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Newly Exposed Membrane Glycoprotein II β (CD41) on Activated Platelets has Higher Affinity for Plasma Fibrinogen that Blocks Anti-CD41 Binding

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Authors' contributions

Author AB carried out all experiments, compiled data, made graphical representations and prepared preliminary draft of the manuscript. Author LKK conceptualized the study, designed the experiments, analyzed the data, discussed the results, prepared final version of the manuscript and addressed review comments in the final revised version.

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ABSTRACT

The density of glycoprotein receptors on platelet membrane is dynamic and increase upon platelet activation by various stimulants to facilitate more ligand binding and functional outcome. Since CD41/61 receptor complex is responsible for platelet to platelet cross-linking, infusion of antagonists/antibodies against these molecules is considered an effective method for inhibiting platelet aggregation in certain clinical conditions. However, binding of anti-CD41 to the platelets stored in bags for transfusion was found to be lower. Probably, upon stimulation the newly exposed receptors may immediately bind fibrinogen and prevent access to the antibody. To prove this hypothesis, binding intensity of FITC-conjugated human fibrinogen to resting and stimulated platelets were compared using flow cytometry. In the presence of plasma fibrinogen, monoclonal antibody binding to the CD41 receptor decreased significantly, both onto the stored and agonist-activated platelets as compared to the resting cells. As compared to ADP-activated platelets,

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binding of anti-CD41 to thrombin-activated platelets was significantly lower. Variable binding intensity of anti-CD41 to platelets activated by different stimulants observed in this study allowed us to suggest that therapeutic antibodies also may not bind to platelets that are stimulated by strong agonists. Our results signify the importance of validating such antibodies under conditions of stronger activation of platelets. We observed that more platelet microparticles (PMP) were shredded upon *in vitro* platelet activation by thrombin as compared to induction by ADP. However, fibrinogen did not bind to the shredded microparticles Therefore; PMP in circulation may not take part in pathological thrombus formation.

Keywords: Platelet glycoproteins; flow cytometry; platelet activation; fibrinogen binding; platelet activation markers.

1. INTRODUCTION

In normal physiological conditions, resting platelets in circulation are non-adhesive even though receptors for intercellular adhesion are present on the membrane surface [1]. Upon vascular damage, platelets get activated, expose/release their granule contents, and adhere to each other as well as to the injured vessel wall to stop bleeding [2]. Though their response to various stimuli is through specific receptors for the agonist, ultimately, adhesive protein receptors are essential for them to stick to each other and to the injured blood vessel. The most abundant adhesion receptor complex is CD41/CD61 (GPIIb/IIIa), which primarily binds fibrinogen (Fib) to mediate platelet-to-platelet aggregation by forming the 'CD41/CD61-Fib-CD41/CD61' complex [3]. The reactivity of the platelets is partly dependent on the surface immobilization of various adhesive ligands, but is principally influenced by the activation-dependent conformation change of integrins CD41 and CD61, as well as CD41/CD61-complex formation [4]. Since the receptors are present in considerable density even on resting platelets, this is not a useful marker for distinguishing activated platelets in circulation. Incidentally, lesser anti-CD41 binding to platelets upon storage-dependent activation was observed in our preliminary analysis. On the basis of this observation, the research question aimed to find out if internal pools of CD41/61 which get relocated on the membrane surface upon platelet activation may have more affinity for Fib and prevent antibody/antagonist binding. Another research question raised was whether the activated platelets shred membrane particles and if the process would result in the removal of CD41 receptors from the surface. Such removal of receptor sites along with microparticles could also be a cause for reduced anti-CD41 binding.

A resting platelet has ~80,000 CD41/CD61 receptors. Weak agonists such as adenosine diphosphate (ADP) increase the numbers of active receptors on the platelet surface slightly, whereas strong agonists like thrombin and collagen can increase the density of CD41/CD61 antigen by 50% [5]. Due to the significant involvement of these receptor molecules in platelet aggregate formation, therapeutic targeting of the CD41 receptor has been attempted in different platelet disorders, especially during interventional cardiology [6]. However, since internal pools of receptors may get relocated to the membrane surface upon platelet activation, in spite of the use of antagonist molecules, microaggregate formation is likely to take place [7]. It has been reported that ~80% receptor occupancy by the monoclonal antibody abciximab effectively inhibits platelet function. However, in activated platelets, additional receptors present in the internal pool get exposed to the surface and may play an inhibitory role in the success of these antagonists [6]. Antagonist binding can be reversible and basically depends on the affinity and the dissociation constant of the drug.

