human fibrinogen (VWF-free) from Enzyme Research Lab (South Bend, IN, USA), and ristocetin from DiaMed AG, (Cressier s/Morat, Switzerland). Recombinant human von Willebrand Factor (VWF) was purified as described^{27,28}. Thrombin receptor-activating peptide SFLLRN (TRAP) was synthesized with a semi-automatic peptide synthesizer (Labortec AG SP650, Switzerland) according to van Scharrenburg *et al.*²⁹ FITC-labeled IgG (Dako A/S) was used as a negative control in the FACS experiments.

Platelet preparation and incubations

Freshly drawn venous blood (40 ml) from healthy volunteers was collected with informed consent into 1:10 v/v 130 mmol/L trisodium citrate. The donors claimed not to have taken any medication during two weeks prior to blood collection. Platelet-rich plasma (PRP) was prepared by centrifugation (200 g, 15 minutes, 22°C). ACD (0.1 volume of 2.5 g tri-sodium citrate, 1.5 g citric acid and 2.0 g D-glucose in 100 ml distilled water) was added to lower the pH to 6.5 and prevent platelet activation during further isolation. The suspension was centrifuged (330 g, 15 minutes, 22°C) and resuspended in Hepes-Tyrodé (137 mM NaCl, 2.68 mM KCl, 0.42 mM NaH₂PO₄, 1.7 mM MgCl₂, and 11.9 mM NaHCO₃, pH 7.2). Platelet count was measured on a Cellcounter AL871 (Molab, Hilden, Germany). The platelet number was adjusted to 450 000 cells/μl for perfusion experiments and to 200 000 cells/μl for the other experiments. Metabolic suppressed platelets (MSP) were prepared by incubating the cells for 40 minutes at 37°C in glucose-free Hepes-Tyrodé (pH 7.2) in the presence of 20 μM antimycin A. To find the optimal storage temperature for MSP, suspensions (2 × 10⁸ platelets in 1 ml buffer) were kept at the indicated temperatures in closed Ependorf tubes (impermeable to gas exchange) without agitation for up to 72 hrs. At the times indicated, 100 μl samples were collected, incubated with 20 mM glucose in Hepes tyrodé buffer for 1 hr at 37°C to restore energy generation and used for the functional assays described in "Results".^{30,31} Controls were platelet suspensions in Hepes-Tyrodé pH 7.2 containing 5 mM glucose stored at 22°C (indicated as Controls 22°C) or immediately cooled to 4°C (indicated as Controls 4°C) under similar conditions as the MSP. Controls 22°C and Controls 4°C were incubated with 20 mM glucose (diluted in Hepes-Tyrodé) (1 hr, 37°C) prior to the functional measurements to account for a possible glucose shortage. Platelet poor plasma (PPP) was prepared from PRP by centrifugation (650 g, 15 minutes, 22°C).

P-selectin- and glycoprotein Ib expression and aggregation

P-selectin expression (PSE) and expression of glycoprotein Ib (GPIb, CD42b) were measured on the FACScalibur (Becton Dickinson S.A., Aalst, Belgium) in double-labeling experiments according to Tibbles *et al.*³² PSE was analyzed before and after stimulation with the activating peptide for the PAR-1 receptor, TRAP (15 μM, 2 minutes, 22°C). Samples were fixed with 2% paraformaldehyde (30

minutes, 22°C) and washed with 500 µl PBS (500 g, 5 minutes, 22°C). To the pellets were added 50 µl PBS containing 1% BSA and 0.01%-Tween-20. Cells were incubated with monoclonal CD42b-FITC and CD62-p-PE (1 µg/ml) for 1 hr at 22°C in the dark. Then, samples were washed in 500 µl PBS (500 g, 5 minutes, 22°C). To the pellets 300 µl PBS was added and analyzed in the FACScalibur by counting 10 000 particles. PSE was expressed as percentage P-selectin positive GPIb expressing particles.

Aggregation was analyzed in stirred suspensions (1000 rev/minute; 37°C) in a multi-channel aggregometer from Chronolog corporation (Havertown, USA) after stimulation with 15µM TRAP or a combination of 1 mg/ml ristocetin and 50 µl autologous PPP as a source for VWF. Data were expressed as maximal aggregation after 10-15 minutes stimulation.

Perfusion studies under flow

Perfusion assays were carried out in a single passage perfusion chamber as described.³³ Suspensions analyzed were MSP stored at 4°C (MSP 4°C) and controls stored at 22°C and 4°C. Washed platelets were reconstituted with red blood cells and autologous plasma to obtain reconstituted blood with 100 000 platelets/µl and a haematocrit of 40%. Red blood cells were obtained by centrifugation (200 g, 10 minutes, 22°C) and twice washed in saline containing 5 mM glucose (2000 g, 10 minutes, 22°C). Adhesion was measured under flow on fibrinogen-coated thermomax-coverslips (10 µg fibrinogen/ml PBS) and VWF-coated glass strips (10 µg VWF/ml PBS) at a shear rate of 800 s⁻¹ at 37°C. Coverslips were fixed in 0.5 % glutaraldehyde in PBS, dehydrated in methanol (5 minutes, 22°C) and stained with May-Grunwald-Giemsa.³⁴ Platelet adhesion was evaluated by Camera linked to computer-assisted analysis with OPTIMAS 6.0 software (DVS, Breda, The Netherlands) and data were acquired from 20 a-selected random areas and expressed as % surface coverage.³⁴

Statistical analysis

Data are expressed as means ± SD with number of observations, n. Statistical analysis was based on a paired t-test or an one-way ANOVA (with post t-test) for comparison between 2 and >2 groups respectively. Differences were considered significant at $P < 0.05$.

Results

Transient metabolic suppression of platelets

To find the optimal conditions for metabolic suppression, platelets were incubated in glucose-free, antimycin A containing medium for 0 to 210 minutes at 37°C. At different incubation times, samples were collected and incubated with 5 mM glucose for 30 minutes and the basal and TRAP-induced PSE were measured (Figure 1). The basal PSE was constant during the first 40 minutes but longer incubation led to a gradual increase in PSE which was not restored by incubation in glucose-rich medium. TRAP-induced PSE fell sharply during the first 40 minutes incubation in energy-free medium but recovered almost completely upon glucose addition (see below). Longer incubation in energy-free medium led to incomplete recovery of TRAP-induced PSE and after 210 minutes incubation the reversibility had disappeared completely. Thus, the optimal time for inducing metabolic suppression without irreversible loss of agonist-induced PSE was 40 minutes.

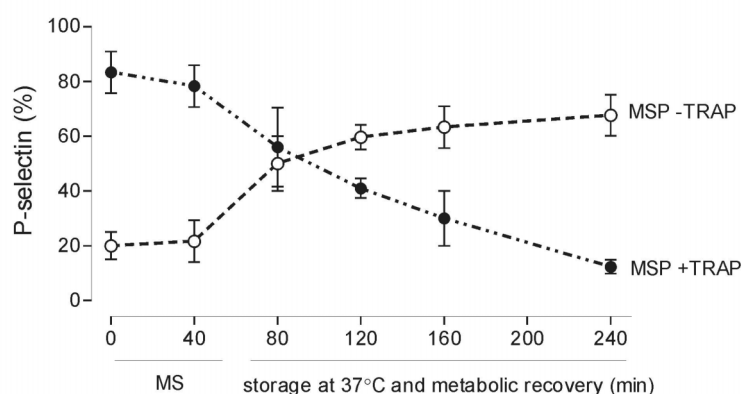


Figure 1. Transient metabolic suppression. To find the optimal incubation period for metabolic suppression, platelets were incubated in glucose-free, antimycin A containing medium for 210 minutes at 37°C. After 50, 90, 130, 170 and 210 minutes incubation, samples were collected and incubated with 5 mM glucose for 30 minutes. Then, PSE of platelets without (open symbols) and with 2 minutes stimulation with 15 μ M TRAP (closed symbols) was measured. The figure illustrates the increase in basal PSE and the fall in TRAP induced PSE after more than 40 minutes incubation in energy-free medium. Data are expressed as P-selectin expression (% of CD62p-PE expressing CD42b-FITC positive particles) and are means \pm SD, n = 3. MS: metabolic suppression.

Storage of metabolic suppressed platelets

Having established the conditions for inducing metabolic suppression, experiments were performed to find the optimal storage temperature of MSP. The 40 minutes incubation in energy-free medium led to a slight increase in basal PSE (Figure 2A). Storage followed by recovery in glucose-rich medium led to a time-dependent increase in basal PSE at all storage temperatures but the increase was faster at higher temperature. A comparison between the different suspensions learned that both control suspensions reached a threshold of >40% PSE after 24 hrs and that MSP stored at 37, 22 and 4°C reached this threshold after about 2, 20 and 48 hrs, respectively.

To study how the MSP preserved their capacity to expose P-selectin upon agonist stimulation, samples were collected and incubated with TRAP. The 40 minutes incubation in energy-free medium

reduced TRAP-induced PSE from about 60 to 10 %. Immediate addition of glucose restored the TRAP-induced PSE almost completely (Figure 2B). After 48 hrs of storage (including recovery with glucose), TRAP-induced PSE recovered to about 60 % in MSP stored at 4°C while the recovery in Control 4°C and Control 22°C suspensions had almost disappeared due to the high basal PSE. Together these data show that a low basal PSE and an optimal TRAP-induced PSE is best preserved in MSP stored at 4°C.

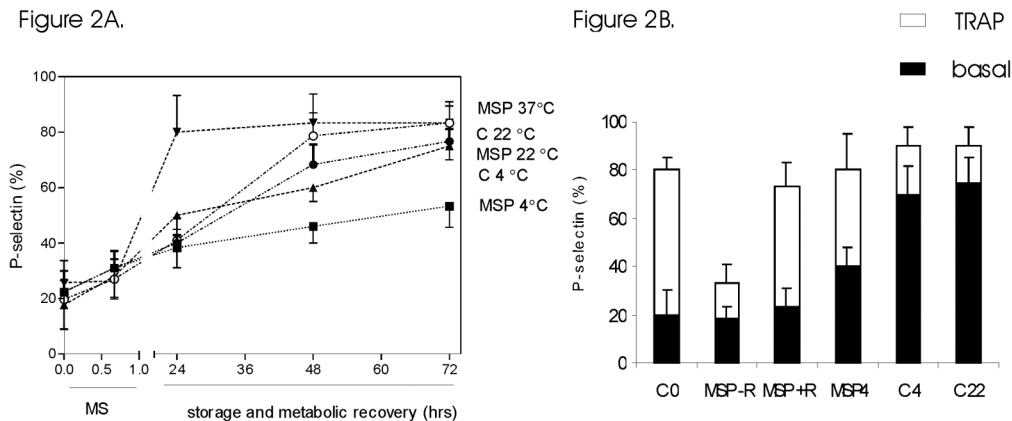


Figure 2. Storage of metabolic suppressed platelets Figure 2A: To find the optimal storage temperature for MSP, platelets were incubated for 40 minutes in glucose-free, antimycin A containing medium (metabolic suppression, MS) and subsequently stored at 37°C (▼), 22°C (▲) and 4°C (■) in the same medium. Concurrently run controls were platelets in glucose-containing, antimycin A free medium kept at 22°C (○) and at 4°C (●). After 24, 48, and 72 hrs, MSP and controls were incubated with 20 mM glucose (1 hr, 37°C). Then, the percentage of CD62p-PE expressing CD42b-FITC-positive particles was measured in unstimulated suspensions. At 24 hrs PSE of MSP 37°C differed significantly from PSE of other suspensions ($P < 0.05$); at 48 hrs MSP 4°C differed significantly from the other suspensions, but the two control suspensions had a similar PSE. Data are means \pm SD for reasons of clarity ($n=3$). Figure 2B: To illustrate the effect of energy depletion, PSE was measured in unstimulated platelets (basal) and in platelets stimulated with TRAP. Shown are data from fresh platelets (C 0), platelets after 40 minutes energy depletion without (MS -R) and with (MSP +R) recovery in glucose-rich medium, MSP after 48 hrs storage with recovery (MSP4), control platelets after 48 hrs storage at 4°C (C 4) and control platelets after 48 hrs storage at 22°C (C 22).

Platelet aggregation and platelet count

Following a 30 % fall in aggregation caused by the metabolic suppression phase, aggregation by MSP stored at 4°C for 24 hrs recovered partially to 55% after incubation with glucose (Figure 3A,B). With longer storage, their aggregability was preserved for at least 72 hrs. The aggregation of MSP stored at 37 and 22°C progressively declined and disappeared completely after 48 hrs. Control 22°C platelets showed a gradual decline in aggregability and after 48 hrs responsiveness was lost. There was a sharp decrease in the number of free platelets in MSP stored at 37°C, a slower decrease at 22°C whereas at 4°C platelet count remained stable. Control 22°C suspensions also showed a decrease in

the number of single platelets. The decrease reflected agglutination and cells could be easily freed by slight agitation (data not shown).

Figure 3A.

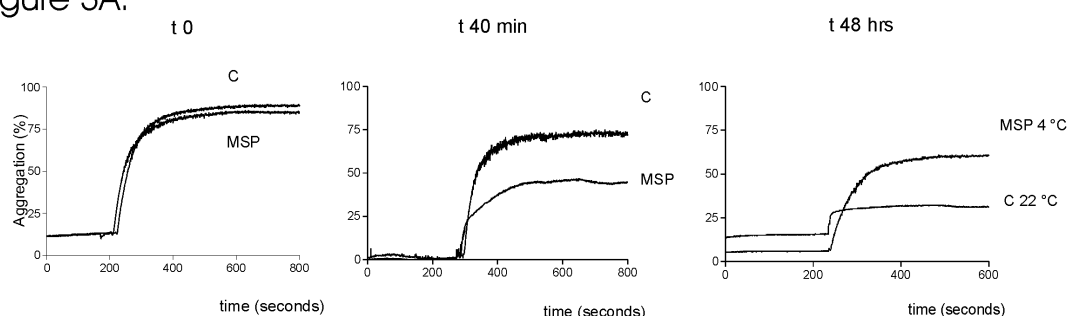


Figure 3B.

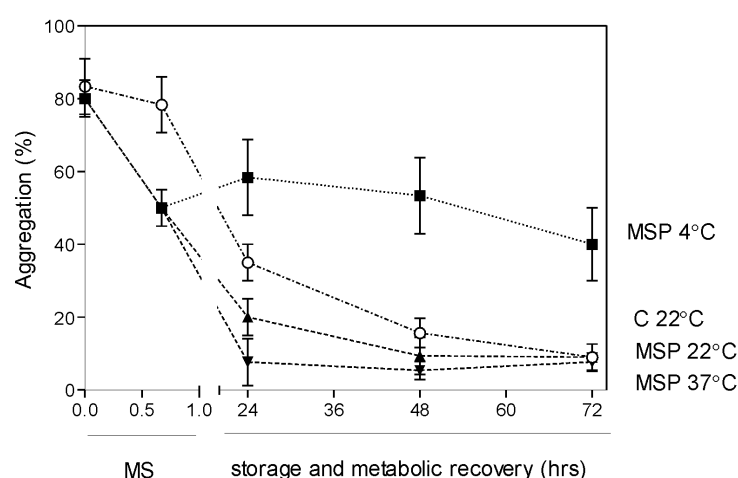


Figure 3 . Platelet aggregation. Aggregation induced by 15 μM TRAP was measured under the conditions described in the legend to figure 2 in stirred suspensions. Figure 3A shows representative tracings of MSP stored at 4°C at the start of the experiment (t0), at the end of the incubation in energy-free medium (t40) and after 48 hrs storage followed by incubation with glucose. Also shown is the aggregation of controls kept at 22°C at these time intervals. Figure 3B shows the aggregation data of MSP suspensions during incubation in energy-free medium (0 – 40 minutes) and subsequent storage and recovery by incubation with glucose. Also shown is the aggregation of controls kept at 22°C at these time intervals. Data are means \pm SD, $n=4$.

Perfusion over VWF- and fibrinogen-coated surfaces

To study the properties of MSP stored at 4°C in more detail, platelet adhesion was measured under flow using VWF and fibrinogen as adhesive surfaces and a shear rate of 800 and 300 s^{-1} , respectively (Figure 4A,B). MSP showed only a minor decrease in adhesion following the 40 minutes incubation in energy-free medium (not shown) and also after 48 hrs storage at 4°C and recovery most of the adhesion was preserved. In contrast, Control 4°C and Control 22°C suspensions gradually lost their adhesive properties both to VWF and fibrinogen resulting in a 60-70 % decrease in surface coverage after 48 hrs. Apparent abnormalities were the lack of a dendritic and spread morphology illustrating major abnormalities in the mechanisms that drive the formation of filopodia and lamellipodia.

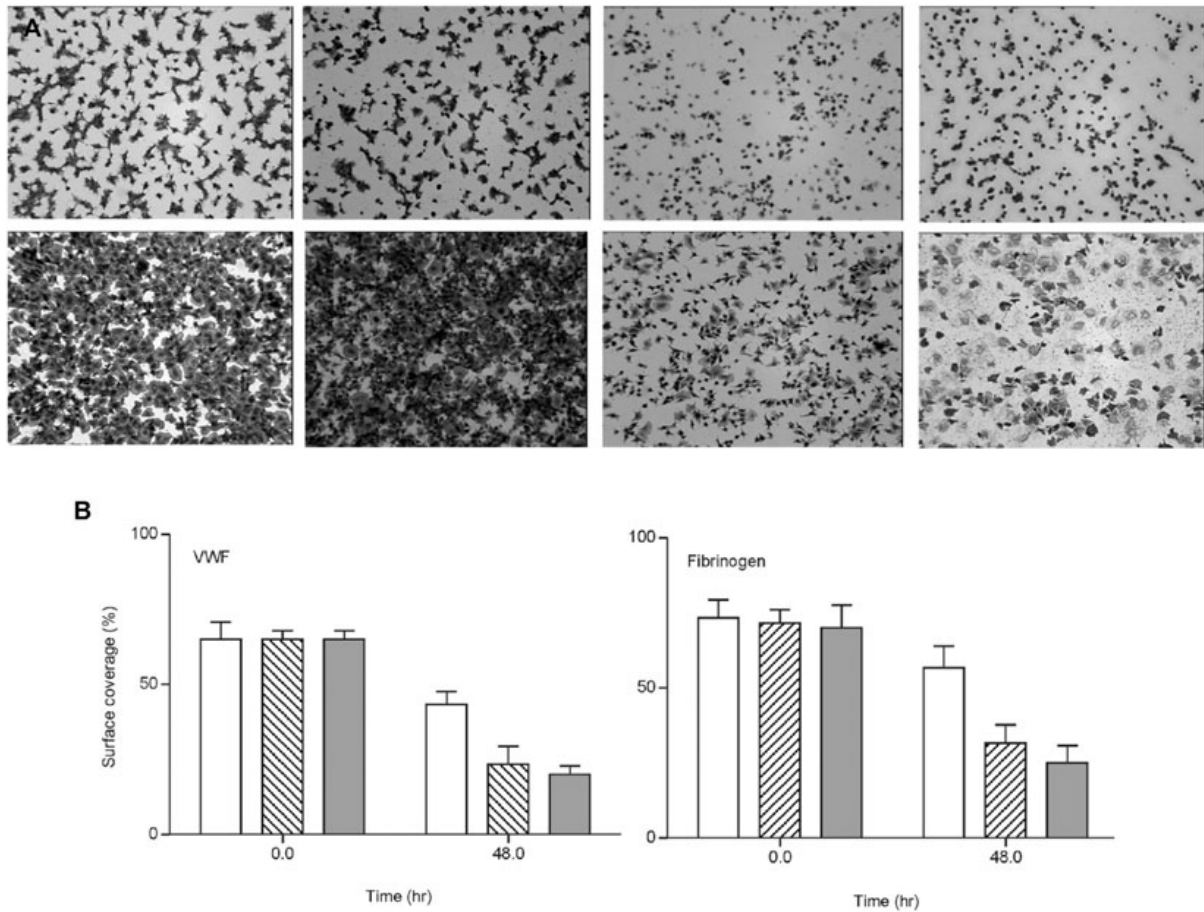


Figure 4 . Platelet adhesion under flow. Reconstituted blood containing MSP, Control 4°C and Control 22°C before and after 48 hrs storage was perfused over a VWF- and fibrinogen-coated surface at a shear rate of 800 s^{-1} at 37°C. Coverslips were fixed and evaluated by light microscopy (figure 4A) and computer assisted analysis (figure 4B). Adhesion was expressed as percentage of surface coverage and data are means \pm SD from 3 independent experiments. At 48 hrs the difference between MSP (4°C) and the two control suspensions was significant ($P < 0.03$).

The observation that MSP stored at 4°C preserved most of their adhesive properties to surface-coated VWF, suggested that these platelets had preserved their receptors for VWF (GPIb). However, there was a slight reduction in VWF/ristocetin-induced aggregation which was already apparent after incubation in energy-free medium (Figure 5A, 5B left panel). In Control 22°C suspensions the fall in aggregation was much steeper resulting in only minor aggregation responses at the end of the 48 hrs storage period. FACS analysis showed that MSP stored at 4°C preserved GPIb for more than 60 %, whereas after storage Controls 22°C showed a decrease in GPIb expression to about 30 % (Figure 5B, right panel).

Figure 5A.

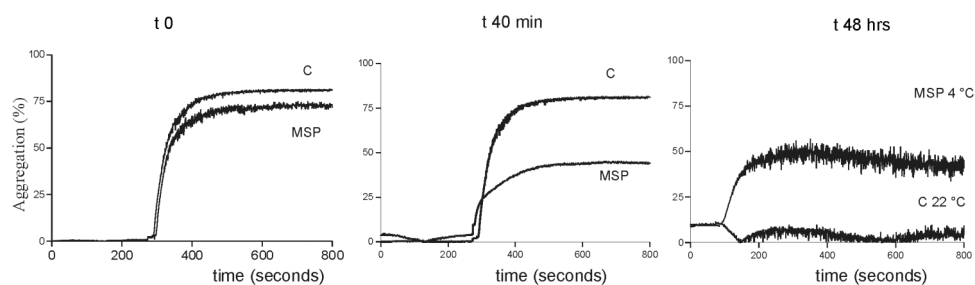


Figure 5B.

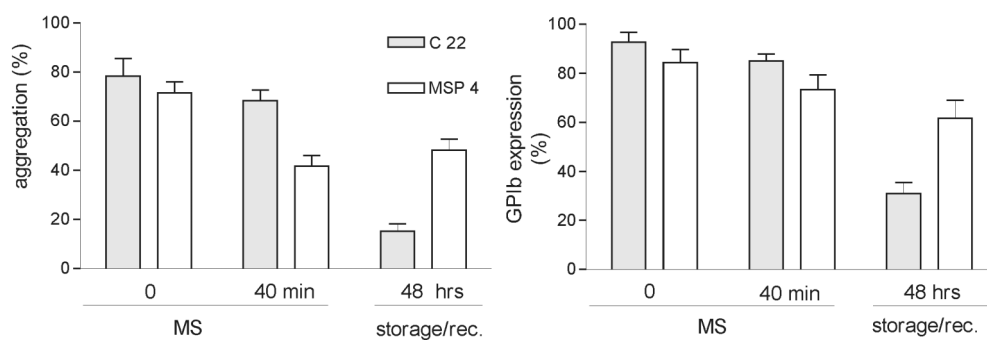


Figure 5 . GPIb expression and GPIb-VWF-induced aggregation. Aggregation induced by ristocetin/VWF was measured under the conditions described in the legend to figure 2. Figure 5A shows representative tracings of MSP at the start of the experiment (t_0), at the end of the incubation in energy-free medium (t_{40}) and after 48 hrs storage at 4°C and recovery. Also shown is the aggregation of controls kept at 22°C at these time intervals. Figure 5B, left panel shows the aggregation data under the same conditions. Figure 5B, right panel shows GPIb expression of CD42b-PE labeled platelets measured by FACS. At 48 hrs the difference between MSP 4°C and the Control 22°C was significant ($P < 0.05$). Data are means \pm SD, $n=4$.

Discussion

The main finding of the present study is that metabolic suppression followed by storage at 4°C better preserves platelet functions than conditions that support energy metabolism during storage at 22°C or apply low temperature without prior induction of metabolic arrest. Compared with both control suspensions, these MSP better maintain a low basal PSE, show better TRAP-induced PSE and aggregation and show an almost normal adhesion to VWF and fibrinogen under flow. However, there is a slight decrease in GPIb expression and VWF/ristocetin induced aggregation in MSP stored at 4°C. The difference with control suspensions is much smaller when MSP are stored at 22°C and storage of MSP at 37°C triggers a dramatic loss of platelet functions within the first hours of the storage period.

Basis of the concept that metabolic suppression could protect platelets against activating stimuli inflicted during storage are earlier findings showing that platelets rapidly lose their capacity to respond to aggregation and secretion-inducing agents when energy supply and demand are out of balance. Incubation in a glucose-free medium (to block glycolytic energy generation) in the presence of antimycin A (to block oxidative energy generation) leaves only glycogen catabolism as a source for metabolic energy.^{23,24} Previous studies have shown that glycogenolysis alone is unable to meet the energy demands of the resting platelets despite the huge amounts of glycogen stored in the cell.²⁶

The result is a rapid fall in the AEC, $AEC = ([ATP_m] + \frac{1}{2} [ADP_m] + [AMP]) / ([ATP_m] + [ADP_m] + [AMP])$ ("m" refers to the metabolic compartment) to values as low as 0.2 – 0.3 until a new equilibrium is reached.^{23,25,26} Surprisingly, platelets survive a period with a low AEC even though their functionality is transiently lost. Apparently, they set priorities in energy consuming mechanisms and preserve the mechanisms that are essential for survival at the expense of their capacity to aggregate and secrete. Subsequent addition of glucose restores the normal AEC and platelets regain their aggregating and secreting properties.