The objectives of this study are to estimate Fib and anti-CD41 binding to platelets using flow cytometry to prove that (i) Fib binds with more affinity to the newly exposed internal pools of CD41 which are mobilized onto the activated platelet surface; (ii) in presence of plasma fibrinogen, anti-CD41 binding to activated platelets is drastically reduced; (iii) CD41 receptors may be removed with shredding of platelet microparticles, which is a consequence of platelet activation; and (iv) to identify if platelet microparticles (PMP) possess CD41 receptor.

Stored platelet concentrates (PC) and washed platelets (WP) were used as *in vitro* study models along with agonists which produced

differently activated platelets to estimate CD41/fibrinogen binding on activated cells.

2. METHODS

2.1 Comparison of Aggregation Potential and Receptor Density

This experiment was carried out to correlate functional response of stored platelets with activation marker exposure. For this, platelet samples were obtained from PC bags collected at a licensed Blood Bank and stored for assuring quality of cells upon storage with approval from Institutional Ethics Committee of Sree Chitra Tirunal Institute for medical Sciences & Technology (IEC-SCTIMST). Aliquots of PC were aseptically removed from the bag after specified periods of storage at 22±2ºC (i.e., 0h, 24h, 48h, & 72 h). The PC was diluted with platelet poor plasma (PPP) to obtain platelet rich plasma (PRP) containing an adequate number of platelets. Response of the platelets to the agonist in samples collected after different storage periods (i.e., 0h, 24h, 48h, & 72h) was estimated by the optical method using an aggregometer (Chronolog-700 Aggregometer, USA) according to the manufacturer's instructions by adding either 10µM ADP or 1µg collagen as agonist. The PPP obtained from the same bag was used for setting the baseline to 0% light transmission. In brief, 450 µl of count adjusted (2.0-2.5 x 10^8 /ml) PRP was prepared from the PC storage bag by diluting with PPP, the agonist was added, and the change in percentage light transmission was recorded. The response was recorded for 3 min in each sample; slope and amplitude was determined using AGGRO/LINK8 software from Chronolog (USA). Six PC bags were evaluated for platelet activation by CD62 estimation and loss of function by aggregometry.

For estimation of the receptors on the platelet surface, the freshly washed platelets and stored cells were stained with FITC-conjugated anti-CD62 and/or PE-conjugated anti-CD41 obtained from Beckman Coulter (USA). The manufacturer has declared that the monoclonal anti- CD41 recognizes the active CD41/CD61 complex as well. Briefly, 1.5-2.0 x10^5 platelets/100 µl were incubated with 2 µl antibodies at room temperature for 30 min in dark conditions, fixed with 3.7% formaldehyde and diluted in phosphate buffer saline (PBS). An analysis protocol was developed using a single laser (488 nm) 3-color flow cytometer (Epics XL Beckman Coulter, USA). Unlabelled resting platelets were used for adjusting forward scatter (FSC) & side scatter (SSC) and negative gate for FITC/PE fluorescence was marked. To ensure that the cells in the gated region were platelets, FITC-conjugated anti-CD42 stained cells were analyzed separately and their position was marked. Whenever dual labeling was done, single labeled cells were used for compensation. The percentage of fluorescent cells was estimated by counting 50,000 events using Beckman Coulter, System II^{TM} XL DOS based software.

2.2 Preparation of Activated PRP and WP

Fresh buffy coats discarded from blood bank (with the approval by IEC, SCTIMST 'for exemption from review') were used for obtaining resting platelets for the study. After centrifugation of the buffy coat at 1000 *g*, the top layer of cells was collected and centrifuged at 300 *g* for removing contaminant RBCs. The count of platelet suspension was adjusted between 1.5-2.0 x 10^8 /ml and activated with different concentrations of ADP ranging from 100nM-800nM. To wash the platelets free of plasma proteins, an earlier established procedure was employed [8]. Briefly, 0.1 vol of acid citrate dextrose (ACD) was added to the PRP and this was centrifuged at 500*g* for 5 min. This step was repeated 2 times after re suspension of platelet button in Tyrode's buffer to ensure complete removal of the plasma proteins. The final washed platelet (WP) pellet was re suspended in Tyrode's buffer, $CaCl_2$ was added to get 5 mM concentration, and the count was adjusted to 1.5-2.0 x 10^8 platelets/ml. The WPs in the suspension were activated with different dose of ADP (100nM to 800nM) or thrombin (0.2 to 1.0 IU/ml).

2.3 Preparation of Fluorescent-labeled Fib

Cryo-precipitated Fib (in-house prepared) was labeled with FITC (Sigma Cat No F7250). Briefly, Fib (5mg/ml) was dissolved in an alkaline buffer (0.1M sodium carbonate, pH 9), 1 mg/ml FITC in dimethyl sulfoxide (DMSO) was added slowly (5 µl aliquots at a time) with gentle mixing, and the mixture was incubated for 12 h in dark conditions at 4℃. Ammonium chloride was added to get the final concentration of 50mM which was incubated for 2h at 4℃. Unbound FITC and few low molecular weight plasma proteins were removed by gel filtration chromatography using Sephadex G25 beads. A fraction of conjugated protein was collected and the ratio of spectral peaks of FITC (F) and protein (P) was calculated by measuring

absorbance at 485 nm and 280 nm. Conjugate with F/P ratio < 0.5 was used for estimating Fib binding to the WP.

2.4 Detection of Fib Binding to Platelets and Microparticles

The WP were activated with different concentrations of ADP and stained with FITC-Fib and/or PE- anti-CD41. In brief, WP was activated with graded concentrations such as 100nM, 200nM, 400nM and 600nM of ADP or with thrombin to get the final concentration of 0.1/0.2/0.4/0.8/1.0 IU. After activation, ~1.5-2.0x10^5 platelets/100µl was incubated for 30 min at room temperature with 5µg FITC-Fib and PE-anti-CD41 (2µl). A protocol was developed to analyze $CD41^+$ cells alone, Fib^+ cells alone, and dual positive cells. Standard sized beads (0.2 µm, from Beckman Coulter) were used for quality control and gated the micro particle(s) population. The percentage of $CD41^+$ microparticle(s) was estimated by counting 50000 events using Beckman Coulter, System II™ XL DOS based software. The same protocol was used to detect competition of Fib for CD41; unlabelled Fib and PE-conjugated anti-CD41 were added together and percentage of fluorescent particles was estimated.

2.5 Statistical Analysis

Data was analyzed by one-way analysis of variance. Results are expressed as mean ± SD of six sets of observations. Replicate samples used for each assay are specified in the figure legends.

3. RESULTS

3.1 Agonist Response of Stored Platelets and Receptor Density

Tracings of the aggregatory response of samples from a single representative PC bag, drawn at different time intervals of storage, are shown in (Fig. 1). The response of the platelets to both agonists (ADP and collagen) indicated that the function of platelets in PC deteriorated when the storage period progressed (Fig. 2). Compiled data from 6 PC bags showed that the ADP induced- and collagen induced- aggregation response deteriorated in an almost similar pattern, and reached a bare minimum by 72 h. However, better response to ADP as compared to collagen was seen by the end of the storage period; large donor-to-donor variation was also found.

However, binding of CD41 estimated on different days of the storage period in PC bags showed no increase in receptor density with aging related activation. But as the storage period increased, P-selectin exposure increased, but not to a remarkable extent (Fig. 3A). High donor-to-donor variability was seen and the increase was not distinctly different for different days of storage. Though $CD62^+$ cells increased steadily, the percentage of $CD41^+$ cells decreased. On the initial day of study, binding of CD41 to the platelets was highest and it reduced as the storage period progressed till 48 h (Fig. 3B). At the end of 72 h of storage, the percentage of $CD41^+$ cells remained steady without any significant increase or decrease.

3.2 Agonist Response and Receptor Density in Fresh Platelets

A similar trend was observed even for the agonist-activated WP, i.e., with a higher dose of ADP/thrombin, the percentage of $CD62^+$ cells increased, whereas the percentage of $CD41^+$ cells decreased (Figs. 4 and 5). The increase in the percentage of $CD62^+$ cells was proportionate to the agonist dose, but not much effect was observed in proportion of $CD41^+$ cells in the ADP-activated platelets. In the case of 1.0 IU thrombin-activation, anti-CD41 binding was reduced to ~50% compared to that in the resting platelets (Fig. 5). Therefore, it may be confirmed that the strength of the agonist negatively influenced the percentage of $CD41^+$ cells; when the agonist dose was stronger, the anti-CD41 binding was reduced.

3.3 Fibrinogen Binding to Activated Platelets

Binding of Fib to platelets upon activation was estimated to show that CD41 receptors on activated platelets could be occupied. The washing of platelets 3 times removed maximum possible Fib from platelet suspension. Upon activation of WP using ADP which is a weak agonist, at any dose, the estimated $CD41^+$ platelets was more than 90% (Fig. 4), but with the strong agonist thrombin, it was ~50% at the highest dose (Fig. 5). When FITC-Fib was added to even 0.2 IU thrombin activated WP suspension, the proportion of Fib^+ cells was ~80% (Fig. 6A), which further increased when the agonist dose was higher. Therefore, it is clear that more receptor sites are exposed upon thrombin activation, but anti-CD41 does not bind to them because Fib occupies the sites with higher affinity. This is more evident when anti-

CD41 and conjugated Fib were added together. Antibody binding was practically absent as compared to Fib binding (Fig. 6B). When the agonist dose was increased, the percentage of Fib^+ platelets was higher.