Despite the better preservation of MSP compared with controls illustrated in the present report, it is clear that the preservation of platelet properties is far from complete. A clear change is the increase in basal PSE in these cells resulting in >40% positive platelets after 48 hrs storage at 4°C. P-selectin is a protein embedded in the alpha-granule membrane and its surface expression reflects a gradual secretion of alpha-granule contents during the storage period. In earlier work the metabolic energy required for complete secretion was estimated at 5.2 ATP equivalents / 10^{11} platelets.³⁵ It is unlikely that under the suppressed metabolic conditions applied to MSP this energy is readily available. Instead, the expression of P-selectin might reflect cell damage especially since it is accompanied by an increase in phosphatidylserine exposure and Annexin-V binding (data not shown). After transfusion of the PC's, platelets bind strongly to monocytes and neutrophils, probably as a result of P-selectin expression³⁶. P-selectin exposure has been taken as a marker for platelet viability *in vitro*,^{19,15} and *in vivo*.³⁷ Holme et al. 1997 found a poor correlation between P-selectin expression and platelet recovery post transfusion and a somewhat better correlation with platelet survival.¹⁸

Another disturbance is the fall in the capacity to aggregate upon stimulation by TRAP and ristocetin/VWF. Apparently the protection by metabolic suppression is incomplete and some damage to the mechanisms that drive platelet aggregation cannot be prevented. In contrast, there is little change in the adhesion to VWF and fibrinogen by MSP stored at 4°C. Normal adhesion to VWF

accords with the almost normal GPIb expression in these cells stored at 4°C for 48 hrs. At high shear GPIb mediates the rolling and adhesion of platelets on a VWF-coated damaged vessel wall and initiates signal transduction leading to platelet activation.^{38,39} Exposure of platelets to high shear stress causes aggregation via VWF and GPIb.⁴⁰ This interaction leads to platelet activation and binding of fibrinogen to $\alpha_{IIb}\beta_3$, which forms a firm bridge between aggregating platelets.³⁸ Adelman *et al.*³⁹ showed that platelet storage at 22°C is accompanied by a decrease in GPIb due to proteolysis. We found a similar decrease in our Control 22°C suspensions and could reduce the fall in GPIb expression by 30 % by adding epsilon-aminocaproic acid (1.0 mM), confirming the proteolytic nature of this decrease (not shown). GPIb expression in MSP 4°C was unchanged by this treatment illustrating minimal proteolysis during storage at low temperature. The almost normal adhesion to fibrinogen indicates that the receptor for fibrinogen, integrin $\alpha_{IIb}\beta_3$, is well preserved.

Platelet storage at low temperature is accompanied by a rise in cytosolic Ca^{2+} , actin filament fragmentation,⁴¹ activation of Ca^{2+} -dependent proteases^{41;42} and clustering of phosphoinositides in the plasma membrane. These changes are thought to cause cold-induced actin filament rearrangements and platelet rounding.^{12;41} Depletion of intracellular Ca^{2+} stores appears to increase the permeability of the plasma membrane for Ca^{2+} ions.⁴² These changes are accompanied by tyrosine phosphorylation of the 130-kDa protein vinculin.⁴² Whether these changes are prevented by prior induction of metabolic arrest remains to be investigated.

Storage at low temperature has long been considered incompatible with platelet transfusion since cold-activation led to alterations in platelet shape⁴¹ and P-selectin expression^{21;43} thought to trigger rapid removal from the circulation. The use of pharmacological agents that preserve the disc shape at low temperature² and studies in P-selectin-deficient mice⁶ have made clear that neither changes in platelet morphology nor surface expressed P-selectin are triggers for platelet removal post transfusion. Recent studies in animals show that the cold-induced clustering of GPIb might be an important trigger for the removal of chilled/rewarmed platelets from the circulation through binding to $\alpha_M\beta_2$ receptors expressing macrophages.^{12;13} The possibility that metabolic suppression interferes with clustering of GPIb molecules and might thereby suppress the recognition by macrophages is an interesting subject for further studies.

The fact that MSP kept at 4°C better sustain a 48 hrs storage period than control platelets kept at 22°C suggests that metabolic deprivation might be a suitable means to improve the quality of PC's stored under blood banking conditions. It is obvious that the incubation conditions applied in the present study are worse than those of modern blood banks, where gas-permeable bags in combination with platelet additive solutions and autologous plasma lead to much better platelet preservation than in the present Control 22°C suspensions. However, these suboptimal conditions were applied to all suspensions and used to fasten the appearance of storage-induced platelet defects. It is clear that further studies are required to adapt metabolic suppression to conditions compatible with transfusion in humans making use of current procedures for optimal storage of PC's.

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Chapter 3

Platelet binding and phagocytosis by macrophages

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Abstract

Introduction: We reported earlier that metabolic arrest followed by incubation at 4°C reduces the platelet storage defect (Badlou *et al.*, Transfusion 2005). Here we report that this treatment also reduces binding and phagocytosis by macrophages.

Study Design and methods: Phagocytosis of mepacrine-labeled platelets by macrophages changes the latter into bright fluorescent particles easily detected by FACS.

Results: In combination with conventional binding analysis we found that binding to PMA-matured THP-1 cells is primarily regulated by platelet P-selectin expression and phagocytosis by combined phosphatidylserine (PS) exposure and glycoprotein (GP) Iba α clustering. We found that trapping of platelet Ca²⁺ and raising cAMP reduces phagocytosis by lowering PS exposure. Chilling of platelets leads to an increase in binding and PS- and GPIb α -mediated phagocytosis. Prior depletion of platelet energy stores prevents this increase by preserving low Ca²⁺ concentration, PS exposure and PS-mediated phagocytosis.

Conclusion: These data characterize the individual factors that control platelet binding and phagocytosis and might help to define conditions that improve the survival of stored platelets after transfusion.

Abbreviations: GP, glycoprotein ; MSP, metabolic suppressed platelets; GlcNAc, N-acetylglucosamine; AnnV, annexin V; BAPTA, 1,2-bis-(2-aminophenoxy) ethane-NNN'N'-tetra-acetic acid

Introduction

The storage of platelet concentrates at room temperature facilitates bacterial growth and introduces changes in platelets indicative for activation and initiation of apoptosis. Improvements have been sought in lowering the storage temperature but this treatment severely reduces the survival of transfused platelets. A main feature of platelet storage is the gradual increase in surface expression of P-selectin (CD62_P), a component of α -granule membranes. The counter receptor for P-selectin, P-selectin glycoprotein ligand 1 (PSGL-1), is present on leukocytes and the inverse correlation between P-selectin expression and platelet recovery post transfusion is sought to reflect platelet destruction through coupling of these receptors. However, the contribution of P-selectin expression to platelet destruction can only be small since transfused human platelets lose P-selectin in the circulation¹ and P-selectin deficient mice platelets survive normally.^{2,3} A second feature of platelet storage is the membrane appearance of negatively charged phospholipids such as phosphatidylserine (PS), which is a property of activated platelets^{4,5} and cells going into apoptosis^{3,6}. PS exposure may form recognition sites for destruction of senescent platelets and a similar mechanism might remove stored platelets from the circulation⁶.

Attempts to reduce the surface expression of recognition sites for platelet destruction were based on prostacyclin, which inhibits secretion and PS exposure,⁷ arrest of glycolytic and oxidative energy generation, which inhibits ATP resynthesis required for platelet functions^{8,9} and storage at low temperature, which slows down platelet metabolism and in addition suppresses bacterial growth. Chilling of platelets has long been thought to cause irreversible cell damage reflected by loss of the discoid shape and an increase in cytosolic Ca²⁺ and causing poor post-transfusion survival.^{10,11} However, treatments that preserve platelet shape and a low Ca²⁺ level failed to improve *in vivo* survival.^{12,13} Recently, Hoffmeister *et al.*¹⁴ showed that the chilling of platelets triggers the rearrangement of glycoprotein (GP) Ib α , CD42b into clusters that are recognized by α M β ₂ (CR3/Mac-1) receptors on liver macrophages triggering platelet destruction. A resting platelet contains about 20 000 GPIb α molecules bound to GPV and GPIX in a 2:1:2 stoichiometry randomly distributed over the plasma membrane.^{11,15,16} GPIb α serves in the binding of platelets to activated von Willebrand Factor at sites of vascular damage slowing down their velocity in flowing blood and enabling other receptors to firmly attach platelets to the wound.¹⁶⁻¹⁹ Interestingly, cold induced formation of GPIb α clusters leaves the haemostatic functions of GPIb α undisturbed.²⁰

An alternative means to prolong the storage of platelets is by metabolic suppression prior to cold storage. We showed earlier that platelets sustain a period of metabolic arrest without losing their adhesive properties and their capacity to aggregate and secrete granule contents.²¹ During metabolic arrest platelets fail to respond to platelet activating agents such in agreement with the energy requirement of aggregation and secretion.

In the present study we investigated the mechanisms that mediate binding of platelets to macrophages and initiate their destruction. The results reveal major roles for surface P-selectin in binding and exposed PS and clustered GPIb α in phagocytosis. We also show that chilling of platelets induces binding and phagocytosis and that prior metabolic arrest protects platelets against

these cold-induced changes. This protective effect can be explained by suppression of PS exposure providing a means to interfere with surface expression of phagocytotic signals through metabolic intervention.

Materials and methods

We obtained antimycin A, phorbol 12-myristate 13-acetate (PMA), cytochalasin B, BAPTA-AM, N-acetylglucosamine (GlcNAc), mepacrine (Quinacrine) and Fura-2 AM from Sigma Chemicals (Mannheim, FRG, Germany), the anti human CD42b (GPIIb α PE (R7014), anti-human CD14-FITC antibody, annexin V-PE, annexinV-FITC, anti human CD62p (P-selectin) and FITC-labeled IgG as negative control from Dako A/S (Glusdorp, Denmark), serum free cell culture media RPMI-1640 from Corning Inc. (Corning, NY, USA), fetal calf serum (FCS) from Cambrex (Viers, Belgium), penicillin, streptomycin sulfate and trypsin from Gibco invitrogen corporation (Grand Island, N.Y. USA). Prostacyclin (PGI₂) was from Cayman Chemical Company (Ann Arbor, MI, USA) and the stable prostacyclin analogue iloprost from Schering A.G. (Berlin, Germany). Annexin V was a generous gift from Dr. W.L. van Heerde, Department of Hematology, Radboud University Nijmegen.

Platelet isolation and storage

Freshly drawn venous blood from healthy, medication-free volunteers (40 mL) was collected with informed consent into 1:10 v/v 130 mmol/L trisodium citrate. Platelet-rich plasma (PRP) was prepared by centrifugation (200g, 15 minutes, 20 °C). ACD (0.1 volume of 2.5 g tri-sodium citrate, 1.5 g citric acid and 2.0 g D-glucose in 100 mL distilled water) was added to lower the pH to 6.0 and prevent platelet activation during isolation. The suspension (10 mL) was washed by centrifugation (330g, 15 minutes, 22 °C) and resuspended in glucose-free Hepes-Tyrode (137 mM NaCl, 2.68 mM KCl, 0.42 mM NaH₂PO₄, 1.7 mM MgCl₂, and 11.9 mM NaHCO₃, pH 7.2) to a final concentration of 2 x 10⁸ platelets/mL. Platelet count was measured on a Cell counter AL871 (Molab, Hilden, Germany). Platelets in glucose (5 mM)-containing Hepes-Tyrode were stored at 22°C (designated as C22°-platelets), a storage temperature currently used in blood banks, and at 0°C (designated as C0°-platelets), a condition known to induce clustering of GPIIb α binding to macrophage $\alpha_M\beta_2$ and platelet destruction after transfusion.²² Metabolic suppressed platelets (MSP) were prepared by incubation in glucose-free Hepes-Tyrode solution containing 20 μ M antimycin A for 40 minutes at 37°C to deplete energy stores²³. MSP were stored at 4°C for the indicated periods. Suspensions were stored in closed tubes impermeable for gas exchange without agitation. Prior to each measurement, MSP and C22°-platelets were incubated with 20 mM glucose for 1 hour at 37°C to optimally restore energy generation; the C0°-platelets were warmed for 15 minutes at 37°C.²³

Preparation of mepacrine-labeled platelets and PMA-matured THP-1 cells

Immediately before the binding and phagocytosis experiments, 100 μ L platelet suspensions were labeled with 1 μ M mepacrine in Hepes-Tyrode (pH 7.2, 5 minutes, 22°C). Mepacrine is a fluorescent polyphenolic compound, which emits at 519 nm which is in the range of the emission of FITC (530 \pm 15 nm). Free mepacrine was removed (5 minutes, 350g, 22°C, with soft mode) while preventing platelet activation with 10 ng/mL PGI₂. Monocytoid THP-1 cell lines were cultured to a density of (2 - 4) $\cdot 10^5$ cells/mL in RPMI 1640 containing 10% FCS, 2 mM glutamine, penicillin (10 U/L) and streptomycin (1 μ g/L) at 37°C. THP-1 cells were counted in a Burker-Turk chamber and 1 mL suspension containing 1×10^6 cells was added to a well of a 48 wells plate (Corning incorporated, Corning, NY, USA). Maturation was induced by incubation with 500 nM PMA for 24 hours at 37°C unless stated otherwise. In a few experiments free PMA was removed from the THP-1 cells prior to incubation with platelets to prevent platelet activation, as indicated.

Platelet – macrophage interaction

The binding of platelets to macrophages was a modification of the procedure described by Hoffmeister *et al.* ¹⁴ In short, 2×10^6 platelets in Hepes-Tyrode buffer (pH 7.2) were added to a well containing 1×10^6 PMA-matured THP-1 cells in 1 mL RPMI 1640 medium and the mixed suspensions were incubated at 37°C without agitation. Wells were gently washed with HBSS buffer (0.3 mM KH₂PO₄, 13.7 mM NaCl, 417 mM NaHCO₃, 31 mM Na₂HPO₄ and 0.5 mM KCl in aqua dest) and free platelets collected in the wash medium were isolated by centrifugation-resuspension under protection of PGI₂. Wells were incubated with 200 mL HBSS buffer containing 5 mM EDTA for 15 minutes at 0°C, reconstituted with free platelets removed during the wash step and thereafter 100 μ L suspension was incubated with 2 μ g/mL anti-human CD42b-PE and 2 μ g/mL anti-human CD14-FITC antibody for 15 minutes at 37°C. Then, HBSS buffer was added and 20 000 particles were measured by flowcytometry (FACS Calibur, Becton-Dickinson, San Jose, CA, USA). FACS data were analyzed with WinMDI software. Binding of platelets to macrophages was expressed as the percentage of CD42b/CD14 positive particles of the total number of CD42b and/or CD14 positive particles. Phagocytosis of platelets by PMA matured THP-1 cells was measured by FACS analysis of mepacrine positive CD14 cells that were inaccessible to the anti CD42b-PE antibody and expressed as percentage of total number of CD14 positive, CD42b negative particles.

In a few experiments THP-1 cells (1×10^6 cells) were allowed to attach to glass, mildly matured by incubation with 100 nM PMA (15 minutes, 22°C) and incubated at 22°C with platelets stored for 48 hours at 22°C. Binding and phagocytosis were measured by real-time analysis with an Orthoplan fluorescence microscope (Leica, Heidelberg, Germany) with a 100x objective connected to a charge-coupled device (CCD) camera system (Jai, Copenhagen, Denmark) coupled to a personal computer with Optimas 6.0 DVS software (Breda, the Netherlands).

Interference with binding and phagocytosis

Platelets stored at the indicated conditions were incubated with P-selectin antibody (17 $\mu\text{g/mL}$), annexin V (17 $\mu\text{g/mL}$), cytochalasin B (10 μM), BAPTA-AM (10 μM) and iloprost (10 μM) for 1 hr at 22°C. Additions were removed by a wash step prior to mixing with THP-1 cells and analysis of binding and phagocytosis. The role of GPIIb α clusters was evaluated by measuring phagocytosis of the cold platelets in the presence of GlcNAc (100 mM).

Measurement of P-selectin and PS expression

Platelets were incubated with glucose, cytochalasin B, BAPTA-AM or iloprost for 1 hr at 37°C and P-selectin and PS expression were measured on a FACScalibur^{23,24}. Samples were fixed with 2 percent paraformaldehyde (30 minutes, 22°C) and washed with 500 μL of phosphate-buffered saline (PBS; 500g, 5 minutes, 22°C). Pellets were resuspended in 50 mL PBS containing 1 % BSA and 0.01 % Tween 20 (5 minutes, 22°C) and platelet suspensions were incubated with annexinV-FITC and CD62-p-PE (1 $\mu\text{g/mL}$ each) for 1 hour at 22°C in the dark. Samples were washed in PBS and 10,000 particles were analyzed using WinMDI software.

Measurement of $[\text{Ca}^{2+}]_i$

Platelets were incubated with 0.2 μM Fura-2-AM for 45 min at 37°C (light protected) and free Fura-2 was removed by a wash step under protection of PGI₂ (10 nM). Fura-2 fluorescence was recorded at 37°C in F-4500 fluorescence spectrophotometer (Hitachi Ltd., Tokyo, Japan) with excitation wavelengths of 340 and 380 nm and emission at 510 nm. Basal $[\text{Ca}^{2+}]_i$ was calculated according to the method of Grynkiewicz et al.²⁵

Confocal microscopy

Human washed platelets (10×10^6) were incubated with moab CD42b-PE (2 $\mu\text{g/mL}$) (AN51-PE, DAKO, Denmark) and mepacrine (50 $\mu\text{g/mL}$) for 15 minutes at 37°C. THP-1 cells (5×10^6) were stimulated with PMA for 15 minutes at 37°C on coverslip. Then platelets were mixed with THP-1 cells and incubated for 30 minutes at 22°C. Samples were first identified using a confocal laser scanning fluorescence microscope (Leica TCS 4D, Heidelberg, Germany) as defined.²⁶ A 63x objective, 0.75 NA oil immersion lens (Zeiss, Thornwood, New York) was used to illuminate the sample and zoom 4 (31 x 31 μm) images were collected.

Statistics

Data are expressed as means \pm SEM with number of observations n. Statistical analysis was based on a paired t-test or One-way ANOVA (with post t-test) for comparison between 2 and more groups. Differences were considered significant at a *p* value of less than 0.05.

Results

Binding and uptake of mepacrine-labeled platelets by macrophages

Figure 1 shows pictures of real time video microscopy of the interaction at 22°C between platelets stored for 48 hours and mildly matured THP-1 monocytic cells, a condition that was optimal for morphological analysis. A picture taken after 5 minutes shows a number of free platelets and two macrophages covered with platelets (panel A). Shown is also one macrophage that has already phagocytosed one or more platelets leading to the release of mepacrine into the cytosol making the macrophage a bright and strongly fluorescent particle. After 15 minutes more bound platelets were engulfed and taken up by the macrophages and destructed (B). After 30 minutes all three macrophages were strongly fluorescent illustrating that they had completed the phagocytosis phase (C). A macrophage that had taken up a few platelets before starting their destruction is shown in D. Also shown are the separate fluorescence of a mepacrine-labeled macrophage (panel E), PE-labeled platelets (panel F) and the merge (panel G).

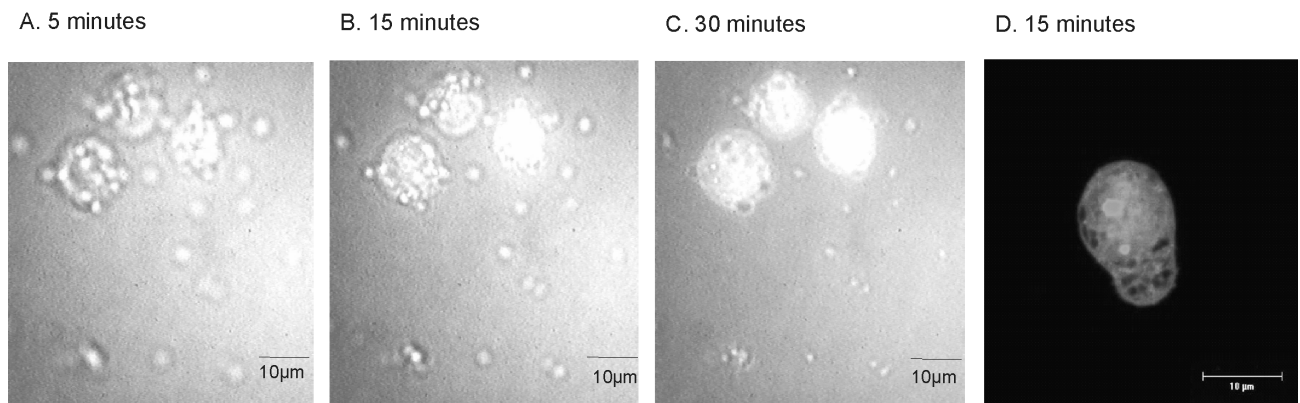


Figure 1. Binding of mepacrine-labeled platelets to mildly matured THP-1 cells followed by their phagocytosis. Photographs taken after 5 (A), 15 (B) and 30 (C) minutes incubation at 22°C. Note the bright fluorescence of THP-1 cells after digestion of mepacrine-labeled platelets. Figure 1D shows mepacrine positive platelets trapped in a THP-1 cell prior to be destroyed. Also shown are the separate fluorescence of a mepacrine-labeled macrophage (panel E), PE-labeled platelets (panel F) and the merge (panel G).

FACS analysis of mixtures of platelets and macrophages

To define the fluorescent signals of the individual cells, complexes of platelets bound to macrophages and macrophages that had taken up mepacrine-labeled platelets, separate suspensions and mixtures were analyzed by FACS. The fluorescence signals of platelets labeled with CD42b-PE (Figure 2A), mepacrine (Figure 2B), double labeled with CD42b-PE/ mepacrine platelets (Figure 2C) were well separated. CD14-FITC labeled macrophages in the absence (Figure 2D) and presence (Figure 2E) of unlabeled platelets could also easily be recognized. When CD14-FITC labeled macrophages were incubated with mepacrine, there was a clear shift to higher fluorescence intensity (Figures 2D and 2F). When platelets were stored for 48 hours at 22°C and thereafter incubated with macrophages for 30 minutes at 37°C, part of the platelets had bound to macrophages without being incorporated. These

bound platelets remained accessible to CD42b-PE labeling. Another part had bound and was thereafter taken up becoming inaccessible for CD42b-PE labeling and increasing the CD14-FITC fluorescence by releasing mepacrine into the macrophages (Figure 2G). A similar incubation at 0°C showed that at low temperature phagocytosis was aborted resulting in more platelet-macrophage complexes accessible to CD42b-PE (Figure 2H).

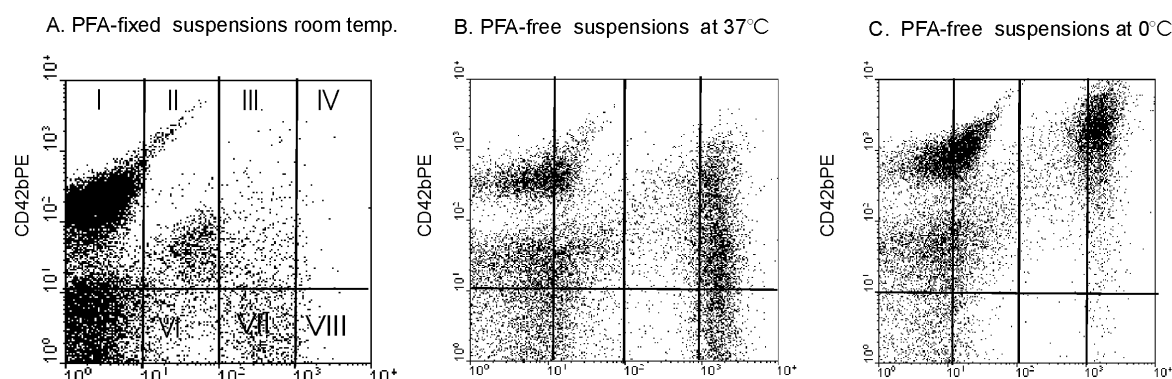


Figure 2. FACS analysis of platelet-macrophage interaction. Suspensions of platelets labeled with CD42b-PE (A), mepacrine (B) and double labeled with CD42b-PE/ mepacrine (C). CD14-FITC labeled macrophages in the absence (D) and presence (E) of unlabeled platelets. CD14-FITC labeled macrophages incubated with mepacrine show a shift to higher fluorescence intensity (F). Platelets stored for 48 hours at 22°C were labeled with mepacrine and incubated with macrophages for 30 minutes at 37°C. FACS analysis shows binding and phagocytosis (G). A similar incubation for 30 minutes at 0°C shows that phagocytosis is aborted (H).

Phagocytosis of stored platelets

To investigate how storage of platelets changes their interaction with macrophages, freshly collected and platelets stored for up to 72 hours at 22°C were analyzed for binding and phagocytosis. Binding of freshly isolated platelets was rapid reaching about 40% after 10 minutes interaction with macrophages. In a PMA-free mixture binding was < 20% indicating that platelet activation was a prerequisite for platelet-macrophage interaction, such in accordance with previous findings^{27,28}. The binding of platelets to macrophages in the presence of PMA remained constant in platelets stored for different periods of time which enabled detailed analysis of phagocytosis at a constant and maximal binding. (Figure 3A).