3.4 Receptor Density on Shredded Microparticles

The microparticles in the selected gate were estimated and it was found that the $CD41^+$ particles concentration increased with higher agonist dose; both in the case of thrombin and ADP activation (Fig. 7). However, nearly 10 times more $CD41^+$ PMP were detected in the thrombin-activated platelet suspension as compared to the ADP-activated suspension at the highest dose. However, when Fib was added into the suspension before adding the anti-CD41, there was no significant effect on the percentage of the $CD41^+$ particles.

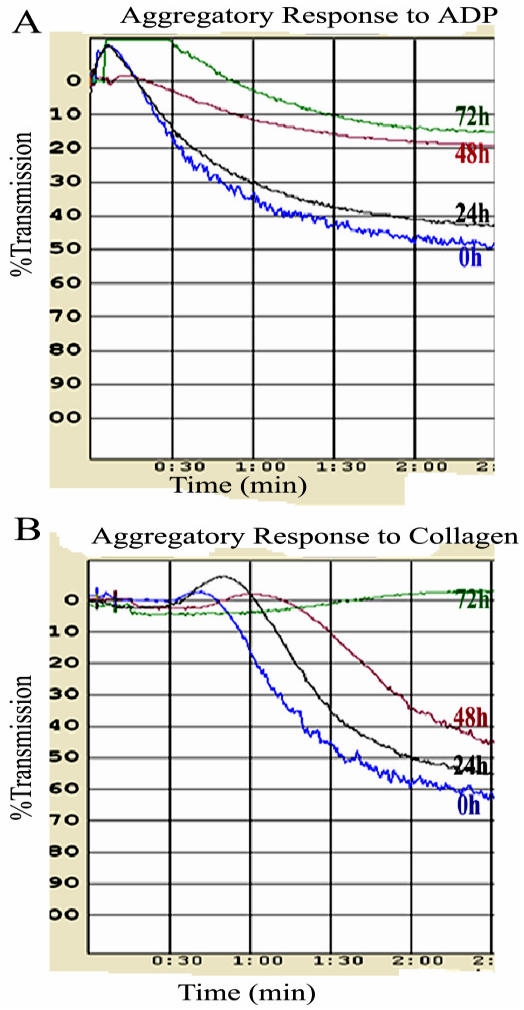

Fig. 1. Representative tracings of aggregatory response of stored platelets

All tracings shown are of platelets from the same bag at different periods of storage. A, response of platelets to ADP; B, response of platelets to collagen. Count was adjusted in PRP to 2-2.5x10^8 platelets/ml and platelets in suspension were then challenged with ADP (10µM) or Collagen (1µg). The x-axis represents time scale of measurement after adding agonist to platelet suspension and the y-axis represents % light transmittance. As platelets aggregate, the light path gets cleared, increasing the percent transmittance. Clearly, the percent transmission was low as the storage period increased, which indicates that all platelets in the suspension were not recruited into aggregates when the platelets get older and activated

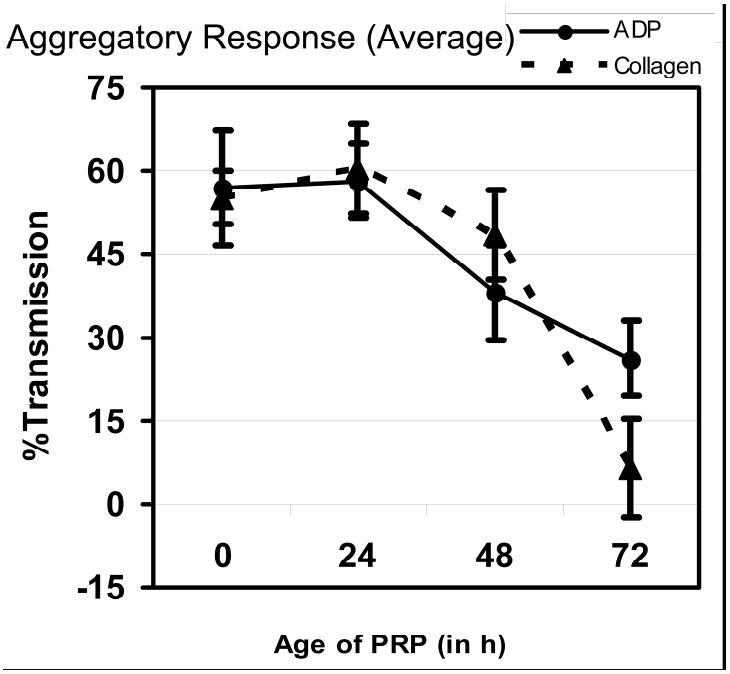

Fig. 2. Compiled data of aggregatory response of PC

Data on response platelets in stored PC to agonists; ADP and Collagen at each time intervals was computed and is represented in graphical format. The profile shows similar pattern of loss of response to both agonists with progress in storage period. All data shown are mean ± SD (n=6). Donor-to-donor variability is reflected in the S.D