There was little phagocytosis of freshly isolated platelets, but platelets stored for 48 – 72 hours were rapidly phagocytosed leading to 50% mepacrine positive and GPIIb-negative macrophages (Figure 3B). A plot of binding and phagocytosis performed at different temperatures further emphasized the different kinetics of the two processes (Figure 3C). Binding of fresh platelets was constant between 0 and 37°C but prolonged storage led to an increase in binding at low temperature. Thus, in addition to the PMA-induced platelet binding to macrophages additional factors became apparent at low temperature that further increased binding. In contrast, phagocytosis increased rapidly at increasing temperature (Figure 3D). Collectively, these results demonstrate that binding and phagocytosis are

controlled by different regulatory mechanisms and that storage of platelets induces changes in the platelets that make them prone to phagocytosis.

Regulation of platelet binding and phagocytosis by macrophages

The inverse relation between P-selectin expression and platelet survival after transfusion²⁹, the role of PS exposure in destruction of apoptotic cells^{6,30} and the destruction of chilled platelets through clustering of GPIIb/IIIa suggest that these factors contribute to platelet binding and phagocytosis by macrophages.

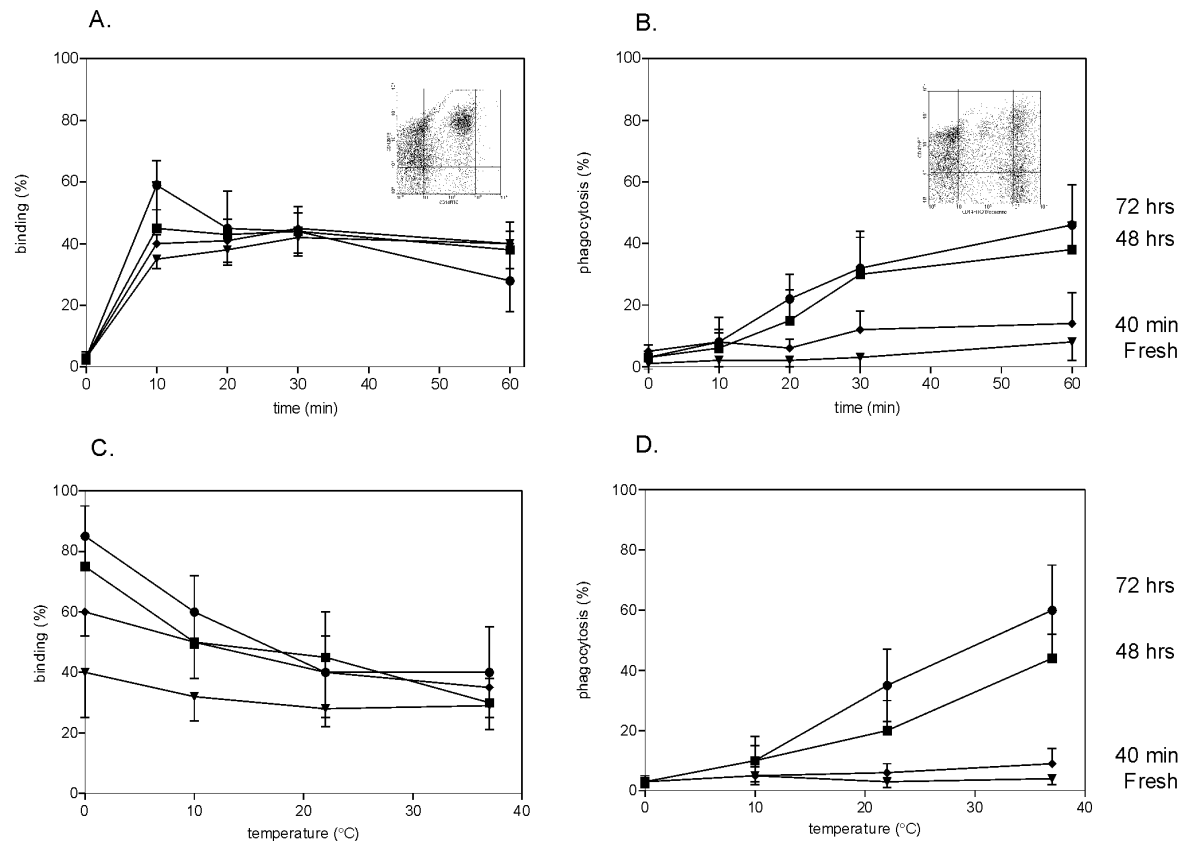


Figure 3. Kinetics of platelet binding to and phagocytosis by THP-1 cells. Binding (A) and phagocytosis (B) of platelets stored for 0 – 72 hours (hrs) in buffer with 5 mM glucose at 22°C. (C,D) platelets incubated with THP-1 cells at the indicated temperatures for 30 minutes, 37°C. The inserts show 48-hours stored platelets without (C) and with (D) mepacrine labeling to specifically illustrate binding (C) and binding with phagocytosis (D). Data are means \pm SEM, n = 6. Statistics shows comparisons between 72 hours- stored platelets at 10 minutes and 40 minutes-stored platelets at 60 minutes (A). 48-72 hours stored platelets at 10 and 60 minutes (B) 72 hours-stored platelets at 0 and 20°C (C) and 10 and 37°C (D). ns = not significant.

To clarify the contribution of these factors, platelets stored for 48 hours at 22°C were incubated for 1 hour with anti P-selectin antibody to neutralize surface expressed P-selectin, with annexin V to block exposed PS and with GlcNAc to interfere with the interaction between GlcNAc residues on GPIIb/IIIa clusters and $\alpha_M\beta_2$ on the macrophage. Under these conditions there was little effect of PMA, which accords with the maximal secretion of platelets stored for a prolonged period. As shown in Figure. 4A, treatment with anti P-selectin antibody reduced binding to about 50% suggesting an important but not

exclusive role of P-selectin in binding. In contrast, neither annexin V nor GlcNAc interfered with binding. Interference with P-selectin expression also reduced phagocytosis, illustrating that binding is a rate limiting step in platelet destruction. Treatment with annexin V triggered a more than 60% decrease in phagocytosis, indicating that PS exposure is a major determinant of platelet phagocytosis. As observed in chilled platelets^{14,20} GlcNAc interfered with phagocytosis inducing about 50% inhibition (Figure 4B). These data demonstrate that exposure of PS and clusters of GPIb α are major determinants in the phagocytosis of platelets by macrophages.

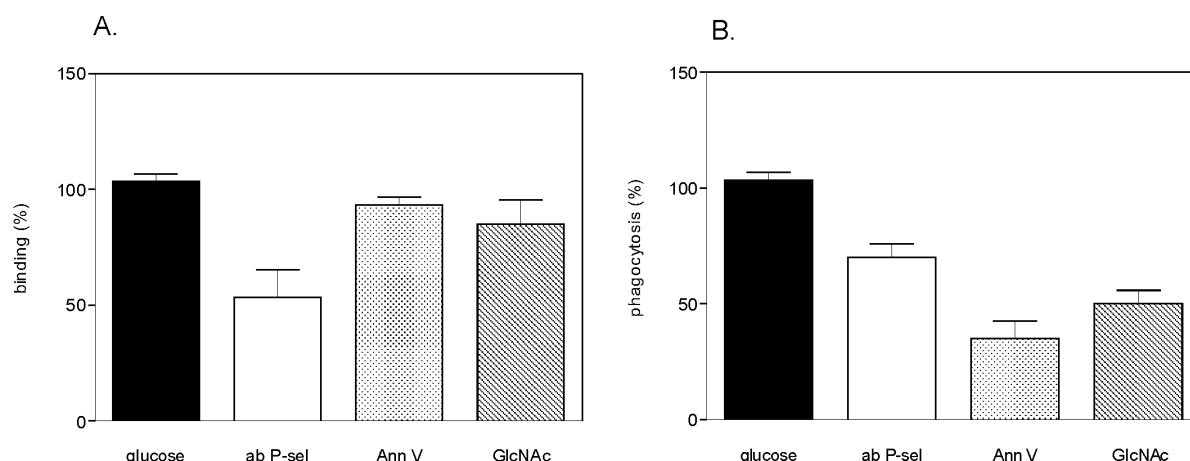


Figure 4. Interference with binding and phagocytosis. Binding (A) and phagocytosis (B) of platelets stored for 48 hours at 22°C and incubated (1 hour, 37°C) with 100 mM glucose (a control for 100 mM GlcNAc), anti P-selectin antibody (ab P-sel), annexin V (Ann V) and N-acetyl glucosamine (GlcNAc). Prior to incubation with THP-1 cells (30 minutes, 37°C) platelet suspensions were washed to remove extracellular additions. Data are means \pm SEM, n = 4. ns = not significant.

To investigate the contribution of platelet metabolism to phagocytosis, platelets were stored for 48 hours at 22°C and subsequently incubated with cytochalasin B, an inhibitor of barbed end actin assembly, BAPTA-AM, a Ca²⁺ chelator and iloprost, a cAMP raising agent. Thereafter the platelets were washed and binding and phagocytosis were measured. None of these treatments affected binding (data not shown) and P-selectin expression (Figure 5A), in accordance with the maximal P-selectin expression of 48 hours stored C 22°-platelets²³ and the irreversible nature of the secretion response. Cytochalasin B also left PS exposure unchanged. In contrast, BAPTA-AM reduced PS exposure by about 70 (Figure 5B). Also iloprost slightly reduced phagocytosis but the difference was not significant. Phagocytosis was not affected by cytochalasin B treatment which accords with observations in chilled platelets.^{14,22} These data indicate that cytosolic Ca²⁺ and cAMP contribute to the regulation of phagocytosis of platelets. To investigate whether the lower phagocytosis in the presence of these inhibitors was the result of interference with GPIb α clustering or PS expression, studies were repeated in the presence of annexin V to block exposed PS and with GlcNAc to interfere with phagocytosis through clusters of GPIb α . The presence of annexin V rescued phagocytosis of platelets pretreated with BAPTA-AM and iloprost (Figure 5C).

In the presence of GlcNAc the effect of the inhibitors remained the same (Figure 5D). In addition, cytochalasin B reduced phagocytosis by 55%. Since this inhibitor failed to interfere with PS exposure, this points to the involvement of other factors that contribute to phagocytosis regulation under conditions that suppress the clustering of GPIb α . Together with the reduced surface expression of PS in the presence of these inhibitors, these findings indicate that BAPTA-AM and iloprost reduce phagocytosis by lowering the exposure of PS without interfering with the formation of GPIb α clusters.

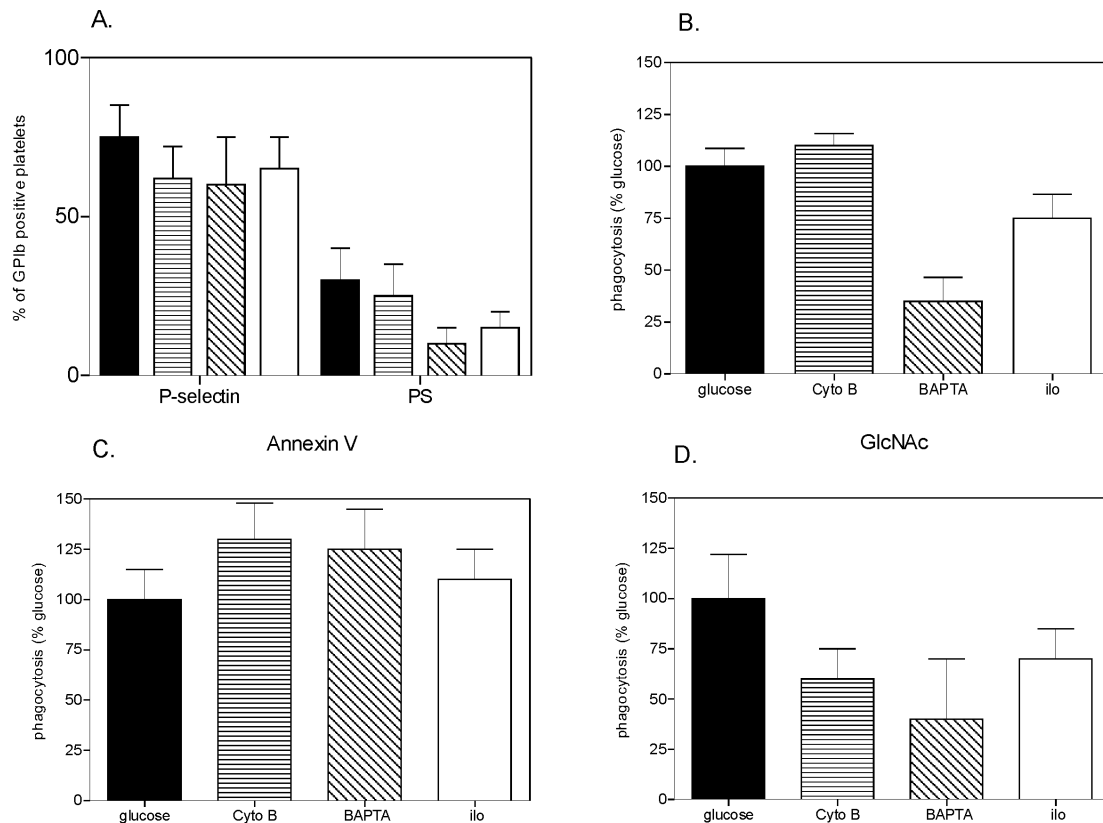


Figure 5. Metabolic interference with platelet phagocytosis. (A) The effect of glucose (5 mM), cytochalasin B (Cyto B, 10 μ M), BAPTA-AM (10 μ M) and iloprost (10 μ M) for 1 hour at 37°C was studied on surface expression of P-selectin and PS exposure in platelets stored at room temperature for 48 hours. (B) The effect of these inhibitors on phagocytosis. BAPTA-AM interfered with PS exposure and phagocytosis; iloprost had a slight effect. (C) The same incubations in the presence of annexin V (10 μ g/ mL) to neutralize exposed PS and (D) in the presence of GlcNAc (100 mM) to block GPIb α -mediated phagocytosis. Data are means \pm SEM, n = 6. ns = not significant.

Optimal preservation of platelets

Our earlier observation²³ that metabolic arrest followed by storage at low temperature and recovery with glucose at 37°C better preserves platelet adhesion and aggregation than platelets stored at 22°C and 0°C led to the question whether this treatment also reduced binding and phagocytosis. MSP were prepared by incubating washed platelets in glucose-free, antimycin A containing medium for 40 minutes at 37°C to reduce glycolytic energy supply and abort mitochondrial resynthesis of metabolic

ATP. Then, the platelets were stored at 4°C for 48 hours and thereafter energy generation was restored by incubation with glucose at 37°C. After 40 minutes incubation in energy-rich medium platelet showed 20% binding to macrophages in a PMA-rich milieu.

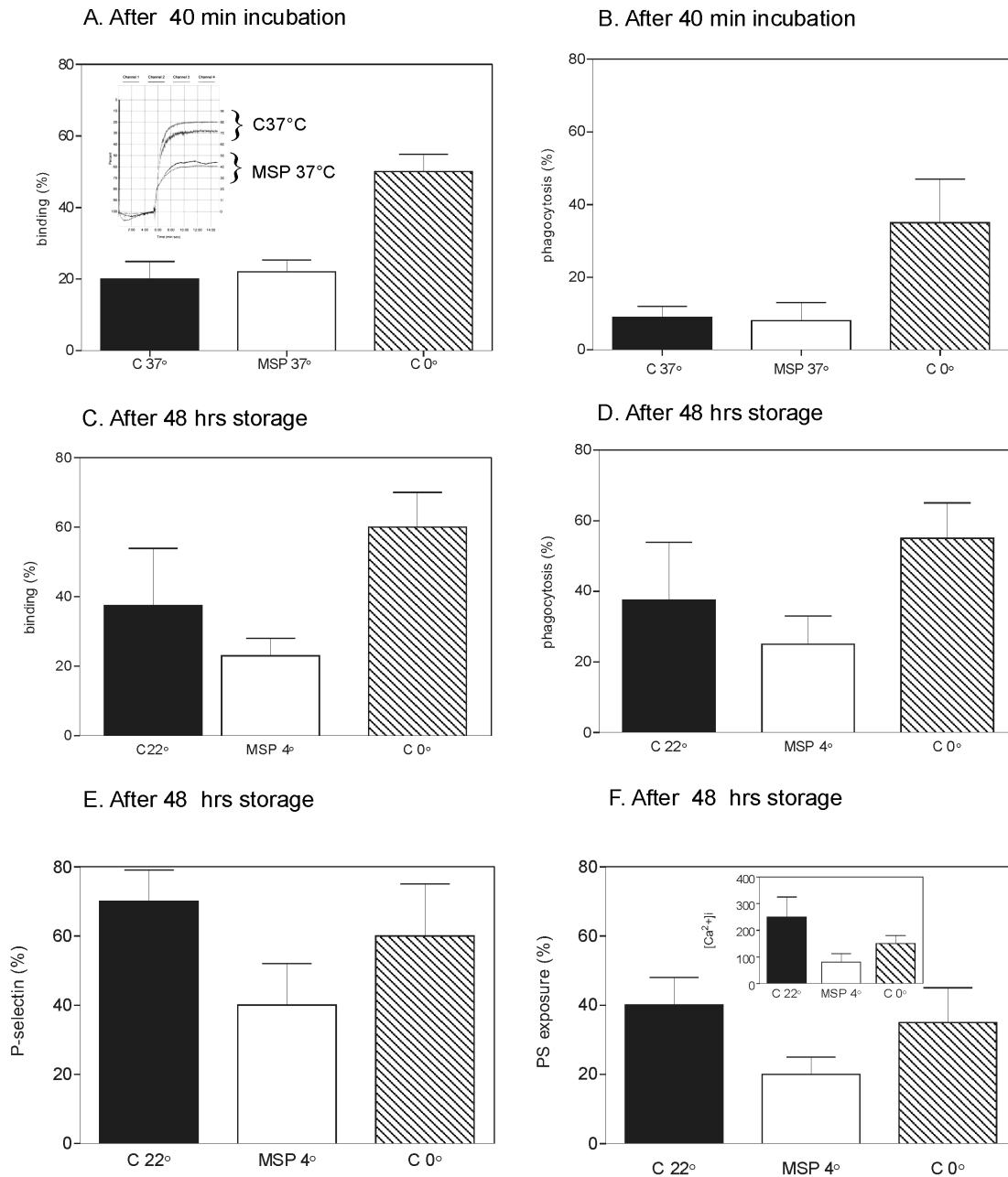


Figure 6. Binding and phagocytosis of platelets stored under different conditions. (A) Binding and (B) phagocytosis of platelets incubated for 40 minutes at 37°C in glucose containing, antimycin A - free medium (controls, C37°) and glucose free, antimycin A containing medium (MSP 37°) and on ice (C0°). The inset in (A) shows that metabolic suppression decreases TRAP-induced aggregation in MSP incubated for 40 minutes at 37°C. Suspensions were stored for 48 hours at the indicated temperatures and analyzed for (C) binding, (D) phagocytosis and (E) surface expression of P-selectin and (F) PS. The inset in (F) shows the Ca^{2+} concentration in platelets stored for 48 hours before addition to THP-1 cells. Further details as in Fig. 5. Data are means \pm SEM, n = 6. ns = not significant.

A similar incubation in energy-free medium induced a similar binding indicating that under these conditions lowered energy content does not affect binding although it reduced aggregation (Figure 6A, and inset). In contrast, platelets chilled on ice without metabolic interference showed a 2.5-fold increase in binding compared with controls. When phagocytosis of the three platelet preparations was analyzed, similar differences were observed. Platelet stored at 22°C and MSP's showed about 10% phagocytosis (Figure 6B). Chilling induced almost 3 times more, illustrating the induction of phagocytosis by a temperature fall as described earlier.¹⁴ Subsequent storage at 22°C increased binding but not at 4°C and 0°C (Figure 6C). In contrast, phagocytosis increased during prolonged storage in all three storage conditions (Figure 6D). These differences were accompanied by similar changes in P-selectin expression and PS-exposure. Compared with room temperature-stored and chilled platelets MSP appear better protected against the storage-induced changes that trigger PS-expression, probably because of their capacity to preserve a low $[Ca^{2+}]_i$ (Figure 6F, inset). Together these data show that differences in binding explain a major part of the differences in phagocytosis observed between the three suspensions. Similar studies in the absence of PMA showed lower binding and phagocytosis after 40 minutes storage but the differences between the three suspensions remained the same. After 48 hours storage removal of PMA had little effect (data not shown). To investigate the contribution of PS exposure and GPIb α clustering in MSP and chilled platelets in more detail, phagocytosis was measured in the presence of annexin V to block the role of expressed PS and GlcNAc to block phagocytosis through clusters of GPIb α . In addition, MSP were analyzed before and after the recovery phase with glucose to address the role of metabolic energy (Figure 7).

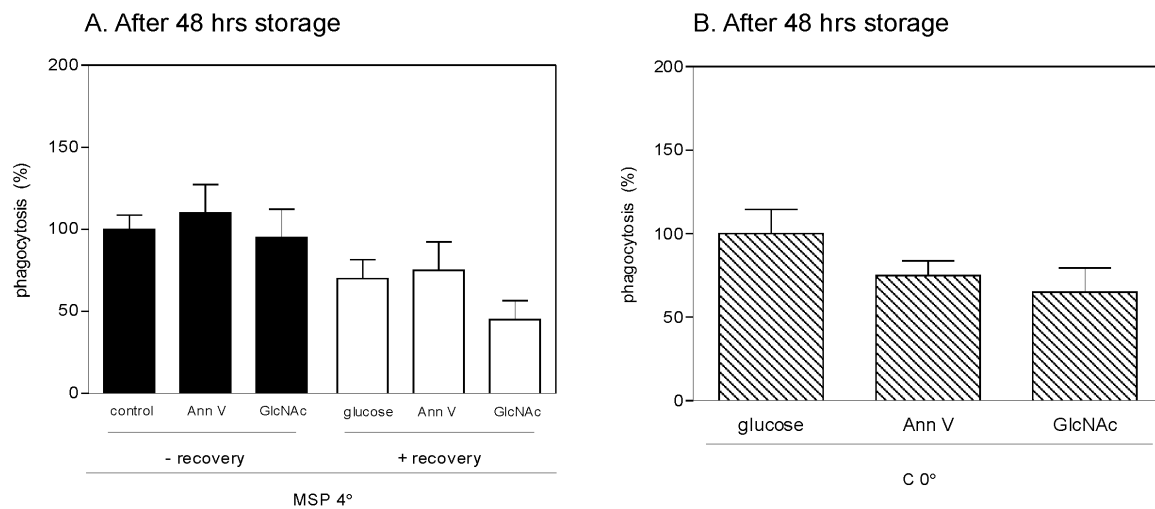


Figure 7. PS- and GPIb α - mediated phagocytosis of MSP and chilled platelets. (A) Phagocytosis of MSP stored at 4°C for 48 hours before (0 mM glucose) and after (20 mM glucose) in the presence of annexin V and GlcNAc. Recovery reduces phagocytosis and restores the inhibition by GlcNAc but not by annexin V. (B) Chilled platelets (C 0°C) stored on ice for 48 hours and treated with 100 mM glucose, annexin V and 100 mM GlcNAc. C° platelets remain sensitive to GlcNAc. Data are means \pm SEM, n = 4. ns = not significant.

MSP that were still in the energy-depleted phase were phagocytosed by macrophages as seen with MSP after the recovery phase. However, neither annexin V nor GlcNAc interfered with phagocytosis of these cells, indicating that other processes than PS exposure and GPIb α clustering were responsible for destruction. Recovery with glucose induced a 30% lower phagocytosis. This number was unchanged in the presence of annexin V but the GlcNAc induced a fall in phagocytosis as observed in 22°-platelets. Platelets chilled to 0°C and stored for 48 hours showed lower phagocytosis in the presence of GlcNAc which is similar as seen with platelets stored at room temperature.

Discussion

The present study shows that binding of platelets to macrophages and the phagocytosis that follows are regulated by different mechanisms. Freshly collected platelets in the absence of platelet activators show little binding and PMA treatment is required to induce rapid and complete interaction with macrophages. The PMA-induced binding remains constant when platelets are stored at 22°C which makes it possible to investigate the regulation of phagocytosis. The binding appears for a major part mediated through P-selectin expressed on the platelet surface since (i) it is absent in platelets with blocked secretion by prostacyclin treatment⁷, (ii) present in platelets which express P-selectin^{29,31} and, (iii) inhibited by an anti P-selectin antibody (this study). Inhibition by anti P-selectin is incomplete suggesting that an additional mechanism might be involved that has yet to be identified. In fact, platelets survive normally in P-selectin deficient mice indicating that P-selectin is not an exclusive mediator of platelet-macrophage interaction². Candidates for P-selectin independent binding to macrophages are CD36, the $\alpha_v\beta_3$ vitronectin receptor³² and the ligand-receptor pair CD40-CD40L.³³

P-selectin and PSGL-1 regulate the initial interaction between the vessel wall and leukocytes and between activated platelets and leukocytes. P-selectin is a transmembrane protein present in Weibel-Palade bodies of endothelial cells and α -granules of platelets and rapidly translocates to the cell surface after cell activation.³⁴ Together with $\alpha_M\beta_2$ integrin, PSGL-1 regulates leukocyte adhesion to the endothelium.³⁵ PSGL-1 is also present on matured THP-1 cells³⁶ and the coupling with P-selectin might be a first step in the process that lead to platelet destruction. PSGL-1 mediated monocyte activation leads to conformational activation of $\alpha_M\beta_2$, synthesis and release of various cytokines, chemokines and reactive oxygen species and tissue factor expression.³⁷ It also triggers surface expression of PS contributing to PS-mediated activation of the coagulation system³⁷. If a similar mechanism is operational in macrophages, stimulation of PSGL-1 would initiate both release of chemokines and phagocytosis of platelets.