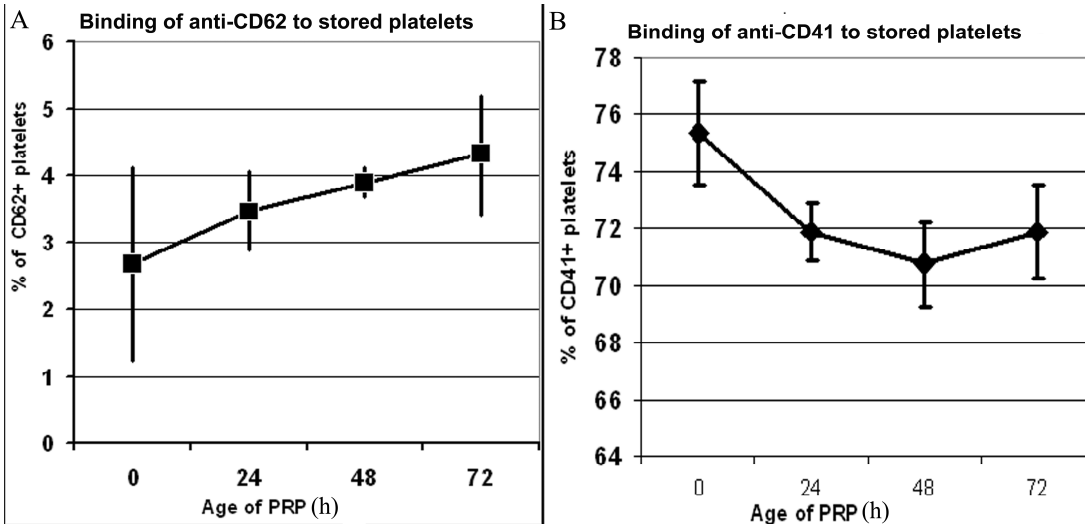

Fig. 3. Profile of exposure of glycoproteins in stored PC

A, percent of CD62$^+$ platelets after different storage intervals; B, percent of CD41$^+$ platelets after different storage intervals. The percentage of positive cells was calculated by using Beckman EPICS XL, and is shown (y axis) against the storage period (x axis). All data shown are mean ± SD (n=6)

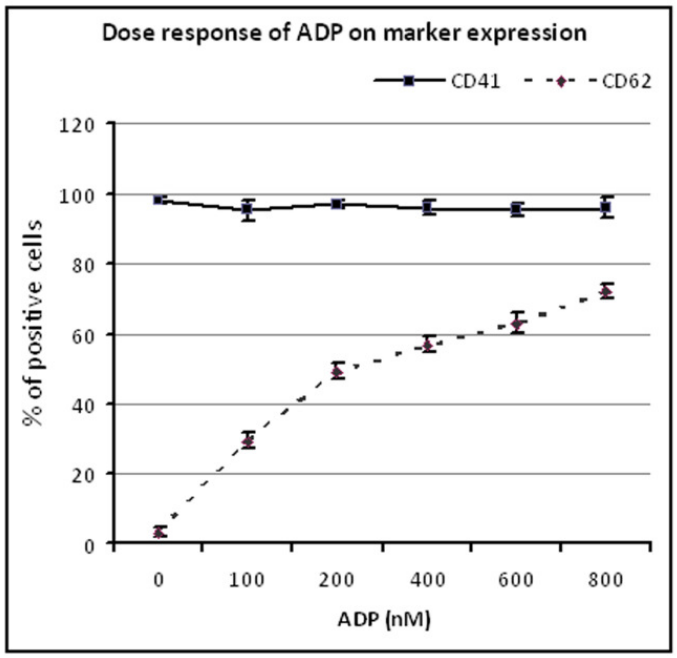

Fig. 4. Profile of anti-CD41 and anti-CD62 binding in ADP activated washed platelets

Percentage of positive cells was calculated by using Beckman EPICS XL, and is shown (y-axis) against agonist (ADP) concentration (x-axis). All data shown are mean ± SD (n=6). Agonist was added from stock to get the final concentration as marked in the graph