Phagocytosis of platelets depends on surface exposure of PS and clustering of GPIIb α and neither one appears involved in binding. Also the binding of lymphocytes, Jurkat cells and neutrophils to macrophages is independent of PS.³⁸ Hoffmann *et al.*⁶ postulated that regardless of the receptors engaged on the phagocyte, ingestion does not occur in the absence of exposed PS. The present study shows that neutralization of exposed PS by annexin V leads to more than 50% inhibition of platelet phagocytosis, indicating that exposed PS is a crucial component in the phagocytotic pathway such in agreement with findings in lymphocytes or neutrophils^{38,3,30}. The asymmetric phospholipid distribution in plasma membranes is normally maintained by energy-dependent lipid transporters that translocate different phospholipids from one monolayer to the other against their respective concentration gradients³⁰. When platelets are activated or enter apoptosis, lipid asymmetry might be perturbed by scramblases that shuttle phospholipids non-specifically between the two monolayers³⁰ in a process depending on ATP and $[Ca^{2+}]_i$. In stored platelets PS exposure may function in the same way generating a signal for ingestion.^{6,14} Recognition of PS depends on PS receptor⁶ on the macrophage. The scavenger receptors LOX-1³⁹, CD36⁴⁰, SRB-1⁴¹ and integrin $\alpha_v\beta_3$ may serve a role in PS recognition. The signalling cascades initiated by the PS counter receptor may involve the small GTPases Rac⁴² and cdc42⁴³ which are implicated in membrane ruffling.⁶

GPIb α clustering through sugar linkages has been demonstrated in platelets that were rapidly chilled on ice and is considered a main cause for the short survival of cold-stored platelets after transfusion. The present data show that GPIb α -mediated phagocytosis by macrophages also occurs during prolonged storage at room temperature, which is daily practice in blood banks. Clustering of GPIb α is independent of barbed end actin assembly since it occurs in the presence of cytochalasin B such in agreement with earlier observations.^{14,22} Interference with platelet metabolism after prolonged storage did not change the binding properties but reduced phagocytosis. Since GPIb α clustering appears not to be affected by intracellular mediators,²⁰ it is likely that the reduced phagocytosis in the presence of BAPTA-AM and iloprost reflect interference with PS exposure. BAPTA binds cytosolic Ca²⁺ and suppresses Ca²⁺ rises after platelet activation⁴⁴ and storage. BAPTA left binding unchanged but reduced phagocytosis by more than 50%. Earlier studies have demonstrated that PS exposure is a reversible process although the mechanism that restores the positioning of PS on the inner leaflet of the plasma membrane is unexplained⁴⁵. Iloprost is a stable prostacyclin derivative and induced a slight inhibition of phagocytosis but did not interfere with binding. Prostacyclin is known to raise cAMP, which through protein kinase A inhibits many steps in the platelet activation cascades. It also suppresses an increase in [Ca²⁺]_i and the decrease in phagocytosis induced by iloprost might well reflect the inhibition of Ca²⁺ signaling thereby lowering PS expression⁴⁶.

Storage at room temperature also led to a decrease in CD42b-PE fluorescence. A similar decrease was found in platelets stored at 4°C in the presence of proteolysis inhibitors (data not shown). Whether this change in antibody binding reflects changes in GPIb α involved in phagocytosis has yet to be investigated.

To investigate how different storage conditions induced binding and phagocytosis, C 22°-platelets were compared with C 0°-platelets and platelets with metabolic arrest (MSP). At the end of the 40 minute starvation period at 37°C, binding and phagocytosis of MSP was not different from platelets incubated in a normal milieu. In contrast, chilling of platelets to 0°C increased binding and phagocytosis. Chilling induced phagocytosis has been reported earlier¹⁴ and the present findings suggest that increased binding contributes to the high phagocytosis of these cells. Subsequent storage for 48 hours further increased binding of C 22°-platelets but not of C 0°-platelets. Analysis of P-selectin expression confirmed the differences in binding between these suspensions. In contrast, after 48 hours storage all suspensions showed more phagocytosis. Again, differences in phagocytosis between suspensions were accompanied with similar differences in PS exposure confirming the role of PS as a major phagocytotic signal. Thus, the advantage of low temperature storage is suppression of binding and phagocytosis signals during storage. The advantage of metabolic suppression lies in the protection against cell alterations inflicted during the fall in temperature. Separate analysis of PS- and GPIb α -mediated phagocytosis learned that metabolic suppression attenuated the rise in [Ca²⁺]_i during platelet storage, resulting in a lower PS-expression and PS-mediated phagocytosis compared with 22°- and 0°-platelets. GPIb α -mediated phagocytosis remained present provided that energy metabolism was restored. In addition to PS-mediated phagocytosis chilled preserved PS-mediated phagocytosis in accordance with the higher level of [Ca²⁺]_i and exposed PS seen in these cells. A limitation of this study that should be acknowledged is its restriction to *in vitro* conditions. Thus,

although the relevance of our findings for platelet survival *in vivo* remains unanswered, they may help to define conditions that improve the survival of platelets after transfusion.

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Chapter 4

ROLE OF GLYCOPROTEIN Ib α IN PHAGOCYTOSIS OF PLATELETS BY MACROPHAGES

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Submitted

BACKGROUND: Platelet storage at 0 – 4 °C suppresses bacterial multiplication, but induces clusters of glycoprotein Ib α (GPIb α) that trigger their phagocytosis by macrophages and reduce their survival after transfusion. We investigated whether cold storage affected the binding of an anti-GPIb α antibody and a possible relation with platelet binding and phagocytosis by macrophages *in vitro*.

STUDY DESIGN AND METHODS: Human platelets were isolated and stored for 1 - 48 hrs at 0°C. Binding of AN51-PE antibody to amino acids (AA) 1-35 on GPIb α (CD42b) was compared with binding of platelets to matured monocytic THP-1 cells and phagocytosis of mepacrine-labelled platelets by these cells analyzed by FACS.

RESULTS: Fresh platelets detected as AN51-PE positive particles showed normal binding and < 5% phagocytosis. Cold-storage decreased AN51-PE binding and increased phagocytosis. N-acetylglucosamine, known to interfere with macrophage recognition of GPIb α clusters, restored AN51-PE binding and suppressed phagocytosis.

CONCLUSIONS: We conclude that binding of an antibody against AA 1-35 on GPIb α reflects changes in GPIb α that make platelets targets for phagocytosis *in vitro* by macrophages.

ABBREVIATIONS: moAb: monoclonal antibody; AN51: moAb CD42b (GPIb α) clone R7014; AA: amino acids; GlcNAc= N-acetyl glucosamine

Introduction

The storage of platelet concentrates at room temperature facilitates microbial multiplication and introduces changes in platelets indicative for activation and apoptosis.¹⁻³ Improvements have been sought in lowering the storage temperature thereby reducing bacterial growth and opening ways to prolong the storage period.⁴⁻⁷ A disadvantage of cold storage is that it induces the so called cold-induced platelet lesion which is accompanied by changes in glycoprotein (GP) Ib,⁸ shape change and actin assembly,^{9;9} myosin activation and an increase in $[Ca^{2+}]_i$.¹⁰ The main drawback of cold storage is the induction of clusters of the von Willebrand Factor (VWF) receptor GPIb α , which are recognized by integrin $\alpha_M\beta_2$ (Mac-1, CR3) receptors on liver macrophages triggering rapid platelet destruction *in vitro* and their removal from the circulation after transfusion.^{5;8;11} GPIb α clusters expose N-acetylglucosamine (GlcNAc) to which $\alpha_M\beta_2$ binds through the integrin's affinity for N-linked glycans. Enzymatic galactosylation of chilled platelets blocks $\alpha_M\beta_2$ recognition and prolongs the circulation of cold stored platelets.

GPIb α is a member of the GPIb-V-IX complex and a major sialoglycoprotein that contains different N- and O-linked oligosaccharides.^{12;13} The complex is formed in the endoplasmic reticulum and transported to the golgi for further modification.¹⁴ GPIb α serves in the binding of platelets to activated VWF at sites of vascular damage slowing down their velocity in flowing blood and enabling other receptors to firmly attach platelets to the wound.^{13;15} Although chilled-rewarmed platelets form clusters of GPIb α , this property does not change the haemostatic functions of the receptor.⁸ The VWF binding site is located within amino acids (AA) 36-200 at the N-terminal part of GPIb α , which contains the 7-leucine-rich repeats. This is part of glycocalicin, a region of GPIb α rapidly released by proteolytic degradation.^{16;17} It is also the site for factor XI binding on the activated platelet.^{17;18} Cleavage of glycocalicin impairs factor XI binding and affects the cessation of bleeding.¹⁸ The expression of GPIb on the platelet surface is readily detected by FACS analysis following incubation with a PE- or FITC-labelled monoclonal antibody (moAb).¹⁹ Storage at room temperature is known to inflict changes on the platelet surface that decrease antibody binding to GPIb α leading to a shift to lower fluorescence intensity.¹⁹ This is generally sought to reflect proteolytic cleavage of GPIb through the metalloproteinase tumor necrosis factor- α converting enzyme (TACE) or ADAMS-17.^{20,21} ADAMS-17 is a plasma constituent and present in platelet concentrates prepared in mixtures of additive solution and plasma.²¹ GPIb α shares the sensitivity to proteolytic damage with other platelet surface receptors such as P-selectin, CD40 ligand, and GPV. Glycocalicin, which is released from GPIb α , and GPV are present in plasma, where they may serve as feedback inhibitors limiting the development of thrombi.²² Proteolysis decreases sharply at lowering the temperature and one would expect GPIb to remain intact in cold-stored platelets. However, GPIb α expression decreases after prolonged storage at 4°C. In the course of our studies on platelet storage we found that the binding of a PE-labelled moAb directed against amino acids (AA) 1-35 in the N-terminal flank of the leucine-rich region of GPIb α termed AN51 decreases during storage at 4°C. This is a condition in which platelets express properties that make them targets for phagocytosis by macrophages. In the present study we

investigated the nature of the affinity loss for AN51 and its possible impact on the mechanisms that control the binding of platelets to macrophages and their subsequent destruction.

Materials and Methods

We obtained phorbol 12-myristate 13-acetate (PMA), mepacrine (Quinacrine), mannose, N-acetylglucosamine (GlcNAc) from Sigma Chemicals (Mannheim, FRG, Germany). The anti human CD42b (GPIb-PE (R7014 clone AN51 directed against AA1-35 of the N-terminal flank of GPIb), unlabelled AN51, anti-human CD14-FITC and FITC-labeled IgG as a negative control were from Dako A/S (Glusdorp, Denmark). Serum free cell culture media RPMI1640 was from Corning Inc. (Corning, NY, USA), fetal calf serum (FCS) from Cambrex (Viers, Belgium), penicillin, streptomycin sulfate and trypsin from Gibco invitrogen corporation (Grand Island, N.Y. USA), prostacyclin (PGI₂) from Cayman Chemical Company (Ann Arbor, MI, USA). The FACSaria sorter was from BD Biosciences Pharmingen (USA). The stable prostacyclin analogue iloprost was a kind gift from Schering A.G. (Berlin, Germany). N (R)-[2-(hydroxyaminocarbonyl)methyl]-4-methylpentanoyl-L-alanine amine TNF- α protease inhibitor (TAPI) was from Calbiochem (Louisville, KY, USA). D-glucose was from BDH analaR (Poole Dorset, UK). The moAb 6D1 against AA 104-128 of the VWF binding domain was a generous gift of Dr. Ruggeri from the Department of Molecular and Experimental Medicine, The Scripps Research Institute (La Jolla, CA, USA). MoAb 6B4 directed against AA 201-268 partially overlapping the binding sites for thrombin and the VWF-ristocetin complex and moAb 10H9 against AA 276-282 adjacent to these binding sites have been described.^{23;24}

Platelet isolation and storage

Freshly drawn venous blood from healthy volunteers (40 mL) was collected with informed consent into 1:10 v/v 130 mmol/L trisodium citrate. The donors claimed not to have taken any medication during two weeks prior to blood collection. Platelet-rich plasma was prepared by centrifugation (200g, 15 minutes, 20 °C). ACD (0.1 volume of 2.5 g tri-sodium citrate, 1.5 g citric acid and 2.0 g D-glucose in 100 mL distilled water) was added to lower the pH to 6.0 and prevent platelet activation during further isolation. The suspensions were centrifuged (330g, 15 minutes, 22 °C) and resuspended in Hepes-Tyrodé (137 mM NaCl, 2.68 mM KCl, 0.42 mM NaH₂PO₄, 1.7 mM MgCl₂, and 11.9 mM NaHCO₃, pH 7.2) with 5 mM glucose to a final concentration of 2×10^8 platelets /mL. Platelet count was measured on a Cell counter AL871 (Molab, Hilden, Germany). Platelets were stored on ice (termed C0 suspension) for up to 48 hours, a condition known to induce clustering of GPIb α and binding to and phagocytosis by macrophages.⁸ The suspensions were stored in closed tubes impermeable for gas exchange without agitation. Prior to each measurement, the C0 suspensions were re-warmed for 15 minutes at 37°C, as described.²⁵

Analysis of GPIb binding to AN51-PE antibody

The expression of GPIb α was measured by incubating 0.1 mL platelet suspension (2×10^8 platelets /mL) with PE-labelled AN51 antibody for 15 minutes at 37°C at the indicated concentrations. Subsequently, 300 μ l PBS was added and 10000 events were analyzed on a FACScalibur (BD

Biosciences, USA). The distribution of AN51-PE fluorescence on fresh platelets was gated and termed M1 fraction. Loss of AN51-PE binding during storage resulted in a shift to lower fluorescence, termed M2 fraction. Quantitative measurements of changes in GPIb α fluorescence were evaluated by WinMDI software. Some experiments were performed after prior incubation for 30 minutes, 37°C, with 5 mM EDTA, 110 nM TAPI, 100 mM GlcNAc, 100 mM mannose and 100 mM glucose. To investigate which regions of GPIb α contributed to affinity changes for AN51-PE binding, platelets were pre-incubated with 10 μ g/mL of the following moAb's: AN51 (unlabelled), 6D1, 6B4 and 10H9 without and with GlcNAc for 60 minutes, 37°C, prior to incubation with 2 μ g/L AN51-PE.

Binding and phagocytosis assay

The binding and phagocytosis assay carried out as described. THP-1 monocytic cell lines were cultured to a density of (2 - 4) $\cdot 10^5$ cells/mL in RPMI 1640 containing 10% FCS, 2 mM glutamine, penicillin (10 U/L) and streptomycin (1 μ g/L) at 37°C.^{26,28} THP-1 cells were counted in a Burkert-Turk counting chamber and 1 mL suspension containing $1 \cdot 10^6$ cells was added to a well of a 48 wells plate (Corning incorporated, Corning, NY, USA). Maturation of monocytes to macrophages was induced by incubation with 500 nM PMA for 24 hours at 37°C. Before the binding and phagocytosis experiments, 100 μ L platelet suspension was labeled with 1 μ M mepacrine in Hepes-Tyrosine (pH 7.2, 5 minutes, 22°C).²⁵ Mepacrine is a fluorescent polyphenolic compound, which emits at 519 nm that is in the range of the emission of FITC (530 \pm 15 nm). Free mepacrine was removed by a washing step (5 minutes, 350g, 22°C, with soft mode) while preventing platelet activation with 10 ng/mL PGI₂. Then the pellets were resuspended in 25 μ L Hepes-Tyrosine buffer (pH 7.2) containing $2 \cdot 10^5$ platelets/mL were added to $1 \cdot 10^6$ macrophages in 1 mL per well and 2 mM CaCl₂ and MgCl₂ were added (diluted in HBSS) and incubated for 30 min, 37°C. Subsequently, all wells were gently washed 3 times with HBSS buffer (0.3 mM KH₂PO₄, 13.7 mM NaCl, 417 mM NaHCO₃, 31 mM Na₂HPO₄ and 0.5 mM KCl in aqua dest) . To each well 200 μ L HBSS was added followed by EDTA (5 mM diluted in HBSS) and incubated for 15 min on ice. Samples were incubate with MoAb AN51-PE for 15 min, 37°C. To all samples 300 μ L PBS was added and immediately 20,000 events were measured on a FACScalibur. Quantitative analysis was performed using WinMDI software. Binding of platelets to macrophages was expressed as the percentage of CD42b/CD14 positive particles of the total number of CD42b and/or CD14 positive particles. Phagocytosis of platelets by PMA matured THP-1 cells was measured by FACS analysis of mepacrine positive CD14 cells that were inaccessible to the anti CD42b-PE antibody and expressed as percentage of total number of CD14 positive, CD42b negative particles.

Sorting of platelets with high- and low affinity AN51-binding

To separate suspensions with high- and low AN51-binding, platelets were stored at 0°C for 48 hours. Then, suspensions were incubated with AN51-PE for 15 minutes at 37°C and 10×10^6 platelets were sorted on a BDFACS Aria cell sorter. Suspensions were captured in 15 ml plastic tubes containing Hepes-Tyrode buffer pH 6.5 to prevent platelet activation. The distribution of AN51-PE positive platelets was analyzed as defined above. For analysis of the interaction with macrophages, aliquots of sorted suspensions were first incubated with mepacrine (1 $\mu\text{g/mL}$, 5 minutes, 22°C) followed by a wash step in the presence of PGI_2 (10 ng/ml). The pellets were resuspended in Hepes Tyrode buffer (pH 7.2) containing 5 mM glucose. Platelets were incubated with THP-1 macrophages for 30 minutes at 37°C, for analysis of binding and phagocytosis, as described.²⁵

Statistics

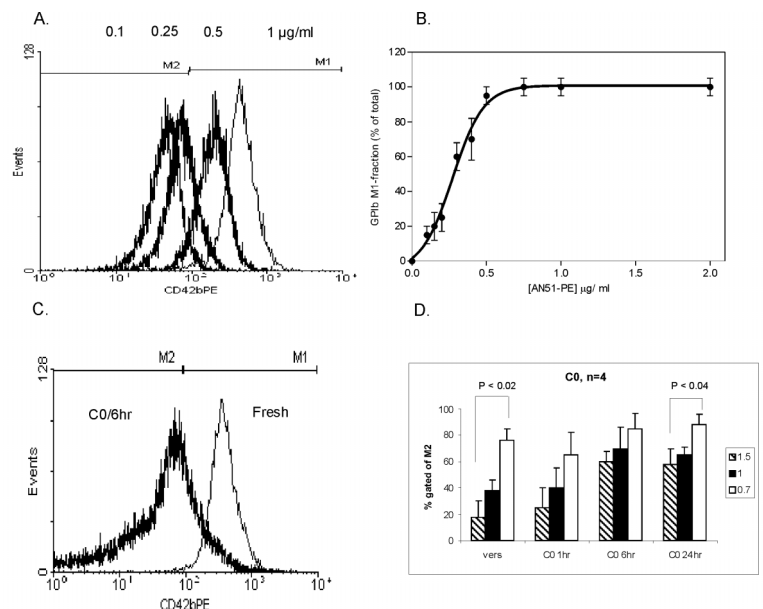
Data are expressed as means \pm SEM with number of observations, n. Statistical analysis was based on a paired t-test or One-way ANOVA (with post t-test) for comparison between 2 and more groups. Differences were considered significant at a p value of less than 0.05.

Results

AN51-PE binding after prolonged platelet storage at 0 °C

When freshly collected platelets were washed and incubated with 2 µg/mL AN51-PE antibody, FACS analysis showed a single population of platelets, which was gated and named M1 fraction. A step wise decrease in antibody concentration at a constant platelet count resulted in a step-wise decrease in fluorescence intensity revealing a range of antibody concentrations that bound to the platelet without inducing maximal fluorescence intensity, named M2 fraction (Figure 1A). Analysis of AN51-PE binding using a constant, suboptimal antibody concentration and platelets stored for different periods at 0 °C showed a similar decrease in fluorescence, probably as a result of changes in GPIb α that decreased its affinity to the AN51-PE antibody and subsequently, changing GPIb α positive fractions from the M1 to the M2 fraction (Figure 1B). Indeed, within a small window of antibody concentration (1- 2 µg/ mL) AN51-PE binding was a sensitive means to detect storage-induced changes in GPIb α (Figure 1C, D).

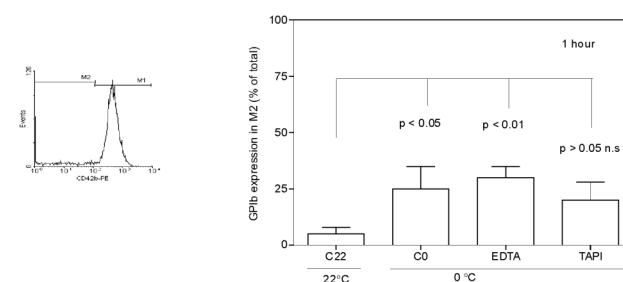
Figure 1. Analysis of GPIb α expression by FACS. (A) shows the distribution of AN51-PE on fresh platelets stored at room temperature for 1 hour using different concentrations of antibody. (B) platelets stored on ice for 6 hours (C0/ 6 hrs) lose binding of AN51-PE and shift from the M1 to the M2 region. (C) shows the effect of AN51-PE concentration on the detection of the M1 fraction. (D) shows the effect of different concentrations AN51-PE on the peak shift form M1 to M2 during storage at 0°C for 24 hours. Data are means \pm SEM, n = 4.



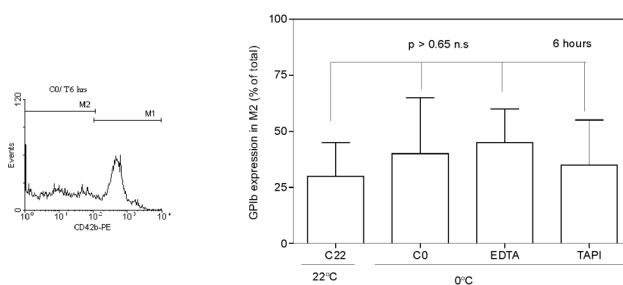
To investigate whether the decrease in antibody binding observed during cold-storage was the result of proteolytic damage of GPIb α , incubations were repeated in the presence of EDTA which inhibits various metalloproteinases²⁷ and fibrinolytic proteinases²⁸ and TAPI, an inhibitor of ADAMS-17. A concurrently run control suspension was stored at room temperature (Figure 2). A first analysis after 1 hour incubation at 0 °C already showed 25% of the platelets in the low affinity fraction M2. Neither EDTA nor TAPI interfered with this expression suggesting that a decrease in GPIb α was not the result of proteolysis. The fresh control suspension kept at room temperature showed little GPIb α affinity loss, indicating that the cooling step was the cause for the fall in GPIb α affinity for AN51-PE. Subsequent storage at 0°C did not prevent a further increase in low affinity GPIb α both in the absence and presence of proteolytic inhibitors. The same increase was found in platelets stored at room temperature.

Figure 2. The shift from M1 to M2 during platelet storage at 0°C and 22 °C in the presence of inhibitors of proteolysis of GPIb α , TAPI and other metalloproteinases (EDTA). Platelets were incubated in the absence (control) and presence of 1mM EDTA and 110 nM TAPI and analyzed after 1 hour (A), 6 hours (B) and 48 hours (C) storage. Data are means \pm SEM of 6 independent experiments.

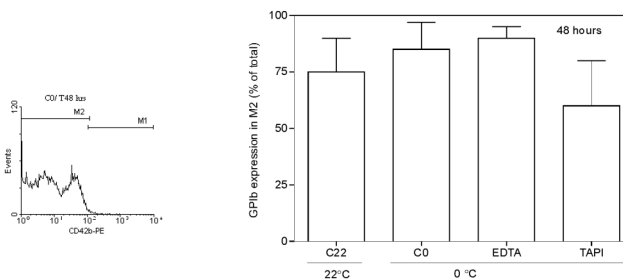
A. Fresh.



B. 6hr



C. 48 hr



Low affinity AN51 binding and phagocytosis by macrophages

Cooling of platelets to 0°C is known to rearrange surface GPIb α into clusters. These clusters are recognition sites for integrin $\alpha_M\beta_2$ which mediates their uptake and destruction by liver macrophages.⁷ To evaluate the possibility that the affinity loss of GPIb α during cold storage was a reflection of clustering of GPIb α , cold-stored platelets were incubated with PMA-matured macrophages and binding / phagocytosis was analyzed in the absence and presence of an excess of GlcNAc (Figure 3). This sugar is known to interfere with phagocytosis through competitive inhibition with GlcNAc exposed on clusters of GPIb.^{7,29} Cold storage hardly changed the binding of platelets to macrophages. (Figure 3A,C). In contrast, there was a gradual increase in phagocytosis, which was accompanied by an increase in platelets with reduced AN51-PE binding (Figure 3A). Treatment with GlcNAc left the binding unchanged but strongly reduced phagocytosis (Figure 3B). The constant binding is caused by the presence of PMA in the matured THP-1 suspensions. This is a trigger for optimal surface expression of P-selectin, a main mediator of platelet binding to macrophages. These findings confirm earlier observations^{30,31} and are in agreement with the concept that GlcNAc interferes with phagocytosis by competition with GlcNAc on clusters of platelet GPIb α .