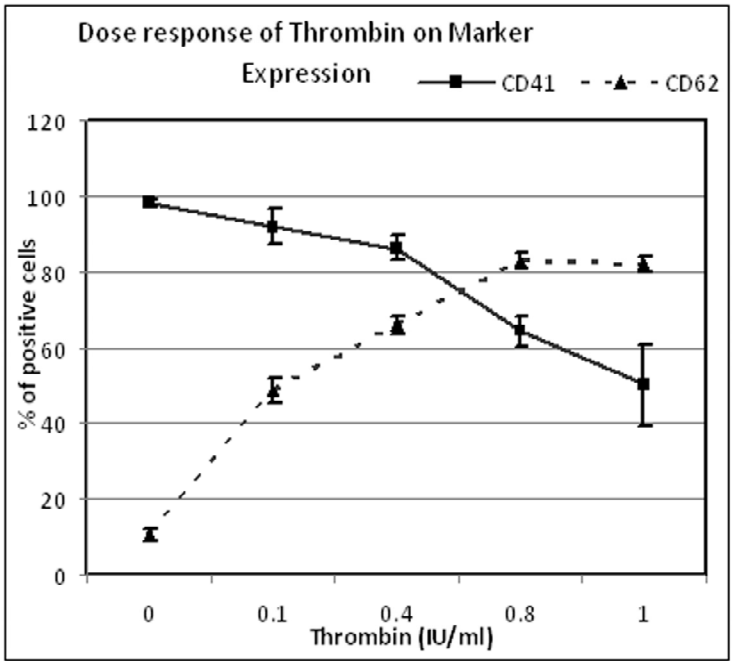

Fig. 5. Profile of anti-CD41 and anti-CD62 binding in thrombin-activated washed platelets

Percentage of positive cells calculated using Beckman EPICS XL is shown (y-axis) against agonist concentration (x-axis). All data shown are mean ± SD (n=6). Agonist was added from stock to get the final concentration as marked in the graph. SD (n=6)

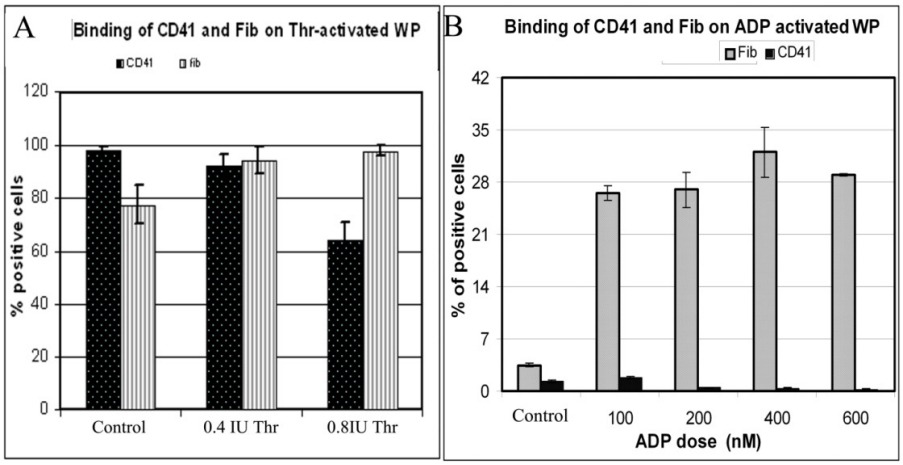

Fig. 6. Profile of Fib and anti-CD41 binding to washed and activated platelets

A, percent of CD41$^+$ platelets and Fib$^+$ platelets when PE-anti-CD41 and FITC-Fib were added in separate aliquots of thrombin- activated suspension; B, percent of CD41 and Fib$^+$ platelets when CD41 antibody and FITC-Fib were added to ADP-activated washed platelets simultaneously Percentage of positive cells calculated using Beckman EPICS XL is shown (y-axis) against agonist concentration (x-axis). All data shown are mean ± SD (n=6). Agonist was added from stock to get the final concentration as marked in the graph