Effect of GlcNAc on AN51-PE binding to GPIb α

We next investigated whether GlcNAc interference of platelet phagocytosis by macrophages involved binding to GPIb α . Fresh platelets showed a single population of cells with high affinity binding of AN51-PE (M1 fraction, Figure 4A). This binding was unchanged after treatment with GlcNAc illustrating that the GlcNAc did not change the properties of the antibody. Cold storage again introduced a shift to lower antibody binding (M2 fraction), although some platelets remained in the high affinity range. Interestingly, when platelets were stored for 6 hours at 0 °C and thereafter incubated for 1 hour with GlcNAc (37 °C), the low antibody binding shifted to the high binding observed in fresh platelets (Figure 4B). Also when GlcNAc was added prior to cold storage, the high affinity M1 fraction was preserved (Figure 4C). These data suggest that GlcNAc introduces a conformational change in GPIb α that affects the binding of AN51-PE.

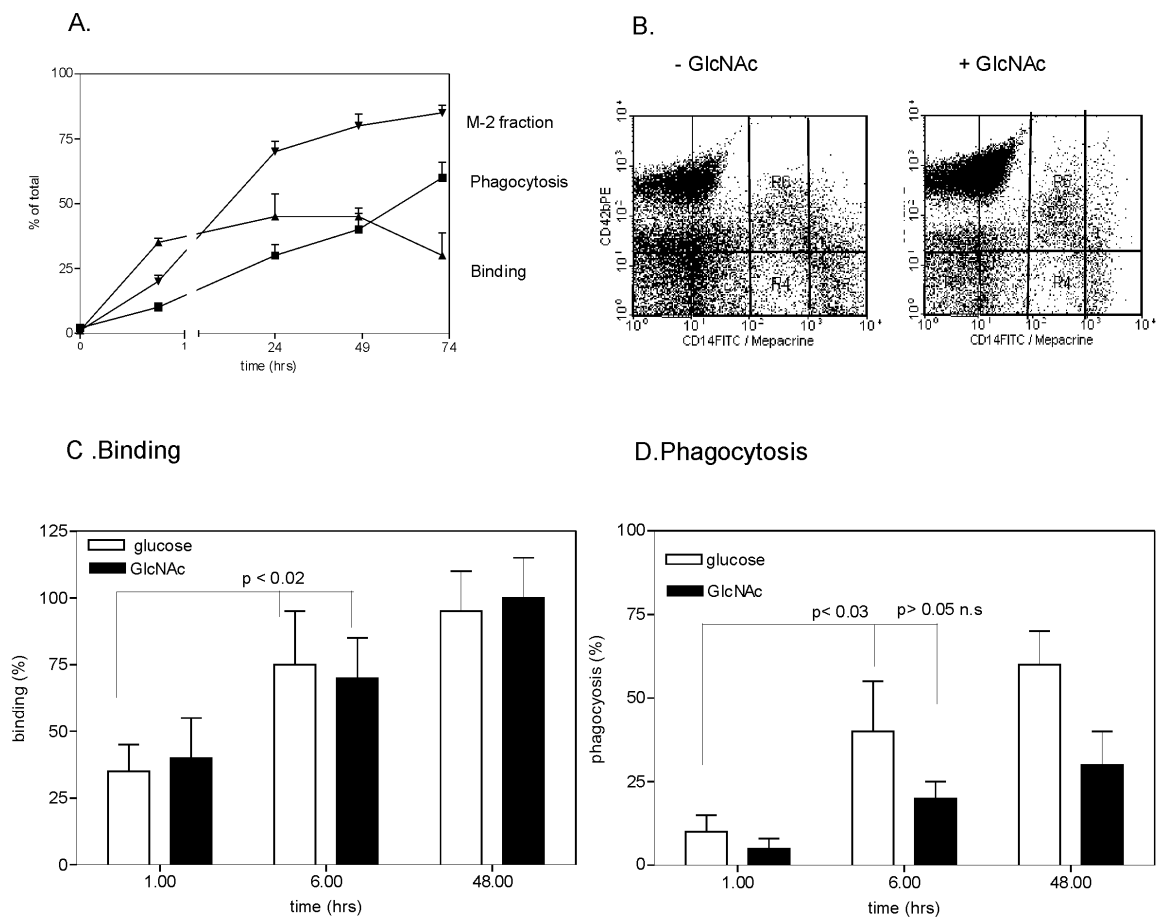


Figure 3. Comparison between the shift from M1 to M2 after 72 hours storage, and their binding and phagocytosis by THP-1 macrophages after 30 minutes at 37°C incubation (A). In (B) is shown scatter plots of binding and phagocytosis of platelets stored for 48 hours followed by 1 hour preincubation at 37°C without and with 100 mM GlcNAc. (C, D) show the effect of GLcNAc on binding and phagocytosis of platelets. Data are means \pm SEM of 6 experiments.

To investigate whether this effect was specific for GlcNAc, studies were repeated with 100 mM glucose and mannose (Figure 4D). Although these sugars showed a slight effect, there interference was much smaller than observed with 100 mM GlcNAc. Separation by cell sorting resulted in isolation of two separate populations which had preserved their typical affinity for AN51-PE binding (Figure 4E-G). Each fraction showed the same binding to macrophages, indicating that the change in antibody binding or sorting procedures did not play a role in platelet binding to macrophages. Phagocytosis was completely absent both in the M2 fraction and the M1 fraction. Apparently, the sorting procedure led to loss of phagocytosis signals on the platelets (data not shown).

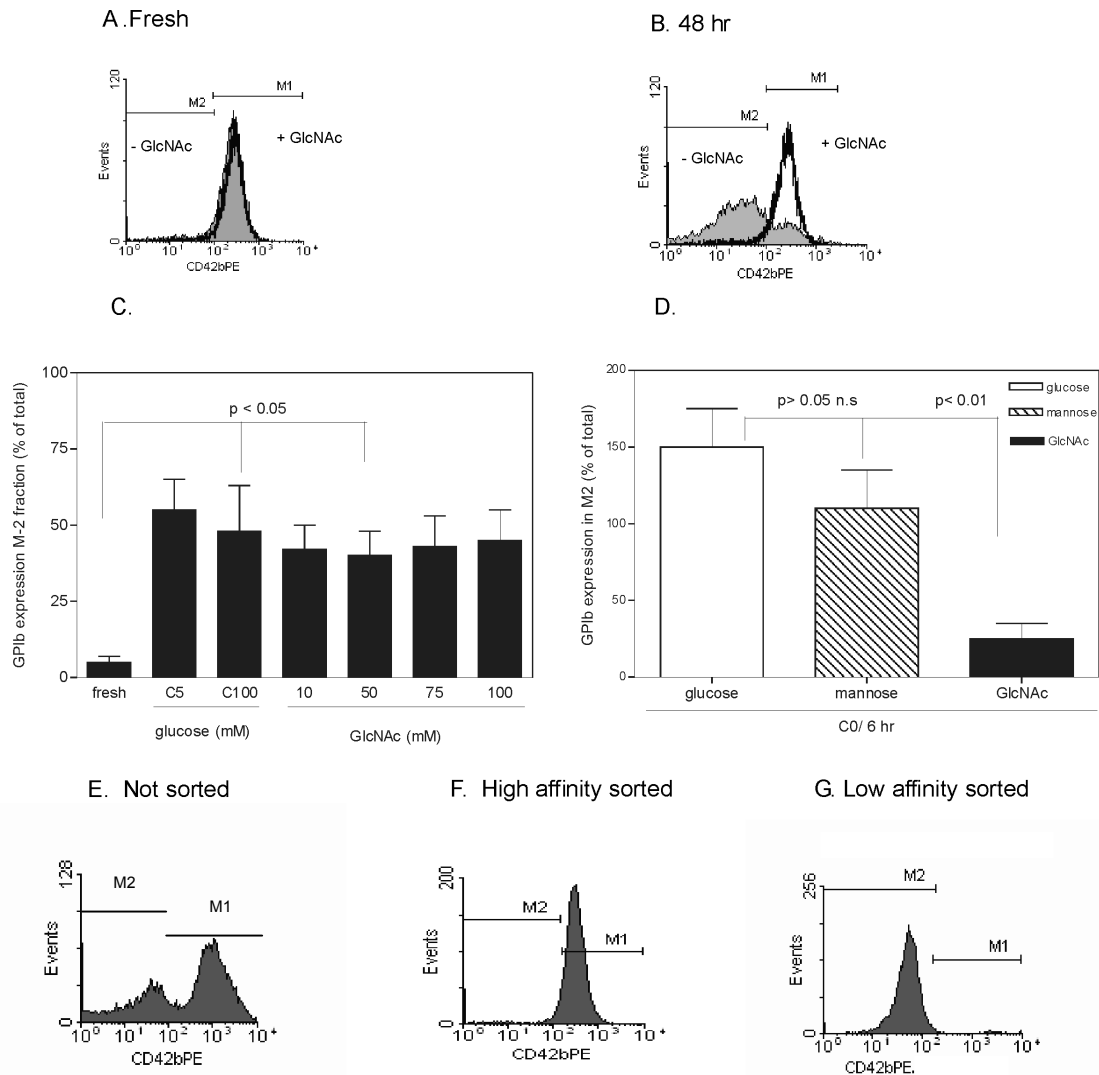


Figure 4. Effect of GlcNAc on AN51-PE binding to GPIIb/IIIa. (A,B) show the effect of GlcNAc addition to fresh (A) and 48 hours stored platelets (B) on AN51-PE binding. GlcNAc restores the high affinity binding in stored platelets. (C) shows the M-2 fraction after 6 hours incubation in the presence of different concentrations of GlcNAc compared to controls with 5 mM (C 5) and 100 mM glucose (C 100). (D) shows the M2 fraction of platelets stored at 0°C for 6 hours and then incubated with 100 mM glucose, mannose and GlcNAc for 1 h at 37°C. In (E-G) is shown the binding of AN51-PE; control sample without sorting only passed through the BD-FACSARIA sorter (Not sorted) (E), and after sorting of platelets (F,G) with high (High affinity sorted) and low density expressing population (Low affinity sorted) bound to AN51-PE antibody.

Interference with AN51-PE binding to GPIb α

In an attempt to understand how conformational changes in GPIb α reflected by AN51-PE binding affected the role of GPIb α as recognition signal for phagocytosis, platelets were incubated with different moAbs with defined binding epitopes on GPIb α and changes in AN51-PE binding were compared with phagocytosis. Again, cold storage led to an increase in the M2 fraction (Figure 5A). Preincubation with unlabelled AN51 induced the expected increase in M2 and served as a control for impaired AN51-PE binding. The moAb 6D1 is directed against AA 104-128 in the binding region for VWF and did not change the M1-M2 distribution. In contrast, moAb 6B4, directed against AA 201-268 in the C-terminal flank of the leucine-rich VWF binding region and overlapping the binding sites for thrombin (AA 216-262) and VWF-ristocetin (AA235-261) greatly enhanced the number of M2 platelets. MoAb 10H9 directed against AA 276-282 adjacent to the thrombin and VWF-ristocetin binding sites had no effect. Thus, the affinity loss for AN51 binding caused by cold storage is enhanced by antibody binding to AA 201-268. Preincubation with 100 mM GlcNAc did not change these effects (Figure 5B). Again 6B4 induced a further shift to the M2 fraction.

There was a slight interference by 10H9 following GlcNAc incubation which was not seen in its absence suggesting that GlcNAc binding induced a change in GPIb α that made an antibody against AA 262-282 interfere with AN51-PE binding.

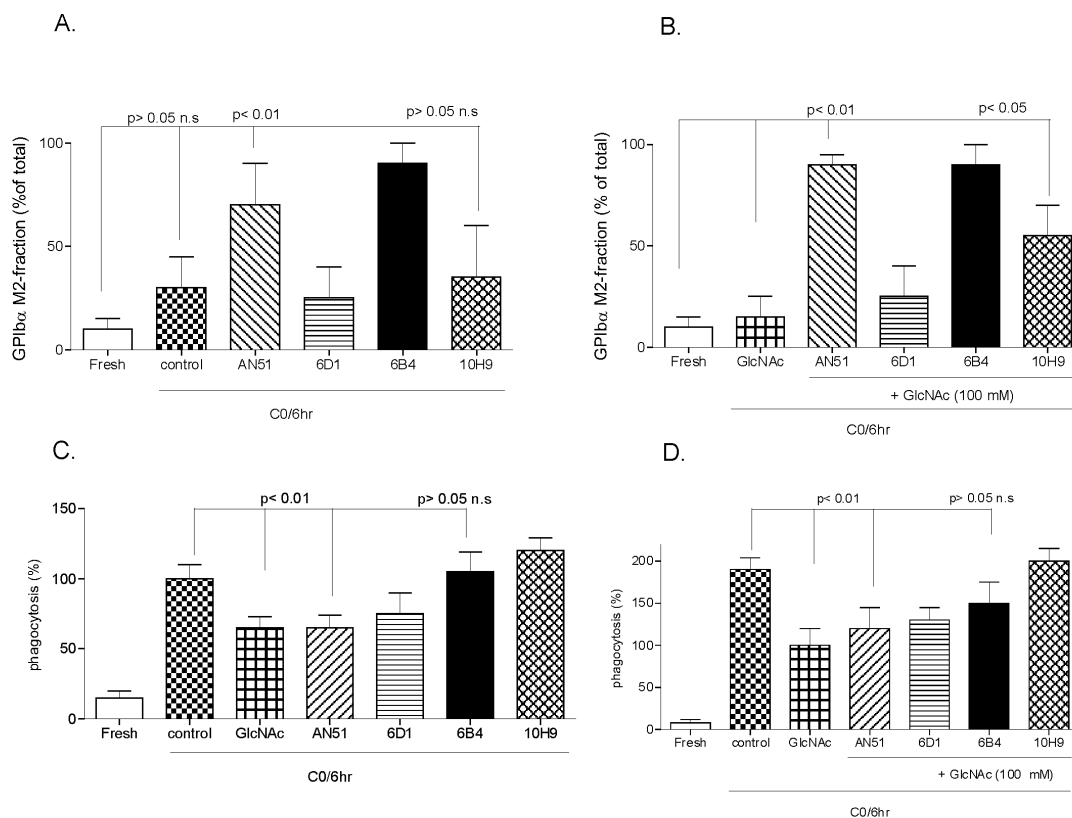


Figure 5. Effect of antibodies against GPIb α on binding of AN51-PE and phagocytosis. (A) Platelets stored for 6 hours at 0°C were incubated for 1 hour at 37°C with moAbs AN51 (unlabelled), 6D1, 6B4 and 10H9 and the binding of AN51-PE was measured. (B) The same samples were first incubated with GlcNAc for 1 hour at 37°C and then with moAbs for 1 hour at 37°C. (C,D) show the effect of the same preincubations on phagocytosis. Data are means \pm SEM of 4 independent experiments.

Concurrent analysis of phagocytosis demonstrated that treatments in the absence, the antibodies had little effect AN51 and 6D1 slightly reduced phagocytosis to the range also found with GlcNAc, whereas 6B4 and 10H9 preserved phagocytosis in the range of the controls (Figure 5C). The unlabelled AN51 antibody did not induce more phagocytosis suggesting that the recognition signals for apoptosis are close to AA 1-35. When the platelets were first treated with GlcNAc and thereafter with the antibodies, the difference between the lower phagocytosis induced by GlcNAc and the higher phagocytosis induced by the antibodies became larger illustrating that these antibodies opposed the mechanisms that reduced the phagocytotic signals on GPIIb α (Figure 5D). Again AN51 had no effect. Treatment with antibodies prior to GlcNAc resulted in similar findings as treatment with antibodies alone, suggesting that the binding of the antibodies is sufficiently strong to resist interference by GlcNAc (data not shown).

Discussion

Here we show that cooling of platelets and subsequent storage at 0°C induces a change in GPIb α that leads to a decrease in binding affinity for a moAb directed against AA 1-35 in the N-terminal flank. High affinity binding is restored by GlcNAc which is known to inhibit phagocytosis of cold stored platelets by macrophages by competing with GlcNAc-exposing GPIb α clusters for binding to integrin $\alpha_M\beta_2$ on macrophages.^{7;29} Detection of these changes in GPIb α depends on a critical antibody concentration as an excess fails to detect a shift from the M1 to the M2 fraction (data not shown), whereas a suboptimal concentration detects all fresh platelets in M2 preventing a further increase upon cold-storage. The observation that GlcNAc restores high affinity antibody binding of cold-stored platelets suggests that in addition to its capacity to interfere with GPIb α - $\alpha_M\beta_2$ interaction, the sugar introduces a conformational change in GPIb α that affects its affinity to AN51-PE. Interestingly, the same treatment reduces phagocytosis, making AN51-PE binding a marker for phagocytosis signals on GPIb α .

Attempts to evaluate separate M1 and M2 populations for their capacity to become phagocytosed were unsuccessful. Both populations could be clearly separated and showed a similar binding to THP-1 cells matured by preincubation with a high concentration of PMA. Previous studies have shown that surface expressed P-selectin is a major intermediate in platelet binding to macrophages suggesting that M1 and M2 platelets showed a similar degree of α -granule secretion, which is the source of P-selectin expression. This might be the result of activation by PMA in the cell suspensions, which is a potent inducer of the platelet secretion response.³²⁻³⁴ Initially, the presence of AN51-PE on the platelets during sorting was thought to be the cause of the abolished phagocytosis. However, addition of AN51 antibody prior to platelet-macrophage contact in stead of the usual addition of antibody at the end of the phagocytosis assay showed that AN51 did not reduced with platelet destruction. Probably, the duration of the sorting procedure and the strain to which platelets are submitted destroys the phagocytosis signals in GPIb α . The finding that AN51-PE binding recognizes phagocytosis signals in GPIb α but does not block with this process indicates that its binding domain AA 1-35 is not the site where changes induced by cold storage lead to recognition by macrophages. Apparently, loss of AN51-PE binding is a sensitive marker for changes elsewhere in GPIb α that become targets for macrophage recognition. These changes are enhanced by moAb 6B4 directed against AA 201-268 overlapping the binding sites for thrombin and the VWF-ristocetin complex and 10H9 against AA 276-282 in the anionic sulfated sequence adjacent to these binding sites. In contrast, moAbs 6D1 against AA 104-128 of the VWF binding domain had no effect. In freshly collected platelet suspensions moAb 6B4 is known to inhibit VWF binding to its binding domain in region in AA 36 – 200^{23;35} suggesting a spatial orientation of GPIb α that brings regions AA 201 – 268 and AA 36 – 200 in close proximity. The present data with cold-preserved platelets suggest such a proximity for the regions AA 201 - 268 recognized by 6B4 and AA 1- 35 to which AN51 binds and factors that interfere with such an interaction such as the binding of an antibody affect the generation of phagocytosis signals. Also moAb 10H9 interfered with AN51-PE binding and phagocytosis although its binding epitope is adjacent of the 6B4 binding site (Figure 6).

Figure 6.

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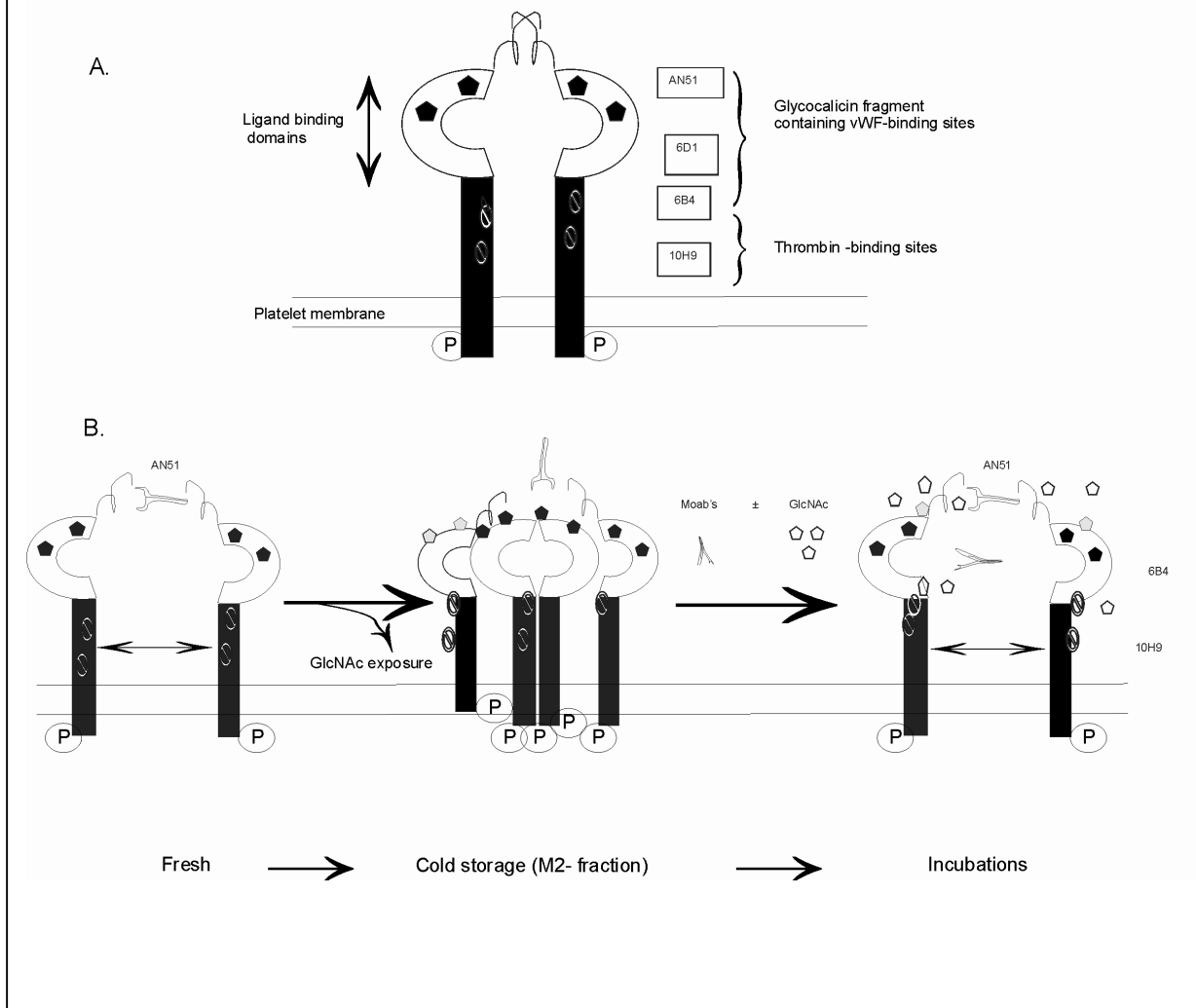


Figure 6. Schematic representation of changes in GPIIb/IIIa during cold storage. (A) model of GPIIb/IIIa and the position of the epitopes for moab binding. (B) cold storage induces a conformational change that causes 6B4 to interfere AN51 binding, GlcNAc restores the conformation of GPIIb/IIIa of fresh platelets. GlcNAc (◡), O-linked oligosaccharides (⊙)

This interference was particularly evident in the presence of GlcNAc with the antibody reducing AN51-PE binding and increasing phagocytosis. Possibly, GlcNAc contributes to these effects by changing the conformation of GPIIb/IIIa thereby increasing AN51-PE binding, interference by 10H9 and reducing phagocytosis. Competition inhibition studies with GlcNAc and anti GPIIb/IIIa antibodies show that GlcNAc interference with GPIIb/IIIa is too weak to interfere directly with antibody binding (data not shown). Further studies are required to elucidate the mechanism through which GPIIb/IIIa regulates the exposure of targets for macrophage recognition and platelet destruction.

Acknowledgment

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Chapter 5

Role of surface markers for binding and phagocytosis of platelets by macrophages

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Submitted

Abstract

Introduction: Using an *in vitro* assay for analysis of the interaction between platelets and macrophages, we previously identified surface-expressed platelet P-selectin as an intermediate in binding and exposure of phosphatidylserine (PS) combined with conformational changes in glycoprotein (GP) Ib α as intermediates in phagocytosis. There is little insight in the relative contribution of these regulators of platelet destruction by macrophages.

Objectives: The aim of this study was to evaluate the quantitative relationships between these markers for platelet destruction by macrophages.

Methods and study design: Platelets in buffer were stored at 22°C for different periods to obtain a wide range of surface expressed P-selectin and -PS measured by FACS. The storage-induced conformational change in GPIb α was deduced from decreased binding of an anti GPIb α antibody, also by FACS. Data from the same platelets were compared with binding and phagocytosis by PMA-matured monocytic THP-1 cells to determine the relative contribution of these parameters to platelet destruction. Correlations established in room temperature stored platelets were then compared with platelets stored at 0°C and metabolic suppressed platelets (MSPs) stored at 4°C to investigate whether changes in storage conditions affected the relation between surface markers and platelet destruction.

Results: Between 0 and 80%, P-selectin expression correlated linearly with binding, which then leveled off. PS exposure showed a threshold of 20% below which phagocytosis was < 10%, but between 20 and 50% PS exposure correlated linearly with phagocytosis. GPIb α conformational change showed a threshold of 30% below which phagocytosis was < 10%, but between 30 and 90% GPIb α change correlated linearly with phagocytosis. A comparison between room temperature-stored, cold-stored and metabolic suppressed platelets (MSPs) showed that at a given degree of PS exposure and GPIb α change, cold stored platelets showed more phagocytosis than the other two suspensions.

Conclusions: These *in vitro* correlations may help to predict the extent of platelet binding to macrophages based on P-selectin expression, and the extent of phagocytosis based on PS-expression and GPIb α change. If platelet destruction parameters measured *in vitro* correlate with platelet destruction *in vivo*, the quality of transfused platelets can be predicted on the basis of these relatively simple FACS analyses.

Introduction

Most investigators in the field of platelet transfusion favor the idea that optimal platelet storage *in vitro* correlates with optimal haemostatic effectiveness and survival *in vivo*.^{1,2} There is indeed evidence that optimal preservation of platelet reactivity results in better platelet functions after transfusion with little binding to white blood cells³ and phagocytosis by macrophages.⁴⁻⁶ Nevertheless, there is a need for platelet tests that under laboratory conditions predict how well platelets behave in circulating blood. A test that predicts the efficacy of transfusions protocols for platelet concentrates (PCs) would have a clear medical benefit and save money. Different markers have been tested as indicators of the so called platelet storage lesion (PSL), such as P-selectin expression (PSE),⁷ expression of CD40/CD40 ligand⁸ and glycoprotein (GP)IIb/IIIa;^{9,10} changes in mean platelet volume, sphere to disc changes;¹¹⁻¹⁵ actin assembly;^{16,17} serotonin release;^{14,18} changes in GPIb-V-IX and GPVI expressions;^{19,20} PS exposure;^{21,22} loss of swirling;²³ osmotic pressure response²⁴⁻²⁷ and changes in platelet count caused by agglutination.^{18,28,28-30} Although each marker provides insight in the activation state of the platelets, none of them is a perfect predictor of the quality of transfused platelets.² An additional problem is the lack of comparative studies with data on sensitivity and specificity of the different markers.

Prolonged storage induces an gradual increase in PSE^{24,31-33} and PS exposure^{5,34-40} and a decrease in GPIb α expression.^{6,41-44} Hoffmeister et al^{45,46} described that cold-induced GPIb clustering correlates positively with phagocytosis and that galactosylation of GPIb α restores normal platelet survival. Leytin et al.⁶ described that PSE showed a positive and GPIb expression a negative correlation with phagocytosis of platelets *in vivo*.

Previously we provided evidence that binding of platelets to macrophages is mediated by surface expressed P-selectin and phagocytosis by exposed PS and changes in GPIb α , the receptor for von Willebrand factor. Changes in GPIb α were detected as a decrease in the binding of a PE-labeled moAb against AA 1 – 35 on the N-terminal flank and thought to be related to the clustering of GPIb α observed after cooling of platelets.^{45,46}

The aim of this study was to investigate how PSE, PS exposure and changes in the conformation of GPIb α that occur during platelet storage correlate with binding and phagocytosis by macrophages in an *in vitro* assay. The results show that each of these parameters contribute in a different manner to the processes that lead to destruction of platelets.

Materials and Method

Platelet preparation

Platelets were prepared as previously described.⁴⁴ In short, freshly drawn venous blood (40 ml) from healthy volunteers was collected with informed consent into 1:10 v/v 130 mmol/L trisodium citrate. The donors claimed not to have taken any medication during two weeks prior to blood collection. Platelet-rich plasma (PRP) was prepared by centrifugation (200 g, 15 minutes, 22°C). ACD (0.1 volume) was added to lower the pH to 6.5 and prevent platelet activation during further isolation. The suspension was centrifuged and resuspended in Hepes-Tyrode (pH 7.2). Platelet count was measured on a Cellcounter AL871 (Molab, Hilden, Germany). The platelet number was adjusted to 200 000 cells/ μ l before the experiments. Metabolic suppressed platelets (MSP) were prepared by incubating the cells for 40 minutes at 37°C in glucose-free Hepes-Tyrode (pH 7.2) in the presence of 20 μ M antimycin A. At the times indicated, 100 μ l samples were collected, incubated with 20 mM glucose in Hepes tyrode buffer for 1 hr at 37°C to restore energy generation. Controls were platelet suspensions in Hepes-Tyrode pH 7.2 containing 5 mM glucose stored at 22°C (C 22°C) or immediately cooled on ice (C 0°C). C 22°C was incubated with 20 mM glucose (1 hr, 37°C) prior to the aggregation tests to account for a possible glucose shortage.

Platelet P-selectin, PS exposure and GPIb α expression measurements by FACS

P-selectin and GPIb α expression was measured as described.⁴⁴ In short, at the indicated time points, 100 μ l sample was incubated in 1 ml plastic tubes with moAb CD62p-FITC and CD42b-PE (R70, clone AN51). For analysis of PS exposure, platelets were incubated with a combination of annexin V-FITC and AN51-PE as described.^{47,48} To all samples 300 μ l PBS was added and immediately 10,000 events were measured on a FACScalibur. Quantitative analysis was done using WinMDI software.

Analysis of platelet binding and phagocytosis by macrophages

The binding of platelets to macrophages was measured as described by Hoffmeister *et al.*⁴⁶ with some significant modifications.⁴⁷ In short, monocytic THP-1 cell lines divided in 24x NUNC multiwell plates (1 ml/ well) matured by 500 nM PMA incubated overnight at 37°C, 5% CO₂, in the cell incubator. 2x10⁶ platelets in Hepes-Tyrode buffer (pH 7.2) were added to a well containing 1x10⁶ PMA-matured THP-1 cells in 1 mL RPMI 1640 medium and the mixed suspensions were incubated at 37°C without agitation. Wells were gently washed with HBSS buffer (0.3 mM KH₂PO₄, 13.7 mM NaCl, 417 mM NaHCO₃, 31 mM Na₂HPO₄ and 0.5 mM KCl in aqua dest) and free platelets collected in the wash medium were isolated by centrifugation-resuspension under protection of PGI₂. Wells were incubated with 200 μ L HBSS buffer containing 5 mM EDTA for 15 minutes at 0°C, reconstituted with free platelets removed during the wash step and thereafter 100 μ L suspension was incubated with 2 μ g/mL anti-human CD42b-PE and 2 μ g/mL anti-human CD14-FITC antibody for 15 minutes at 37°C. Then, HBSS buffer was added and 20 000 particles were measured by flowcytometry (FACS Calibur, Becton-Dickinson, San Jose, CA, USA). FACS data were analyzed with WinMDI software. Binding of platelets to macrophages was expressed as the percentage of CD42b/CD14 positive particles of the total number

of CD42b and/or CD14 positive particles. Phagocytosis of platelets by PMA matured THP-1 cells was measured by FACS analysis of mepacrine positive CD14 cells that were inaccessible to the anti CD42b-PE antibody and expressed as percentage of total number of CD14 positive, CD42b negative particles.

Analysis of correlations

To compare PSE, PE expression and GPIIb α changes with binding and phagocytosis, platelets from 6 to 9 different donors were stored under different conditions for up to 48 hours as indicated in the “results” section.^{44,47,48} The surface markers together with data on binding and phagocytosis from the same donor were expressed as means \pm SEM and curve fitted according to the nonlinear regression (curve fit), the sigmoidal dose-response with the Graph pad program.

Statistics

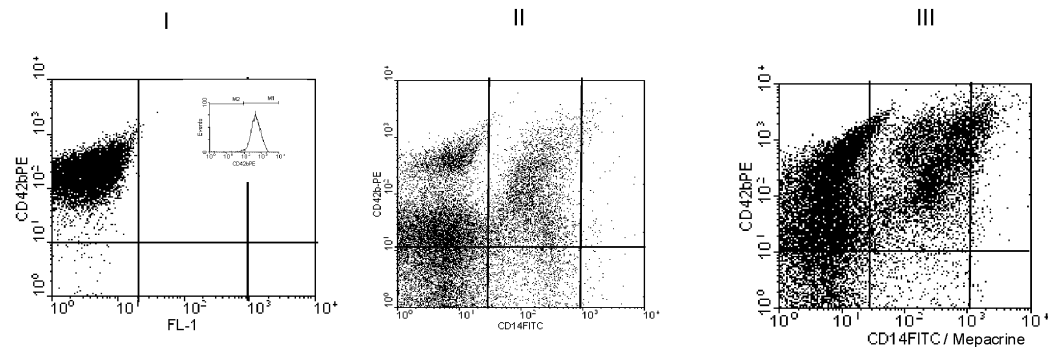
Data are expressed as means \pm SEM with number of observations n. Statistical analysis was by nonparametric Spearman correlation analysis, two-tails with confidence intervals of 95%. Differences were considered significant at a *p* value of less than 0.05.

Results

A decrease in AN51-PE binding to GPIb α correlates with phagocytosis

Figure 1A, B left panels shows the change in binding of AN51-PE antibody after 48 hours storage at 22°C. There is a clear tendency to lower antibody binding. Storage was also accompanied by an increase in platelet binding to macrophages (Figure 1A, B middle panels), and phagocytosis (figure 1A,B right panels). Freshly isolated platelets stored at room temperature for 1 hour showed a single population with high affinity to the AN51-PE antibody, named M1 fraction. After 48 hours storage this fraction decreased by about 40% resulting in a population with low affinity for binding of the antibody, called M2 fraction (Figure 1, inserts). In freshly isolated platelets binding was less than 5 % but after storage for 1 hour, binding to macrophages increased to 25±15% but phagocytosis was less than < 5%. Platelets stored for 48 hours showed an increase in the GPIb M-2 fraction and an increase in binding by about 200% and phagocytosis by 800%.

A C22/ 1hr.



B. C22/ 48hrs

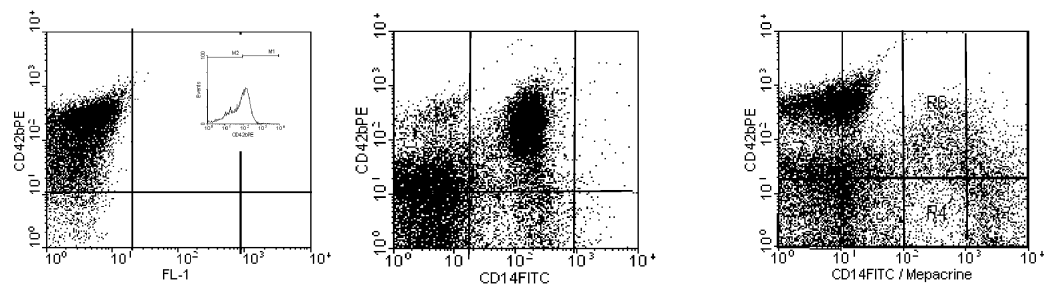


Figure 1. Changes in platelets during storage at room temperature. FACS analysis of room temperature stored-platelets (C22): GPIb α expression after 1 hour (A I) and after 48 hours storage (B I). Binding to THP-1 cells after 1 hour (A II) and after 48 hours storage (B II) in the presence of PMA. Phagocytosis after 1 hour (A III) and after 48 hours storage (B III) in the presence of PMA.

Role of P-selectin expression

In earlier studies we demonstrated that an antibody against P-selectin reduced the binding of platelets by macrophages and consequently their phagocytosis. To define the role of P-selectin in greater detail, we investigated platelet – macrophage interaction without and with removal of the PMA which is normally present to preserve the maturation of monocytic THP-1 cells to macrophages. Fresh platelets showed about 20% P-selectin expression in the absence of PMA and 50% in its presence, confirming the role of PMA as a secretion-inducing agent (Figure 2). Phagocytosis of the same platelets was < 5% with and without PMA removal. After 48 hours storage, there was about 50% P-selectin expression without PMA and 60% with PMA. These platelets showed about 20 and 35 % phagocytosis. These data are in line with the concept that P-selectin is an intermediate in binding and not in the induction of phagocytosis.

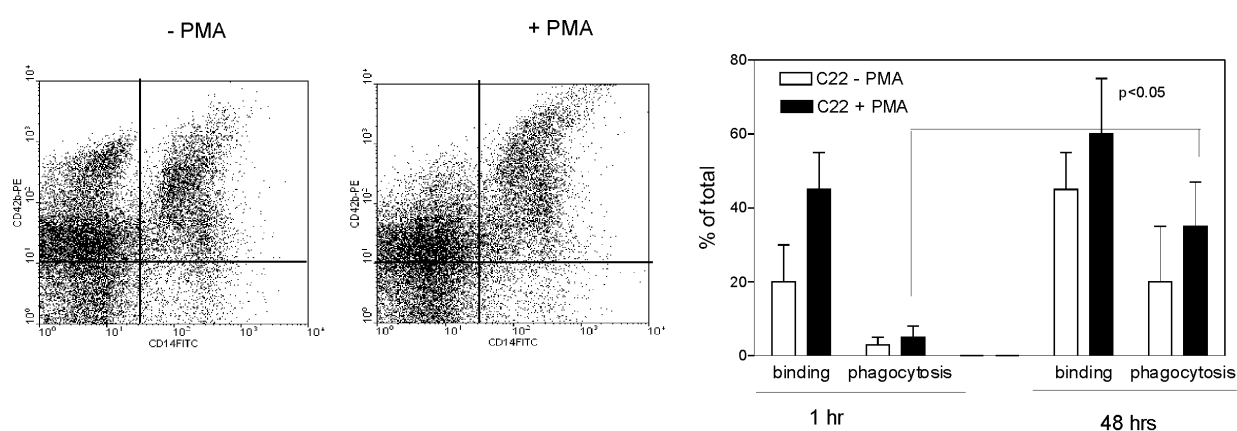


Figure 2. P-selectin expression mediates binding but not phagocytosis by macrophages. Scatter plots show binding of room temperature-stored platelets (C22) without (-PMA,) and with PMA stimulation (+PMA). The right panel shows the binding and phagocytosis of platelets from different donors incubated for 30 minutes at 37°C with macrophages; 20000 events were measured by FACS. Data are means \pm SEM, n=6.

We next studied the correlations between surface markers for platelet destruction and binding and phagocytosis. Platelet suspensions were stored at 22°C for up to 48 hours and at different periods samples were collected for analyses (Figure 3). Between 0 and 80%, P-selectin expression correlated linearly with binding, which then leveled off.

PS exposure showed a threshold of 20% below which phagocytosis was < 10%, but between 20 and 50% PS exposure correlated linearly with phagocytosis. GPIIb α conformational change showed a threshold of 30% below which phagocytosis was < 10%, but between 30 and 90% GPIIb α change correlated linearly with phagocytosis. Thus, an increase in PSE induced more binding almost over the whole range of surface P-selectin.

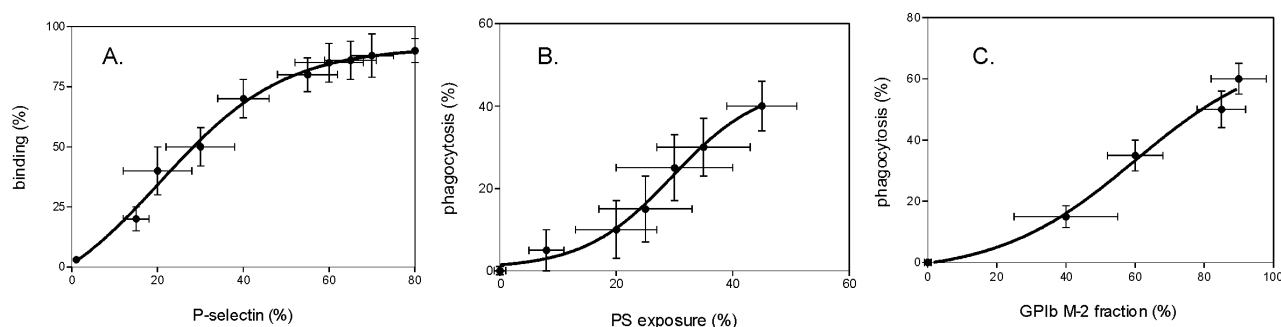


Figure 3. Correlations between P-selectin expression, PS exposure and GPIb α change with binding and phagocytosis in room temperature-stored platelets. (A) shows the correlation between P-selectin expression and platelet binding. (B) Correlation between PS exposure and phagocytosis. (C) Correlation between GPIb α M-2 fraction and phagocytosis. Data are means \pm SEM, $n = 9$.

In contrast, PS exposure and GPIb α change showed a threshold of 20-30% before phagocytosis was initiated, illustrating that a slight degree of PS-exposure and GPIb α change does not immediately lead to phagocytosis. If the regulation of platelet binding and phagocytosis by macrophages are restricted to surface expression of P-selectin and PS and changes on GPIb α , one would expect that correlations established in room temperature-stored platelets are the same in platelets stored under different conditions. To address this issue, platelets were stored at 0°C and at 4°C, the latter after applying metabolic suppression as described ⁴⁴. Correlations between phagocytosis and exposed PS (Figure 4A) and the GPIb α change (Figure 4B) of the platelets from the same donor were approximately the same for platelets stored at 22 °C and MSP.

Platelets stored at 0°C without prior reduction in metabolic energy, however, had a tendency to show more phagocytosis than the other suspensions. This suggests that apart from PS exposure and GPIb α changes other factors contribute to the induction of phagocytosis in cold-stored platelets without metabolic suppression. The nature of these factors has yet to be clarified.

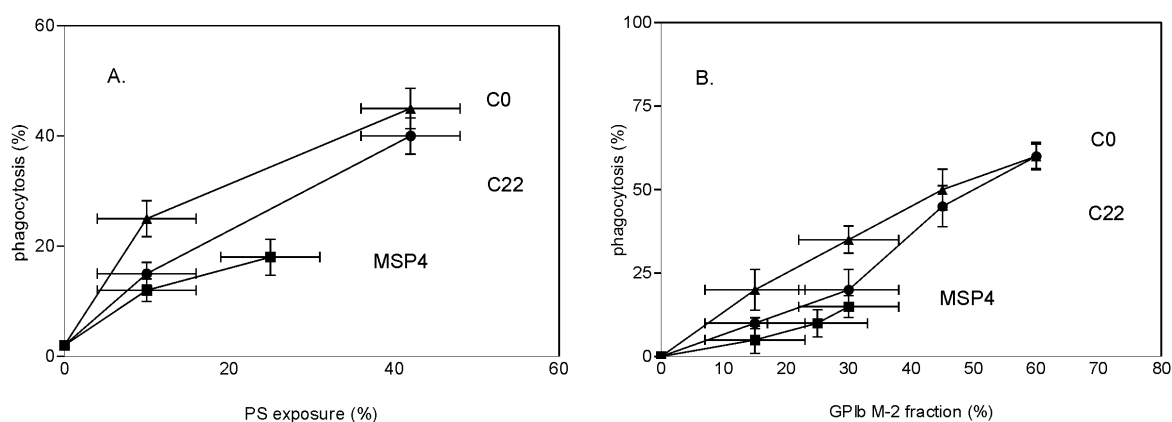


Figure 4. Correlations between destruction markers on platelets and phagocytosis in suspensions stored under different conditions. Correlations between phagocytosis and PS exposure (A) and GPIb α change (B) in platelets from the same donor stored at room temperature (C22), 0°C (C0) and 4°C after prior metabolic suppression (MSP4) after 0, 40min, 48h and 72 h storage

Discussion:

The present data shows that a slight degree of PS exposure (< 20%) and GPIb α change (<30%) do not induce phagocytosis. There appears to be a threshold that protects platelets against phagocytosis and only when surface changes in these parameters exceed this level, platelets become targets for destruction by macrophages. Although this is particularly evident in stored platelets, also fresh platelets show a slight degree of PS exposure and GPIb α change and these platelets are not phagocytosed.⁴⁷ We found no evidence for a threshold for P-selectin expression and even a slight increase in PSE induced binding to macrophages. These differences illustrate that platelet binding to macrophages and their subsequent phagocytosis are different processes, such in agreement with earlier observations.^{6,34,47,49} A certain degree of phagocytosis was accompanied by more PS-exposure than GPIb α change, suggesting that exposed PS is a more powerful destruction signal than the change in GPIb α . However, the two parameters are difficult to compare since each is expressed relative to the total number of platelets in the suspensions and the extend to which they become positive during storage for PS (maximum 50% of total platelets) and GPIb α change (maximum 90% of total platelets) is quite different.

If one assumes that PS and changes in GPIb α are the exclusive intermediates that control the phagocytosis of platelets by macrophages, one would expect that the correlations between surface markers and platelet destruction are independent of the conditions of the storage milieu. This is apparently not true. Cold-stored platelets showed relatively more phagocytosis than platelets stored at room temperature. This might be an indication that other factors contribute to the appearance of

phagocytic signals on the platelets. These factors have yet to be characterized but the difference between cold-stored and room temperature stored platelets might provide a basis for further identification of these factors.

Interestingly, metabolic suppression prior to cold storage greatly suppressed the expression of phagocytic signals. The relation between PS exposure and GPIb α change *versus* phagocytosis appears to follow the correlation established with room temperature stored-platelets, but the low PS expression and GPIb α change hamper reliable calculations. The rationale of applying metabolic suppression prior to storage is that storage conditions trigger activation of platelets and start functions such as secretion and PS-exposure. The activating sequences and the execution of secretion responses in particular are energy-dependent processes. A shortage of metabolic energy would therefore bring the platelets in a “hibernation” state that protects the cells until energy metabolism is restored. The finding that this treatment reduces PS-exposure is not surprising since ATP-driven reactions contribute to the preservation of PS asymmetry and PS flip flop. The finding that metabolic suppression also reduces the change in GPIb α seen during storage is unexpected. GPIb α is the receptor that mediates platelet adhesion to VWF.^{4,46} The cooling of platelets is thought to trigger irreversible GPIb α clustering which provides a signal for rapid destruction by phagocytes.⁴⁶ Hepatic macrophages rapidly remove cold-stored platelets from the circulation through binding of $\alpha_M\beta_2$ integrin to GlcNAc-exposing GPIb α clusters.^{45,46} But also storage at 22°C shows that old platelets are more prone to phagocytosis than young platelets, suggesting that the same mechanism might control the removal of room temperature-stored platelets after transfusion.^{5,6}

At present, it is uncertain whether the decrease in binding of a PE-labeled antibody against AA 1-35 of GPIb α is a reflection of the clustering observed in transmission electron micrographs described before.⁴⁶ If one assumes that such a correlation exists, our findings would imply that there is a role of metabolic energy in the clustering of GPIb α .

It is clear that the present correlations between surface markers for platelet destruction and binding and phagocytosis 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 platelets under *in vivo* conditions. If such a correlation could be established in future studies, the sensitivity of transfused platelets to destruction by macrophages could be predicted on the basis a few, rather simple, FACS analysis.

Acknowledgments

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Chapter 6

Metabolic energy reduction by glucose deprivation and low gas exchange preserves platelet function after 48 hour storage at 4°C

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Submitted

Abstract

Introduction: We showed earlier that metabolic suppressed platelets (MSPs) prepared by incubation in glucose-free, antimycin A medium at 37°C better sustain storage at 4°C than intact controls. However, the use of the mitochondrial inhibitor antimycin A is incompatible with platelet transfusion.

Objectives: The aim of this study was to investigate how energy-reduced platelets (PLTs) could be prepared under blood bank conditions in the absence of antimycin A. **Study design and methods:** PLTs in gas-impermeable bags in glucose-free medium were kept at 22°C for 4 hours to reduce energy stores and thereafter stored at 4°C (ER22-4). Controls were ER22-4 PLTs without prior incubation at 22°C (ER4), MSPs and PLTs in gas-permeable bags with glucose and stored at 22 (C22) and 4°C (C4). **Results:** After 48 h storage ER22-4 were superior to C22 with respect to pH preservation (6.4 ± 0.4 vs 5.3 ± 0.9 , $n=4$), PLT count (800 ± 225 vs $650 \pm 150 \times 10^9$), TRAP-induced aggregation (30 ± 15 vs $10 \pm 5\%$) and glycoprotein (GP)Ib α expression ($60 \pm 15\%$ vs 30 ± 15). GPIb α expression was higher in ER22-4 than in ER4 indicating that energy suppression preserved GPIb α during cold storage. Thus, the effect of antimycin A effect could be mimicked by storage in gas-impermeable bags. **Conclusion:** Energy suppression by methods that approach blood bank conditions is a feasible means to prevent PLT activation during low temperature storage opening ways to improve PLT storage under conditions that attenuate bacterial multiplication.

Abbreviations: MSP4 = Metabolic Suppressed Platelets stored at 4°C, ER = Energy Reduced platelets, C22 = room temperature stored control platelets, C4 = in refrigerator stored control platelets, PSE = P-selectin expression, PLT = platelet

Introduction

The storage of platelet concentrates (PCs) at room temperature facilitates microbial multiplication^{1,2} and introduces changes in platelets (PLTs) indicative for activation³⁻⁶ and initiation of apoptosis.^{7,8} The main feature of the PLT storage lesion is the gradual increase in P-selectin expression,⁶ lactate production with concomitant reduction in pH,⁹⁻¹² and decrease in glycoprotein (GP)Ib expression.^{13,14} Improvements have been sought in lowering the storage temperature from 22°C to 4°C but this condition severely reduces the survival of transfused PLTs.^{15,16} Attempts to reduce the surface expression of recognition sites for PLT clearance have been based on inhibition of granule secretion thereby reducing P-selectin expression (PSE),^{5,6,17-19} preservation of a neutral pH,²⁰ and arrest of glycolytic and oxidative energy generation.^{14,21} A reduction in ATP resynthesis impairs the capacity of PLTs to respond to activating stimuli during storage, which might improve their quality after storage provided that energy metabolism and hemostatic functions can be fully restored. Transient metabolic suppression can be induced by incubation of PLTs with antimycin A, an inhibitor of mitochondrial respiration, and storage in a glucose free medium. After a period of so called “hibernation”, these metabolic suppressed PLTs (MSPs) can be stored at low temperature and subsequent incubation in glucose-rich medium leads to recovery of PLT functions.¹⁴

We showed previously that metabolic suppression better preserve the capacity to aggregate upon stimulation with thrombin receptor (PAR1) activating peptide and preserve a higher GPIb α expression, GPIb-related aggregation and adhesion to von Willebrand Factor (VWF) than controls stored at 4°C.¹⁴ An important drawback of the preparation of MSPs is the use of antimycin A, an inhibitor of cytochrome B, and obviously incompatible with platelet transfusion. In addition, studies with MSPs were performed in closed test tubes, a condition clearly different from the storage of PCs in blood banks.