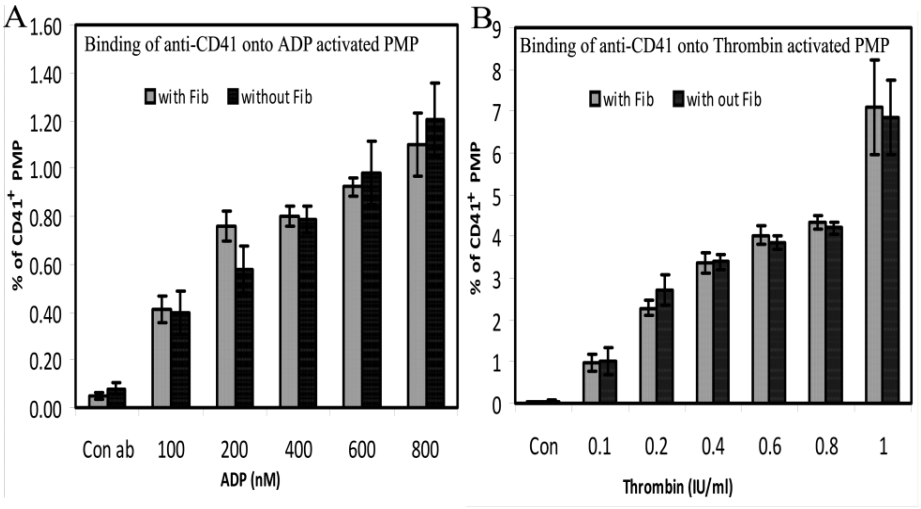

Fig. 7. Profile of anti-CD41 binding to PMP

A, anti-CD 41 binding. to micro particles in ADP-activated washed platelets in the presence and absence of Fib; B, CD 41 binding to microparticles in Thrombin-activated washed platelets in the presence and absence of Fib; The PMP gate was selected using 0.2µm beads obtained from Beckman (USA). The CD41$^+$ PMP was calculated using Beckman EPICS XL. Percentage of CD41$^+$ in PMP is shown (y-axis) against agonist concentration (x-axis). All data shown are mean ± SD (n=6). Agonist was added from stock to get the final concentration as marked in the graph

4. DISCUSSION

Activation of platelets is known to increase the density of CD62, CD41 and CD61 on platelet surface. CD62 is involved in platelet-leukocyte adhesion through P-selectin glycoprotein ligand 1 (PSGL1), whereas CD41/CD61 is involved in platelet-to-platelet interaction mediated by Fib binding. The leukocyte receptor CD62 is located in α-granules of the resting cell and gets exposed to the membrane surface only upon platelet activation. It is therefore an established platelet activation marker and its exposure and density may be quantified by flow cytometry [9]. However, there is no report on the value of CD41 as a platelet activation marker. This study suggests that the percentage of CD41$^+$ cells decreases with platelet activation. The activation

of platelets has been explained since a long time in concentrates stored for transfusion. Such activated platelets lose their ability to get recruited into aggregates even when an agonist is added. The refractoriness of activated platelets to agonists may be quantified using aggregometry which is a specific functional assay. Interestingly, in this study we showed that parallel to the development of refractoriness to agonist, there is reduction in the percentage of anti-$CD41^+$ cells in stored platelets and such reduction could be used as an indicator of platelet activation. More receptor sites are expected to be exposed on activated platelets, but contrary to our expectations, the percentage of $CD41^+$ cells were reduced when storage time progressed. Such reduction in percentage of $CD41^+$ cells were also seen in fresh platelets activated with a strong agonist.

This observation could be explained by several reasons:

i) A new pool of receptors that were exposed on the surface of platelets are not accessible to the CD41 antibody.
ii) Existing receptors on the resting platelets and newly exposed internal pools of CD41 on the activated platelets get masked due to Fib binding caused by the increase of receptor's affinity to Fib during activation.
iii) Microparticles got shredded when activation was high, due to which the receptor sites were lost.

If the occupancy of receptors by Fib is considered the reason for reduced anti-CD41 binding to activated platelets, efficacy of therapeutic antibodies also may be lowered by a similar process. This study attempts to prove that the newly exposed receptors or those are clusterized upon platelet activation with strong agonists have more affinity to Fib and thus prevents binding of specific antibodies.

Platelet aggregation through Fib interaction is the most important and final common pathway that operates during the platelet aggregation, irrespective of the agonist type which initiates platelet activation. Therefore, blocking of the Fib receptor using synthetic antagonists/antibodies has been a widely experimented strategy to prevent *in vivo* platelet aggregation in patients at high risk for thrombotic occlusion during percutaneous coronary intervention (PCI) [10]. The pharmacokinetics of the abciximab administration was studied systematically by Mascelli M et al. [11] and it was proved that 0.5 h after administration of the drug, platelet aggregation by ADP was inhibited to less than 20% of the baseline value. Based on our results it is suggested that, since they used a weak agonist at a low concentration, chances of new pools of CD41/CD61 receptors getting exposed was much lower as compared to the events in other physiological responses such as thrombin and collagen induced activation. Results of this study suggest that new pools of the receptor exposed by weak agonist can be blocked by antibodies, but not receptors exposed by a stronger agonist such as thrombin which is a physiologically significant one. Effective inhibition of aggregation is therefore possible only when the activation is mild; early 100% blockade of receptors was also reported by these authors [12], but larger pools of CD41/CD61 molecules mobilized upon intense platelet activation were not studied. In our study, >90% cells were positive for CD41 in fresh, resting platelets and ~70% in PC on day 0 of storage, which may be a consequence of processing after blood collection. Only if antibodies show higher affinity towards the new pool of receptors as compared to Fib, the aggregation can be blocked by therapeutic antibody. Therefore, the ability of antagonists/antibodies to block the receptor-ligand interaction becomes a challenge. It was shown by EPICS investigators [13] that in high risk coronary angioplasty, intravenous administration of 7E3 Fab (Centocor, Malvern, Pa.) was effective and 35% of patients benefited from the therapy. Thus, up to 65% of patients did not benefit by the use of the antagonist. It may be possible that in the benefited population, agonist activation was mild and antagonist binding was effective, whereas, in the others, activation may have been strong, Fib blocked the newly exposed receptor sites, and the antagonist was not bound. Many similar studies have reported some beneficial effects of Fib receptor antagonists in coronary angioplasty, but none have studied CD41/CD61 exposure/block by the antagonists in the event of stronger activation. Based on our results, it is suggested that the ability of therapeutic antibodies to bind to internal pools which are exposed upon strong agonist activation should be evaluated for determining the efficacy of the prospective drug.