In the present study we searched for modifications for the preparation of MSPs that would open ways to introduce this procedure in blood banks as a first step for further tests on the applicability of metabolic suppression for transfusion. The results showed that antimycin A can be replaced by storage in gas-impermeable bags. Subsequent storage at 4°C of these energy-reduced PLTs (ER22-4) followed by recovery with glucose leads to PLTs that better preserve a number of *in vitro* functions than controls stored without prior metabolic blockade.

Materials and Methods

We obtained: antimycin A from Sigma Chemicals (Mannheim, FRG), moAbs, CD42b-PE (AN51 clone R7014) was from Dako A/S (Glostrup, Denmark). FITC-labeled IgG (Dako A/S) was used as a negative control in the FACS experiments. Paraformaldehyde was from Sigma-Aldrich, (Mannheim, FRG). Thrombin receptor-activating peptide SFLLRN (TRAP, a PAR1 activator) was synthesized with a semi-automatic peptide synthesizer (Labortec AG SP650, Switzerland) according to van Scharrenburg *et al.*²² Sterile ACD-A was from Hemonetics (Braintree, MA, USA). The polyolefin, gas-permeable, regular PVC and gas-impermeable bags (volumes of 100 and 600 ml) were from Fresenius Hemocare AG (Bad Homburg, Germany).

Platelet incubations

Platelet preparations in capped test tubes

The experiments with PLTs in capped tubes were performed as previously described.¹⁴ In brief, freshly drawn venous blood (40 ml) from healthy volunteers was collected into 1:10 v/v 130 mmol/L trisodium citrate in test tubes. The donors claimed not to have taken any medication during two weeks prior to blood collection. Platelet-rich plasma (PRP) was prepared by centrifugation (200 g, 15 minutes, 22°C). ACD (2.5 g tri-sodium citrate, 1.5 g citric acid and 2.0 g D-glucose in 100 ml distilled water) was added 1:10 to lower the pH to 6.5 and prevent platelet activation during further isolation. The suspension was centrifuged (330 g, 15 minutes, 22°C) and resuspended in Hepes-Tyrode (137 mM NaCl, 2.68 mM KCl, 0.42 mM NaH₂PO₄, 1.7 mM MgCl₂, and 11.9 mM NaHCO₃, pH 7.2). Platelet count was measured on a Cellcounter AL871 (Molab, Hilden, Germany). The platelet count was adjusted to 200,000 cells/ μ l before the experiments started in capped test tubes.

Platelet preparations in polyolefin bags

Blood in quantities of 500 \pm 50 mL was collected from volunteers with informed consent, in quadruple bag systems containing citrate phosphate dextrose (CPD) as anticoagulant and saline-adenin-glucose-mannitol (SAGM) as additive solution for the red cells. Following fast spin centrifugation, the blood was separated into plasma, a buffy coat (BC) and red cells. Leukocyte-reduced platelet concentrates (PC's) were prepared from 5 ABO-identical BCs and one unit of plasma. Following soft spin centrifugation, the platelet-rich-plasma was transferred through a leukocyte reduction filter into a platelet storage bag, as previously described.²³ The PCs on average had a volume of about 400 mL. Sterile ACD-A (2.5 g tri-sodium citrate, 1.5 g citric acid and 2.0 g D-glucose in 100 mL distilled water) was added in a 1:10 ratio to PC to lower the pH to < 6.5 and prevent platelet activation during subsequent centrifugation. One PC was divided over 4 plastic bags. To this end, the bag was under sterile conditions connected with a connection device (Terumo, Japan) to each plastic bag and 80 to 100 mL PC was transferred to the second bags. The bags were supported in centrifuge buckets by plastic supports to keep them in an upright position in order to collect the pellets at the bottom of bags and centrifuged (1,250 g, 15 minutes, 22°C). Following centrifugation, the platelet poor plasma was gently removed in a plasma extractor and to the pellets sterile Hepes-Tyrode containing 5 mM glucose was added to the controls and Hepes-Tyrode without glucose to prepare the metabolic suppressed suspensions. All bags were left undisturbed for a minimum of 60 minutes at room temperature. See for further incubations Figure 1.

Incubations

MSPs were prepared as described¹⁴ by incubating washed PLTs in 2 mL capped test tubes in glucose-free Hepes-Tyrode (pH 7.2) C for 40 minutes in the presence of 20 μ M antimycin A at 37° and without agitation. ER22-4 were prepared by first incubating PLTs in gas-impermeable bags in Hepes-Tyrode without glucose for 4 hours at 22°C without agitation to reduce energy stores followed by storage 4°C, again without agitation. As a control for this treatment ER22-4 were prepared without the

incubation at 22°C and immediately stored at 4°C (designated ER4). Additional controls were PLTs suspended in normal glucose-containing buffer in gas-permeable bags and stored at 22°C with agitation (C22) and at 4°C without agitation (C4). A summary of this procedure is outlined in Figure 1.

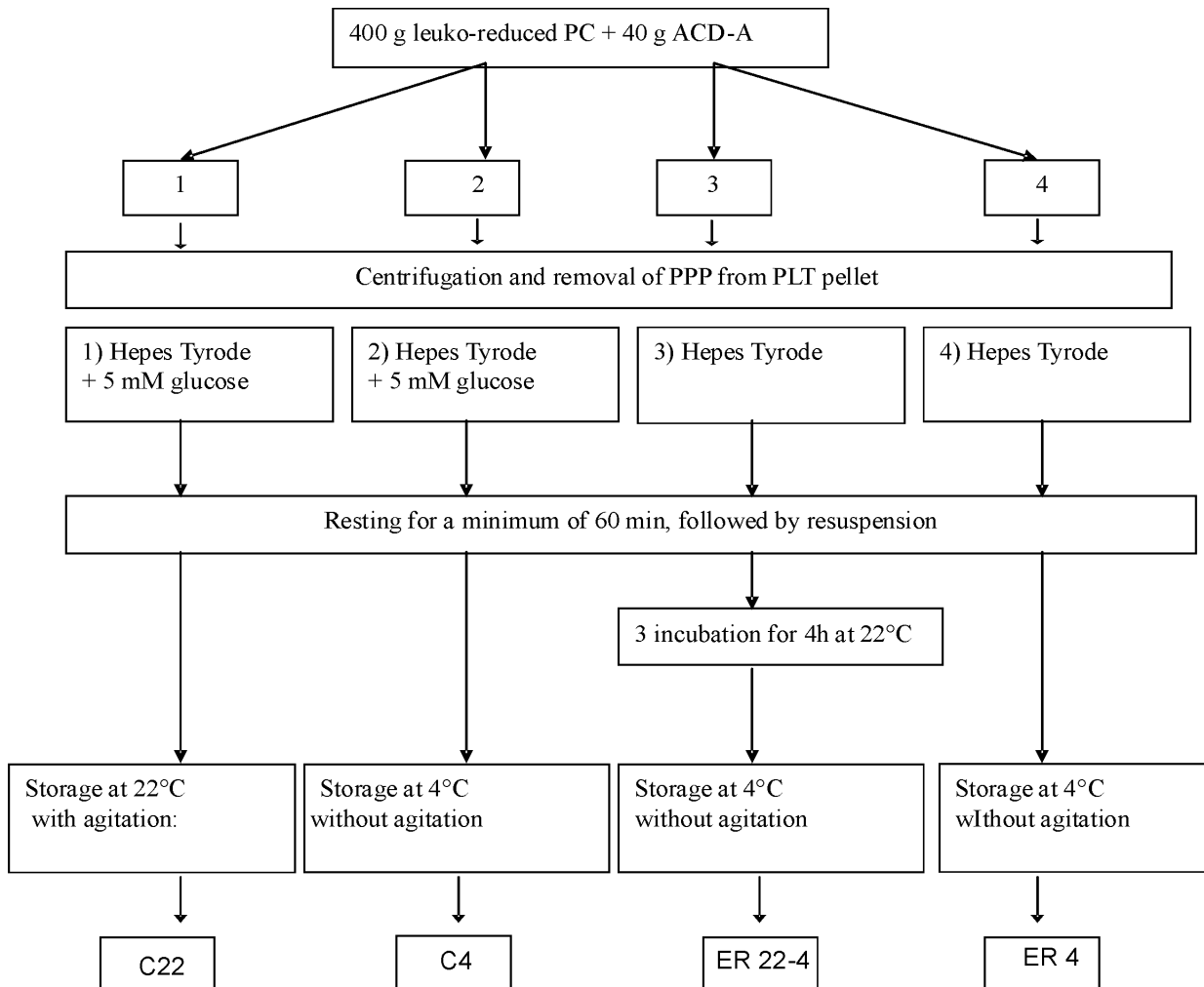


Figure 1. Schematic presentation of the subsequent steps of leukocyte reduced platelet concentrates to prepare the various groups of metabolic suppressed platelets and controls in polyolefin bags. Room temperature controls (C22), stored at 4°C controls (C4), Energy reduced and stored first at 22°C and then at 4°C (ER22-4), energy reduced platelets stored immediately at 4°C (ER 4)

General analyses

At different time points, samples were collected under sterile conditions with a needle and 2-mL syringe through an adapter for analysis of pH, glucose, lactate, pO₂ and pCO₂ on a blood gas analyzer (ABL 705, Radiometer, Copenhagen, Denmark) as previously described.²³ PLT count, and mean platelet volume (MPV) were determined on the Sysmex Cell counter (K1000, TOA, Tokyo, Japan).

Platelet aggregation

At the beginning of the experiments, PLT suspensions, adjusted to about $1000 \times 10^9/\text{L}$ with Hepes Tyrode were placed in polyolefin bags (permeable bags) and in impermeable PVC bags (600 mL). At different time points, samples were collected under sterile conditions with a needle and 2-mL syringe through an adapter and aggregation was measured (500 μL) in stirred suspensions (1,000 rev/minute) at 37°C in a multi-channel aggregometer (Chronolog Havertown, PA, USA) after stimulation with 15 μM TRAP for 15 minutes. Data were expressed as maximal aggregation after 1 hour preincubation without (-recovery), and with 20 mM glucose (+recovery).

Platelet GPIb α expression

GPIb α expression was measured by incubating a 100 μL sample with 200000 PLTs/L with 2 $\mu\text{g}/\text{mL}$ Moab CD42b-PE for 15 minutes at 37°C . To the samples 300 μL PBS was added and immediately 10,000 events were measured on a FACScalibur (BD BioSciences, USA). Semi-quantitative analysis of GPIb α expression was performed using WinMDI software. Fresh platelets show a single population of CD42b-PE positive cells. The gradual decrease in GPIb α expression observed during storage was expressed as percentage of the population measured in fresh platelets.

Results

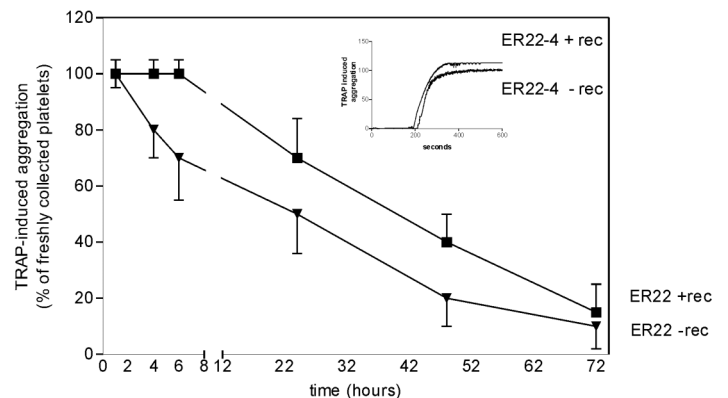
Replacement of antimycin A by O₂-poor conditions

To find conditions in antimycin A-free milieu that led to the same metabolic suppression as observed after 40 minutes incubation in glucose-free, antimycin A containing medium at 37°C (MSPs), PLTs in glucose free-medium were stored in capped tubes with minimal air contact for up to 24 hours at 22°C. During the first 6 hours TRAP induced-aggregation remained close to 100%. Thereafter aggregation decreased to less than 70% as a result of energy depletion but subsequent incubation with 20 mM glucose 1 hour, 37°C) restored the response to 100%. After 24 hours storage, aggregation was reduced to less than 50% and addition of glucose only partially rescued aggregation to about 70%. Thus, the conditions for preparation of MSPs could be replaced by 6 hours storage at 22°C in glucose-free medium with minimal air exchange (data not shown). Similar results were obtained when glucose deprived PLTs were stored in gas-impermeable 600 mL bags. After 4 to 6 hours storage at 22°C ER22 PLTs showed 20 - 40% reduction in TRAP-induced aggregation and recovery with glucose completely restored the responsiveness to TRAP (Figure 2A). At 24 and 48 hours, TRAP-induced aggregation was further decreased and glucose induced only a partial recovery of about 20%. At 72 hours, TRAP-induced aggregation was below 20% and recovery by glucose was absent.

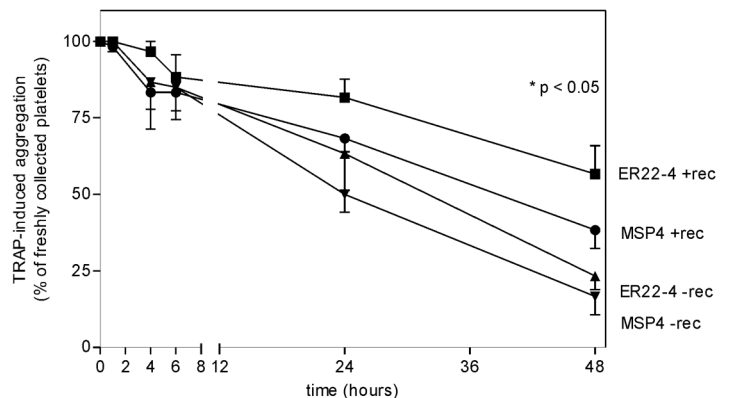
To ensure optimal recovery of ER22, a 4 hours incubation period was chosen to reduce the PLT's energy content while preserving optimal recovery following incubation with glucose.

Figure 2. TRAP-induced aggregation before (-rec) and after (+rec) recovery with 20 mM glucose in antimycin A containing (MSP4) versus PLTs stored in pO₂-poor impermeable PVC-bags. Metabolically suppressed PLTs (MSP) stored in Hepes Tyrode without glucose and with antimycin A were first incubated for 40 min at 37°C and then stored at 4°C in capped test tubes. ER22-4: energy reduced (ER) PLTs stored in Hepes Tyrode without glucose, first for 6 hours at 22°C and then at 4°C in impermeable bags. Data are expressed as mean ± SEM, n = 4.

A.



B.



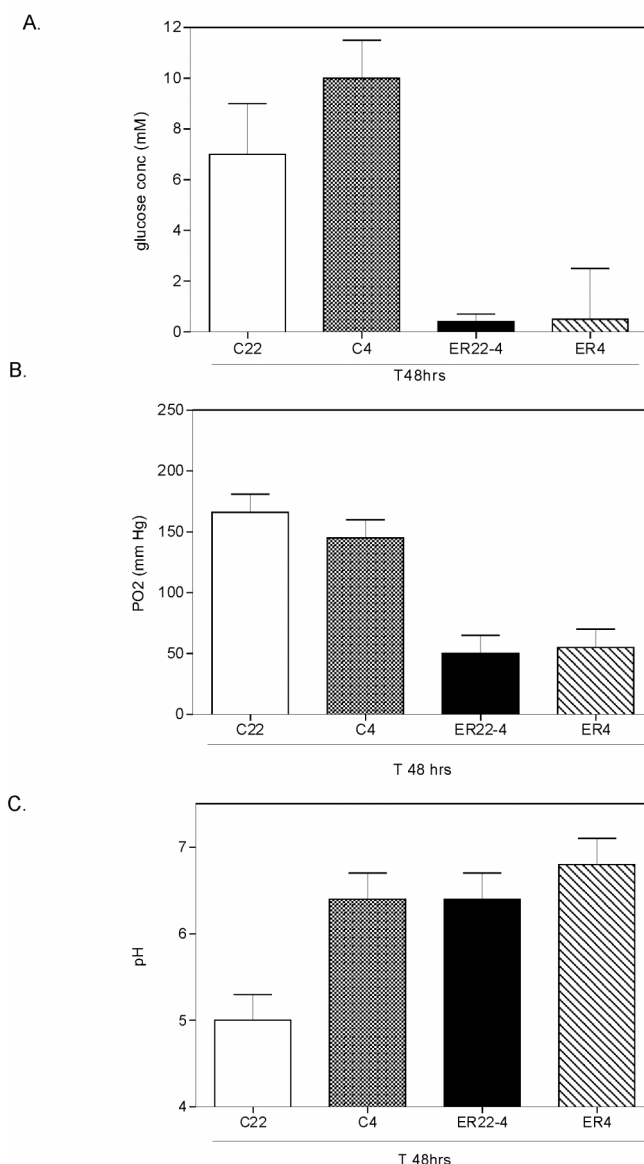
Comparison between MSPs and energy-reduced PLTs in 600 mL polyolefin bags

Next MSPs prepared from PLTs in test tubes (without glucose and with antimycin A) stored at 4°C were compared with ER22-4 prepared from energy-depleted PLTs (without glucose in gas-impermeable bags) and stored at 4°C (Figure 2B). In the first 6 hours, TRAP-induced aggregation decreased about 20% in both suspensions. After 48 hours storage, MSP4 and ER22-4 showed a marked decrease in aggregation prior to recovery with glucose. Following incubation with glucose, recovery of ER22-4 was much better than of MSP4 (Figure 2B). These results suggest that a similar degree of energy depletion is obtained in ER22-4 PLTs as in MSPs and that the absence of antimycin A enables the cells to recover to a greater extent than the antimycin A containing MSPs. They also illustrate that the effect of antimycin A can be replaced by the use of gas-impermeable bags.

General biochemical parameters in energy-deprived PLT suspensions

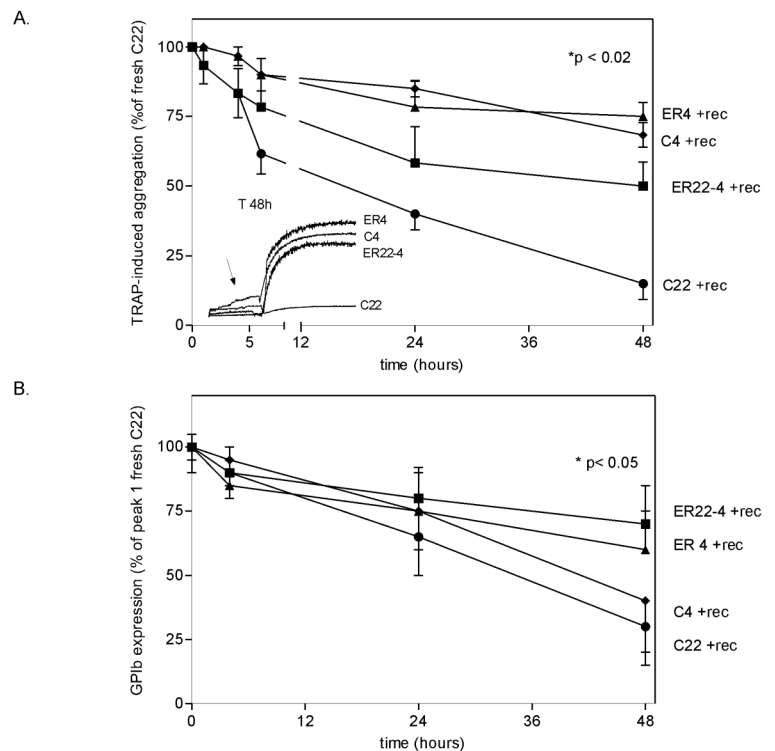
In agreement with the glucose-free conditions, ER22-4 and ER4 suspensions contained little glucose (< 1mM) (Figure 3A) such in contrast with suspensions prepared in glucose containing medium (about 5 to 7 mM). Also O₂-rich and O₂-poor suspensions were found, indicating that the use of gas-impermeable bags reduced pO₂ with about 70% (Figure 3B). All suspensions stored at 4°C better preserved the initial pH illustrating the suppression of energy metabolism in general, and glycolytic lactate production in suspensions containing glucose in particular (Figure 3C). In contrast, C22 PLT suspensions showed a strong decrease in pH in agreement with many observations by other laboratories.^{24,25}

Figure 3. Glucose, pO₂ and pH during storage of different PLTs suspensions in polyolefin bags (600mL)
C22: PLTs resuspended in Hepes Tyrode with 5 mM glucose and stored at 22 °C with agitation; C4: PLTs resuspended in Hepes Tyrode with 5 mM glucose and immediately stored at 4°C without agitation; ER22-4: PLTs resuspended in Hepes Tyrode without glucose and stored at 22 °C without agitation for 4 hours and then stored at 4°C without agitation; ER4: PLTs resuspended in Hepes Tyrode without glucose, and immediately stored 4°C without agitation. Data are expressed as mean ± SEM, n = 4.



To compare the role of energy depletion in greater detail, the aggregation of fresh PLTS was set at 100% and compared with aggregation of PLTS stored for 48 hours with and without energy reduction. After 48 hours storage ER4, C4, ER22-4 and C22 showed about 20, 30, 40 and 90% reduction in TRAP-induced aggregation (Figure 4A and insert). Thus, the energy reduced PLTS stored at 4°C better preserved aggregation than room temperature stored, normal PLTS. However, cold-stored PLTS aggregated better, indicating that under conditions that approach blood bank conditions, a low temperature is beneficial independent of changes in PLT energy content. There was a slight increase in the aggregation curve of ER4 PLTS prior to stimulation, an effect not seen with ER22-4. Possibly, this is a reflection of a slight agglutination. In fresh samples no significant differences in GPIIb α expression were observed (set at 100%). After 4 hour storage in C4, ER4, ER22-4 and C22 a decrease of 10, 20, 20, 22%, respectively, was observed. After 48 hour storage C22 showed a much stronger decrease in GPIIb α expression compared to ER22-4 ($p < 0.05$) (Figure 4B). In ER4 and ER22-4 GPIIb α expression was better preserved with means of 30 and 25%, respectively. We concluded that metabolic suppression by glucose deprivation and storage at 4°C better maintains GPIIb α expression than storage in the presence of glucose, both at 4°C and 22°C. ER4 and ER22-4 showed no significant differences in platelet count ($800 \pm 225 \times 10^9$ /L) and MPV (9 ± 2 fL) (data not shown).

Figure 4. TRAP-induced aggregation and GPIIb α expression before (-rec) and after (+rec) recovery with 20 mM glucose during storage of various groups of PLTs in bags. C22: PLTs resuspended in Hepes Tyrode with 5 mM glucose and stored at 22 °C with agitation; **C4:** PLTs resuspended in Hepes Tyrode with 5 mM glucose and immediately stored at 4°C without agitation **ER22-4:** PLTs resuspended in Hepes Tyrode without glucose and stored at 22 °C without agitation for 4 hours and then stored at 4°C without agitation. **ER4:** PLTs resuspended in Hepes Tyrode without glucose, and immediately stored 4°C without agitation. Data are expressed as mean \pm SEM, $n = 4$.



Discussion

The main finding of the present study is that metabolic suppression of PLTS stored in bags can be induced by glucose deprivation and storage in gas-impermeable bags. Subsequent storage at 4°C better preserved PLT function and GPIb α expression than PLTS stored under conditions that support energy metabolism during storage at 22°C. Compared with control PLTS (C22), the ER4 and ER22-4 showed better preserved TRAP-induced aggregation, GPIb α expression, pH, platelet count and MPV. Earlier observations showed that glucose deprivation in combination with mitochondrial blockade reduced the generation of metabolic ATP.²⁶ After a short incubation under these conditions, energy generation could be restored by addition of glucose addition which led to recover PLT function. In our previous study we demonstrated¹⁴ that for PLTS incubated in test tubes the optimal condition to induce metabolic arrest was a 40 minutes incubation at 37°C without glucose and with antimycin A. Responsiveness to the activating agents TRAP and VWF-ristocetin was significantly reduced during metabolic arrest and addition of glucose induced almost complete recovery to these activating agents. In the current study we tried to find the optimal conditions for replacement of antimycin A and adapted the incubation conditions to procedures that were better compatible with daily procedures in blood banks. We found that the conditions for metabolic suppression when PLT were stored in plastic capped tubes could be mimicked in gas-impermeable plastic bags. PLTS stored in a medium containing a minimal amount of glucose, and stored in an impermeable bag at 4°C showed a decrease in aggregation when they were incubated for more than 24 hours, indicating that glucose deprivation and a low PO₂ reduced platelet responsiveness to activating agents. However, following glucose addition in the first 6 hours TRAP-induced aggregation returned to 100% and between 6 to 48 hours the responsiveness was partially restored. The preservation of platelet function and their recovery was lost after 48 h storage. This is also seen with PLTS stored at 22°C with glucose in gas-permeable bags.

Interestingly, when energy reduced PLTS (ER4) were stored at 4°C for 48 hours; we found almost the same aggregation as in controls containing glucose (C4). This aggregation was better than in both C22 PLTS and in ER22-4 PLTS indicating that low temperature storage better preserves platelet aggregation than storage at 22°C and that following metabolic suppression the recovery is incomplete. These data confirm the data of Hoffmeister et al. that 4°C storage better preserves aggregation than room temperature storage.²⁷ However, changes in GPIb expression show that energy reduction better protects against storage-induced GPIb loss than controls with glucose. Preservation of GPIb α can prolong survival of PLTS in the circulation.^{13,27,28} When PLTS without glucose stored in impermeable bags were first incubated for 4 hours at 22°C and then at 4°C GPIb α expression was still higher than 70%. Application of these conditions in the blood banks may improve PLT storage at 4°C. Another finding was that PLTS stored without or with glucose placed immediately at 4°C showed an increase in spontaneous agglutination, reflected in an increased lag phase of aggregation. This could be an indication of platelet-platelet interaction, cold induced shape change,²⁹⁻³² actin assembly,^{33,34} and an increase in intracellular calcium concentration.³⁵ In PLTS without glucose first stored at 22°C for 4 hour to reduce energy availability and next placed at 4°C the spontaneous agglutination was not observed. Results of previous studies showed^{36,37} that early activation of PLTS promotes rapid binding

and phagocytosis of stored PLTS. We postulate that PLT activation by storage at 4°C should be avoided to prevent rapid phagocytosis by liver macrophages. In conclusion metabolic suppression by glucose deprivation and antimycin A of PLTS in capped test tubes, and incubation for 40 min at 37°C can be replaced by glucose deprivation and incubation for 4 hours at 22°C in bags. Further studies are required to adapt these incubations before the procedure can be introduced in blood banks.

Acknowledgment

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Chapter 7

General discussion

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Different clinical studies have shown that transfusion of stored platelets results in better haemostasis in patients with thrombocytopenia with and without a platelet function defect.¹

Current preservation procedures aim to optimally preserve the metabolic status of platelets during prolonged storage at 4°C.

This thesis describes how metabolic suppression improves the preservation of platelet function. The mechanism of arrest of energy generation prior to cold storage is described (Chapter 2); and how transient metabolic blockade prior to cold storage might be involved in the delay of apoptotic activities and their correlations to platelet binding and phagocytosis (Chapter 3). Furthermore, the different steps in the mechanism of stimulus response coupling are discussed in relation to the role of P-selectin expression, changes in GPIb α expression and PS exposure (Chapter 4). After introduction of metabolic suppression in absence of metabolic inhibitors we adjusted metabolic suppression to conditions compatible with blood bank procedures (Chapter 5).

Platelet storage at room temperature

Currently most Blood banks isolate and store platelet concentrates (PC's) at 22°C. Working at room temperature, has several advantages. It is obviously very simple, and the survival of PC's post transfusion is higher than with chilled-rewarmed PLTs.² These advantages are useful when the blood banks and hospitals have enough patients to use freshly isolated PC's immediately. However, there is never a so called 'demand-supply balance' between the blood banks and hospitals. When more PC's are supplied than demanded, blood banks have to store PC's for sometime.

Storage at room temperature has also dis-advantages. Currently, with PC's stored at room temperature for more than 5 days there is the problem of microbial multiplication.³⁻⁶ This is the reason that so that the FDA limits the storage time to less than 5 days. Another pitfall of storage at room temperature is that it introduces changes in platelets indicative for premature activation, and in GPIb damages⁷ and initiation of apoptosis.^{8,9}

Improvements have been sought in lowering the storage temperature to below 4°C (in the presence of glucose) but this treatment severely reduces the survival of transfused platelets.^{2,10} A main feature of platelet chilling-rewarming is the gradual increase in the sphere to disc shape change, actin assembly,¹¹ a gradual increase in $[Ca^{2+}]_i$ concomitant with spontaneous agglutination, insensitivity to disaggregating agents, and GPIb clustering.⁹⁻¹⁶

Platelet storage under cold conditions

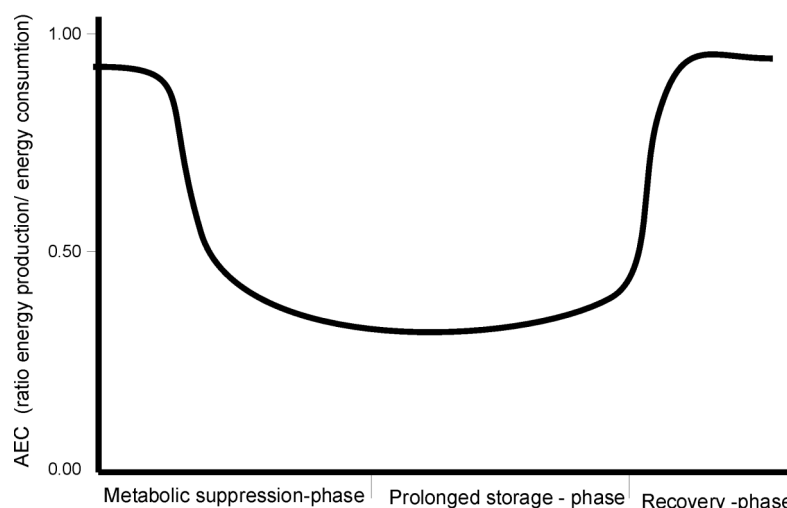
Previous attempts to reduce the surface expression of recognition sites for platelet destruction were based on 1) lowering the storage temperature and freeze-dry techniques,¹⁷⁻¹⁹ 2) room temperature storage with inhibition of secretion using additive solutions containing 10-30% plasma,^{20,21} 3) the use of supplements that increased cAMP and cGMP during storage^{22,23} and 4) cold storage while preventing GPIb clustering by glycosylation.^{10,24} Almost all of these interventions studies which were done at room temperature and in the presence of glucose during storage, failed to preserve platelets responsiveness. Improvements of preparation methods for PC's and the use of sterile storage bags

have resulted in PC's that are effective after seven days.^{25,26} Development of PC's suitable for longer storage has logistic advantages but should meet the present standards at least.

Platelet storage under metabolic suppressed conditions

This thesis objective was to preserve platelet function and prolong their storage time. The main part of study was based on previous results of Akkerman et al.²⁷ who described that successive interventions by transient metabolic suppression of PLTs (so called MSP's) induced by antimycin A and glucose deprivation followed by recovery by 5 mM glucose resulted in better preservation of platelets function, measured at aggregation. During a short storage period of 50 min at 37°C platelets did not respond to activating agents. After recovery, they restored their energy generation and responded again to the same activating agents. An interesting finding was that PLTs set priorities during storage for cell viability and the expense of cell functionality. Apparently, they are able to reorganize and regulate their metabolic energy consumption continuously. Whether in circulation they do the same during hibernation of animals in the winter period is not elucidated yet. It was obvious that a 50 min hibernation period is too short compared with 5 day-storage of PC's in blood banks.

Figure 1. Proposal ratio of adenylate energy charge (AEC) during different phases of platelet storage



To find out the optimal conditions to bring platelets in metabolic suppression, prolong their storage time, and finally restore their responsiveness prior to transfusion a series of experiments was carried out focusing on realization of metabolic suppression prior to storage. Hence, we divided the study in 3 phases:

- 1) realization of optimal transient metabolic suppression (metabolic suppression phase)
- 2) prolongation of storage time in combination with a search for optimal storage temperatures (37, 22 and 4°C) and focused on composition of preserving medium and its influence on platelets functionality, binding and phagocytosis; with primarily focus on glucose addition (during storage phase).
- 3) introduction of an optimal recovery (recovery phase). After metabolic suppression and prolonged storage we wanted to know the impact of the successive interventions on platelet function?

Platelet function studies

We applied a stepwise reduction in energy supply by glucose deprivation and inhibition of mitochondrial respiration by antimycin A. Platelets responded with a step-wise reduction in secretion and aggregation responses. The optimal condition for metabolic suppression was 40 min at 37°C and longer storage led into a gradual increase in P-selectin expression and loss of aggregation responses to TRAP and ADP. Hence, we decided to use for subsequent experiments 40 min at 37°C as optimal time and temperature to introduce metabolic suppression prior to prolonged storage, in capped tubes. The following step was to find out which temperature optimally preserved MSP's during prolonged storage. Higher temperatures than 4°C, induce rapid P-selectin expression, serotonin release and loss of responsiveness to activating agents TRAP and ristocetin.²⁸ Hence, the optimal storage temperature was selected at 4°C.

We confirmed earlier findings²⁷ that complete stop in energy production did not lead to immediate cell death. Then, we searched for ways to optimize the advantages of metabolic suppression for storage of platelets for a prolonged period:

- We prolonged the storage time of washed platelets to at least 48 hours with minimal damage to platelet functions measured in different aggregation, adhesion and secretion studies.
- The arrest of metabolic energy production led to irresponsiveness of platelets to the activating stimuli, during storage at 4°C. This has major advantages for the logistics in the blood banks for delivery of PC's.
- We showed that metabolic suppression prior to storage at 4°C did not affect GPIb expression, a sensitive marker for PLTs survival as described¹⁰. We confirmed that immediate cold storage of PC's with a normal metabolic status, induced rapid changes in GPIb that triggered macrophages to bind and ingest chilled-rewarmed platelets.
- Although we did not measured bacterial multiplication in our samples, there is enough evidence in literature that at 4°C microbial growth is inhibited significantly.^{3,29,30} By applying metabolic suppression we make it possible to place PC's in refrigerators.

Platelet function, binding and phagocytosis studies in vitro

In chapter 2 we investigated in more detail how metabolic arrest prior to cold storage provides a means for better preservation of platelet function during prolonged storage. MSP4 better preserved GPIb-related functions and delayed P-selectin expression. Obviously, metabolic arrest limits the energy sources needed functions. We speculate that limiting energy sources in platelet additive solutions can be used to prevent the platelet storage lesion. Further studies are needed to find out which conditions would optimally to preserve platelet functions.

Pitfalls of preservation of platelet function and survival

Although various publications have referred to the effect of cold on platelets as 'activation', chilled platelets do not resemble platelets, activated by stimuli such as TRAP or collagen. Hence, some research groups and blood banks consider to introduce in cold storage to optimize PC's quality, suppress bacterial contamination and growth while PLTs survival desired to be remained optimal.^{3-5,8,24,29} PC's prepared at room temperature show a better platelet survival after transfusion than PC's stored at 0°C.^{11,31} Hoffmeister et al.¹⁰ showed that galactosylation of GPIb α permits storage of platelets in refrigerator and these platelets show the same survival as room temperature stored PC's. So far, they have not described what happens after transfusion of PC's with synthetic galactosylated GPIb α in humans. Are there any side effects of this galactosylation on immune response of patients? According to literature, the side effects mainly classified under 'unknown effects' after blood transfusion.³²⁻³⁴ Either measured as overreaction of immune response and allergic reaction or as an increase in thrombolytic processes post transfusion.³⁵

However, at this moment they did not it due to the complexity of the problem. First, platelets function is a separate process compared with PLTs survival.² At first chilled rewarmed PLTs that function normally do not circulate and platelets that circulate normally do not show significant haemostatic effectiveness. Moreover, despite in-vitro and in-vivo proper responsiveness of PLTs to activating agents, in survival studies phagocytic cells establish whether transfused PLTs may participate in bleedings episodes or not. In other hand, the most bleeding test at this moment are not very reliable as well.³⁶⁻³⁸ Therefore, is difficult to analyze at once what is the platelets likelihood to remain in circulation and simultaneously, have high haemostatic effectiveness.³⁹

Previous studies described that the *in vivo* viability of platelets have a positive correlation with P-selectin expression, PS exposure,^{40,41} GPIb expression,^{2,42} platelet morphology and shape changes^{11,13,24,43} although this was denied by other groups.⁴⁴ Hoffmeister et al. postulated that cold induced clustering of GPIb α is the major signal for phagocytes to remove chilled-rewarmed fresh platelets. Interestingly, cold induced formation of Gplb α clusters leaves the haemostatic functions of GPIb α undisturbed.² Thus binding to activated vWF and cold-induced binding to Mac-1 of THP-1 cells appear to be separate functions of GPIb α (Chapter 4). Suspensions containing 5mM glucose at 0 or 22°C showed a higher P-selectin expression. It is known that P-selectin plays a pivotal role in platelets binding to phagocytic cells and we confirmed that (Chapter 3 and 6). We show further that P-selectin is not enough for the ingestion of platelets and PS exposure and changes in GPIb expression are a prerequisite for phagocytosis (Chapter 6), as described by other groups.^{2,42,45,46} Furthermore, the major features of GPIb α changes are described focusing on the major role of GPIb α as a sensitive *in vitro*-predictive marker for platelet phagocytosis. The GPIb α on the surface of the platelet exists in linear arrays⁴⁷ in a complex with GPIb β , GPIX, and GPV, attached to submembrane actin cytoskeleton by filamin A and filamin B.⁴⁸⁻⁵² Stimulation of platelets in suspension by thrombin and other agonists causes GPIb α to redistribute in part from the platelet surface into the open canalicular system. This process does not lead to platelet clearance in vivo.^{10,53}

Here rises the question as to the role of GPIb β , GPIX, and GPV in platelets phagocytosis? Quantitative analyzing of GPIb α expression by monoclonal antibody AN51 indicated that fresh

platelets show a high density GPIb α expression (M1-fraction). After short storage at room temperature or on ice the affinity of moab to bind to GPIb α decreases and a second population appears with a low density GPIb α at their surface (M2-fraction).

The appearance of the M2-fraction more in both room temperatures stored and on ice stored platelets populations might be caused by two possible processes induced by storage-induced lesions. First, a fraction of the total platelets show reduced a density of GPIb α , which possibly caused by GPIb α clustering, a concept introduced by Hoffmeister et al.^{10,54} Indeed it positively correlated with phagocytosis (Chapter 5) and was significantly restored after addition of GlcNAc, as GlcNAc reduced phagocytosis. Remarkably, GlcNAc did not have any effects on the binding to macrophages. Obviously, binding is a separate process than phagocytosis. Second, it indicates that both C22 and C0 undergo easier deleterious changes that alter the affinity of the GPIb α binding-epitope to antibody AN51. It is not clear for us as well. Why addition of so high GlcNAc (100mM) restores GPIb α M-1 fraction in room temperature stored and on ice stored platelets? And subsequently how protect them against phagocytosis future studies needed elucidate the mechanism in details.

We investigated whether monosaccharides influence GPIb α with subsequent effects on binding and phagocytosis. Further investigations needed to elucidate why other sugars did not and GlcNAc did decrease GPIb α M2-fraction forming? And thereby reduced phagocytosis?

Transient metabolic suppression prior to cold storage has disadvantages too. The major problem was addition of antimycin A that is not compatible with blood transfusion. To find the optimal conditions to replace antimycin A, inhibitory effect on platelet metabolism and adjust this method to blood bank conditions. We used gas impermeable plastic bags. We found that the conditions for metabolic suppression when PLT were stored in capped plastic tubes could be mimicked in bags 600 mL and storage in a glucose-poor medium for 4 hour at 22°C. Platelets stored in a medium containing minimal glucose (<1mM) and stored in an impermeable bag at 4°C showed a decrease in aggregation when they were incubated for more than 24 hour indicating that glucose deprivation and restricted gas exchange reduced platelet responsiveness to activating agents. However, following glucose addition in the first 6 hour TRAP-induced aggregation returned to 100%. Between 6 to 48 hours, the responsiveness was partially restored. Our conclusion was that also in plastic bags energy restriction did not affect platelet responsiveness, which was reversible up to 100% within the first 6 hour. Furthermore, we confirmed the data of Hoffmeister et al. who described that 4°C at storage better preserves platelet function¹⁰ although it decreases GPIb α expression. Platelets with low GPIb α clustering expression will be rapidly removed in the body by liver macrophages. Therefore, preservation of GPIb α can prolong survival of PLTs in the circulation.^{10,42,54} When platelets stored in impermeable bags without glucose were first incubated for 4 h at 22°C and then at 4°C GPIb α expression was still higher than 70%. Application of these conditions may improve PLT storage at 4°C in the blood banks.

Chapter 6 describes the correlation between platelet storage lesion (PSL) that is often accompanied by the apoptotic and morphological changes, cytoplasmic membrane shrinkage.^{9,23} Both phenomena, nevertheless, are associated with the appearance of P-selectin expression and PS on the cell surface.^{23,37,42} Our approach was an attempt to quantify what the correlation is between P-selectin, PS

exposure and GPIb α and thereby prediction of phagocytosis of platelets based on in-vitro observation. We hypothesized that in-vitro data of binding and phagocytosis could predict in-vivo survival of platelets after transfusion. Markers for the storage lesion were: P-selectin expression, PS exposure, and GPIb affinity changes. Furthermore, to evaluate the function and survival we used our suggestive marker for GPIb clustering which was defined quantitatively by FACS and marked under M2. On the basis of these parameters, one can predict the degree of platelet binding and phagocytosis in vitro. We hope that by expanding this study *in vivo*, this model system can be used in the blood banks, for first attempt for qualify control of PC's.

There is evidence in the literature that show that PC's expressing less than 10% P-selectin, PS exposure, and GPIb clustering survive the first 24 hours of transfusion.^{10,42,53} Leytin et al. described that P-selectin expression on the platelets surface during storage triggers fast P-selectin-mediated clearance *in vivo*,^{44,53} although this was denied by other laboratories.

We and other research groups have previously shown that there is a significant correlation between P-selectin expression and binding and between PS exposure, GPIb peak-2 formation and phagocytosis.^{2,42,45,55-57} Here to verify our model system we induced P-selectin expression in washed platelets by PMA, and investigated the binding and phagocytosis. The binding obviously has positive correlation with high PSE but phagocytosis has not. The important les from this observation was that the platelet binding and phagocytosis are not causally linked processes. As postulated before by Berger et al.⁵³ and Hoffmann PR et al.⁵⁸ binding and phagocytosis of cells to macrophages are separate processes.

PS exposure also plays a pivotal role in the recognition and removal of apoptotic cells via a PS-recognizing receptor on phagocytic cells.²⁴ Direct correlations between PS exposure above 10% and phagocytosis indicate that platelets should not be positive for PS exposure. A decrease of PS exposure by binding to annexin V did not decrease binding but significantly decreased phagocytosis. Based on our studies we hypothesize that for the binding and complex formation studies between platelet and phagocytic cells, PS exposure is not good marker but for phagocytosis can be used properly.

Future plans should focus on direction of cold storage and adjustments of metabolic suppression prior to cold storage with blood bank's compatible supplements. After preparation of sterile PC's with limited energy sources and no microbial contamination prolongation of storage time to more than 2 weeks should be feasible. Pilot studies already show that the pooled Leuko-reduced platelet concentrates stored at 4°C with metabolic reductions optimally preserved platelet function after 18 days.

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Chapter 8

Nederlandse samenvatting

De laatste jaren is de behoefte en belang aan trombocyten transfusie sterk toegenomen. Bij de bloed banken jaarlijks miljoenen donors staan hun dierbare bloed af; voor een ander die misschien binnen kort hun leven kan redden. Indien acuut het ziekenhuis trombocyten concentraten nodig zou hebben worden met behulp van centrifugatie techniek de rode cellen, de witte cellen, de trombocyten en het plasma van elkaar gescheiden en de bloedplaatjes concentraten in een steriele plastic bloedcontainer wordt gestopt en naar het ziekenhuis gestuurd.

Maar ertussen als er geen behoefte zou zijn aan de trombocyten concentraten en/of de verhouding tussen donors en patiënten zodanig wijzigt dat er de afgestane bloed moet worden bewaard dan de bloed banken bewaren de rode cellen, de witte cellen in de koelkast, de trombocyten bij de kamer temperatuur en het plasma bij -80°C .

Bewaren van de trombocyten bij kamer temperatuur heeft veel voor- maar ook nadelen. Het belangrijkste nadeel is microbiële groei and vermeerdering zodat er FDA heeft de regelingen gemaakt dat er niet langer dan 5 dagen trombocyten concentraten mogen worden bewaard die aan de patiënten zou worden getransfuseerd. Omdat de sterke microbiële groei voor de patiënten onder operatie en kwaadaardige ziektes zou dodend kunnen zijn. Er is ook grotere kans dat de trombocyten hun functionaliteit verliezen wanneer ze langer dan 5 dagen bij de kamer temperatuur worden bewaard.

Dus meeste bloedbanken zoeken naar de mogelijkheden die microbiële contaminatie en groei te voorkomen bijvoorbeeld door steriel te werken en geïsoleerde bloed zakjes in de koelkast te bewaren. Maar tegelijkertijd koud bewaren van de trombocyten concentraten leiden tot vroegtijdige activatie en clustering van GPIIb-V-IX complex receptor van de trombocyten waardoor ze niet meer bruikbaar zijn. Verder de koud bewaarde trombocyten concentraten worden na transfusie snel door lever macrofagen verwijderd en geklaard.

Dit proefschrift beschrijft een aantal methoden om de veranderingen van de trombocyten concentraten te meten, analyseren en hen achteruit gaan in de functionaliteit tijdens bewaren en na blootgesteld zijn aan de koud condities en daarna aan de macrofagen te kunnen meten en evalueren voordat ze getransfuseerd zijn. Er is getracht de geschetste problemen in kaart te brengen en zo goed mogelijk oplossingen hiervoor aan te dragen. In dit AIO project hebben we gekozen voor het gebruik van de metabole suppressie en verhongeren van de trombocyten tot zeker moment en pas dan ze in koelkast ze voor langere tijd bewaren.

Het is ons gelukt om activatie vermogen, adhesie en aggregatie functie en bovenal levensduur van de trombocyten voor het laboratoria onderzoeken verlengen van ongeveer 4 tot 6 uur naar 48 uur (ongeveer 10 maal meer). De piloot studies laten zien dat we trombocyten concentraten bewaren kunnen voor 18 dagen, met de bloedbank compatible condities. Verder biedt in rust brengen van de trombocyten tijdens bewaren heel veel mogelijkheid voor logistiek en bespaard veel geld voor de bloedbanken. Deze verlenging van bewaartijd kan wereld wijd miljarden kosten besparen voor de bloedbanken.

In hoofdstuk 2 is beschreven hoe we trombocyten in metabole suppressie toestand brengen met behulp van verhongeren van trombocyten in aanwezigheid van Antimycin A; een inhibitor dat

mitochondriale respiratie remt (Platelet Hibernation). Na lang trombocyten in koud zijn bewaard herstellen we energie toestand van ze met behulp van 20mM glucose voordat de functie testen zijn uitgevoerd.

In hoofdstuk 3 is beschreven we hoe viabiliteit, binding en fagocytose van de trombocyten kan behouden blijven tijdens koud bewaren, mits ze vooraf in rust toestand zijn gebracht en metabolisch gesupprimeerd. Blijkbaar metabool gesupprimeerde trombocyten dat in koelkast zijn bewaard beter clustering van de GPIb-V-IX complex receptor kunnen tegen gaan vergeleken met trombocyten die hun metabole energie intact blijft maar direct in koelkast gezet en/ of kamer temperatuur bewaarde controles.

In hoofdstuk 4 is beschreven hoe GPIb clustering eventueel werkt als fagocytose signaal.

In hoofdstuk 5 is beschreven hoe onze model systeem kan bijna elk willekeurige trombocyten concentraat survival kans voorspellen.

Het is een poging richting opbouwen van een model systeem dat kan voorspellen wat zou de uitkomst zijn van de zeker trombocyten concentraten na transfusie.

In hoofdstuk 6 is beschreven hoe we metabole suppressie aanpassen aan de bloed bank condities. Uiteindelijk in hoofdstuk 7 wordt gediscussieerd hoe uiteindelijk moeten trombocyten concentraten worden bewaard. Is het goed dat ze bij kamer temperatuur te bewaren? Is het beter als ze in koelkast worden bewaard? Hoe kan men de voordelen van het koud bewaren gebruiken en tegelijk de nadelen ervan te voorkomen?

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Curriculum Vitea

Bahram Alamdary Badlou, was born in Tehran-Iran in 1964. After high school in Iran he went to army for two years. In May 1992 started with Medicine study at Free University of Amsterdam (VU-Amsterdam) and after two years has chosen to go further with Medical Biology at Medicine Faculty of University Medical Center of Utrecht and finished his MS in 1999 (Drs. Medische Biologie).

As minor study program for 6 months he studied "Alkyl-Dihydroxyacetonephosphate Synthase, a peroxisomal enzyme involved in ether lipid synthesis" in Chemistry Faculty of Utrecht University (1998).

As major program for 9 months he studied "calcium paradox in rat heart by NMR" and his thesis was a literature study about "the effects of Insulin during and after Heart Failure" (1999). During this period he should develop cardioplegic solutions to prevent damages to the isolated rat heart during heart transportation and transplantation.

He is authorized to work with radioactive materials and equipments degree 4B according to Dutch Law TUDelft Stcrt nr.227at 4 December 1997. He is also authorized to perform animal experiments according to Dutch law article nr. 9. Stb.1985, 336 certified at 7 March 1997. Then he worked for 6 month as Pathology and Phatophysiology teacher in the Assay Opleidingen in Rotterdam North, The Netherlands.

Then he worked as Research Associate on a project of Unilever in association with Chemistry Faculty of Utrecht entitled "The Bioremedy of antioxidants, coming to terms with measuring lipid peroxidation and evaluating antioxidant efficacy" in an ex-vivo and in vitro study with C11-BODIPY^{581/591} and Tecan spectrofluorometer, to prevent ageing of rat-1 Fibroblasts (1999 to 2002). After Feb 2002 till 2006 he was hired as PHD student in Sanquin Blood bank NW to study "prolonged platelet preservation by metabolic suppression". His promoter was prof. dr. J.W.N. Akkerman and co-promoter was dr. W.M. Smid. He succeeds for first time to bring platelets in hibernation condition for more than 48 hours, reversibly. His pilot studies revealed that by his metabolic adjustments in the blood banks, platelet concentrates can be stored for 18 days.

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