Several antagonists (abciximab, eptifibatide, tirofiban, lamifiban, and orbifan) against the receptor have been developed to prevent Fib binding and to inhibit platelet aggregation [12,14]. The use of antagonists has certain side effects such as increase in mortality and bleeding

[15]. It is important to check if the antagonist can compete with Fib, as only then would the treatment be effective. Though we have not used therapeutic antibodies to evaluate their binding to the receptor, our results propose a similar profile when activation is intense. When we treated agonist-activated platelets with Fib alone or anti-CD41 alone, an increase in binding of both molecules was found in a dose-dependent manner, but when both anti-CD41 and Fib were added together, only Fib binding was prominent. In pathological conditions, circulating platelets may become activated [16,17], thus causing the release of the internal pool of receptors. The newly exposed receptors get clustered and are functionally important and support the platelet aggregation because of high affinity for soluble Fib. To date, studies have reported only on the effectiveness of antagonist in conditions of mild platelet stimulation.

Another possible reason for the reduction in the percentage of the $CD41^+$ population when platelets are activated could be the removal of receptor sites through membrane shredding. Analysis of PMP for $CD41^+$ events and fib^+ events showed that $CD41^+$ micro particles were more abundant when the agonist dose was higher, but not many Fib^+ particles were detected. Since anti-CD41 binding was reduced even in the absence of Fib, it is possible that the receptors may be removed when the membrane gets shredded as microparticles upon higher level of platelet activation. Clearly, CD41 is present on microparticles, but not in a conformation which favors Fib binding. Therefore, recruitment of PMP to the platelet aggregates is unlikely to take place. Circulating PMP is considered to be a marker for cardiovascular disease progression. From the results of this study, it may be concluded that though PMP is a marker, it may not participate in thrombus formation because the receptor on the particles may not be in a conformation favorable for binding of the ligand (Fib) and for participation in aggregate formation. Binding of ligand requires specific conformations of the binding pocket which may be different in platelets and in microparticles. In microparticles, this conformation has higher affinity towards anti-CD41, whereas in platelets, there is a greater affinity towards their ligand, Fib.

5. CONCLUSION

To conclude, more than 95% of resting and moderately activated platelets bind anti-CD41. In the absence of plasma fibrinogen, activation by thrombin reduced $CD41^+$ cells to ~ 50%. ADP activation did not influence the percentage of $CD41^+$ platelets much at the non aggregating dose used in this study. More Fib bound platelets are detected upon thrombin-activation. Activation-dependent membrane shredding seems to have also caused removal of $CD41^+$ micro particles resulting in reduced numbers of $CD41^+$ cells. Therefore, both higher affinity of Fib for receptors and removal of CD41 with shredded PMP together lower the percentage of $CD41^+$ cells in thrombin-activated platelet suspension. As Fib does not bind to CD41 present on PMP, shredded microparticles may not participate in thrombus formation. It is essential to validate receptor binding efficacy of antibodies upon activation of cells with strong agonist to predict their therapeutic outcome. Our results indicate that in the event of strong platelet activation, antibody binding to the receptor is unlikely to take place.

CONSENT

No patient material was used for this *in vitro* study; only human donor blood components were used.

ETHICAL APPROVAL

The experiments conducted in this study have been approved by the registered Institutional Ethics Committee of SCTIMST. (Ref: ECR/189/Ins/KL/2013. The human blood components used in this study has been approved against the study title: Blood compatibility evaluation of Biomaterials and Medical Devices (IEC-594), in April 2014. The approval reference no for collection of discarded buffy coat is IEC-546 obtained in Feb 2013.

Both authors hereby declare that all experiments have been examined and approved by the appropriate ethics committee and have therefore been performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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