## Microparticle Formation and Platelet Shrinkage in Type-I Glanzmman Thrombasthenia Platelets

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## Abstract

**Background:** Activated normal platelets undergo many biochemical and morphological changes, some of which are apoptotic. Platelet derived microparticles and shrinked platelets as hallmark of platelet activation and apoptosis disperse surfaces containing procoagulant activity around injured vessels and tissues. This study was conducted to determine microparticles formation and platelet shrinkage in Glanzmann thrombasthenia upon activation.

**Patients and Methods:** Platelets from twelve unrelated type I Glanzmann thrombasthenia patients were examined as washed platelets. Calcium ionophore A23187 was used as agonist to activate the platelets. Flow cytometry was applied to measure platelet-derived micro particles (forward scatter; events <1.0 μm size), and platelet shrinkage (mean-FSC). Anti-CD42b was used as platelet specific marker to distinguish platelets from other likely particles. Annexin A5 Alexa Fluor was used to determine phosphatidylserine exposure and confirm platelet activation and apoptosis.

**Results:** Calcium ionophore A23187, dramatically increased MP formation by type-I GT platelets up to 14.5 fold increase over baseline (Buffer treated: 14.18  $\pm$  5.4% vs. A23187 treated: 34.31  $\pm$  15.2% p<0.005). Also calcium ionophore A23187, increased platelet shrinkage by type-I GT platelets and mean-FSC decreased (Buffer treated: 4.12 $\pm$ 1.3 vs. A23187 treated: 1.67 $\pm$ 0.2 p<0.0024).

**Conclusion:** This study showed that, type I Glanzmann thrombasthenia platelets demonstrate platelet apoptosis considering two apoptotic targets, including micro particles formation and platelet shrinkage. We conclude that in thrombasthenic Glanzmann platelets at least some aspects of normal apoptosis is ongoing, and this may explain normal platelet count among these patients.

Keywords: Apoptosis, Glanzmann thrombasthenia, glycoprotein IIbIIIa, flow cytometry, microparticle.

## Introduction

Glanzmann thrombasthenia (GT) is a rare inherited autosomal recessive platelet disorder characterized by lack of platelet aggregation. The molecular basis is linked to quantitative and/ or qualitative abnormalities in the membrane glycoprotein (GP) IIbIIIa also known as  $\alpha$ IIb $\beta$ 3<sup>1, 2</sup>. GT is divided into three types: type I (complex of GPIIbIIIa platelet level is less than 5% of normal), type II (complex of GPIIbIIIa platelet level between 5-20% of normal) and type III or variant (GP complex of normal quantity but not quality and structural abnormalities)<sup>1</sup>. GT should be suspected in patients with mucocutaneous bleeding with platelet aggregation defect in response to all physiologic agonists, having a normal platelet count and morphology<sup>3</sup>. Diagnosis is confirmed by showing platelet  $\alpha$ IIb $\beta$ 3 deficiency or its dysfunction by flow cytometry or other methods 3. GT is now generally recognized as the most frequent inherited integrin disorder but is a relatively rare platelet abnormality<sup>3</sup>. Toogeh et al.<sup>4</sup> have recorded 394 GT patients In Iran by 2004 and Farsinejad et al.<sup>5</sup> Hemmati et al.

reported another 110 cases in Iran by 2010.

Glycoprotein IIb/IIIa plays a key role in platelet activation and aggregation 6. Deficiency of GPIIbIIIa is associated with decreased thrombin generation which further contributes to inefficient hemostasis <sup>3</sup>. Whether decreased phosphatidylserine (PS) exposure on the surface of activated GT platelets and decreased microparticle formation plays a role in this decreased thrombin generation is not clear. Prothrombinase activity of GT platelets stimulated with combination of physiologic agonists may be decreased<sup>7</sup>. In addition, in GT platelets PS exposure and microparticle formation have been reported to be significantly decreased in some studies or very similar to control platelets in some other studies<sup>8,9</sup>. These discrepant results may be attributed to differences in the techniques or experimental conditions that need to be further evaluated <sup>10</sup>.

activation Platelet is associated with physiological cellular responses, some of which include: PS externalization, mitochondrial inner membrane depolarization, caspase 3 activation, release of Bcl-2 family proteins, microparticle formation, and platelet shrinkage <sup>11</sup>. Platelet vesiculation and MP formation are platelet activation events with presumptive role in hemostasis involving dispersion of procoagulant and anticoagulant surface activity all around injured vessels and tissues<sup>9</sup>. MPs and shrinked platelets are two specific platelet apoptotic markers. Physiologic and non-physiologic agonists activate platelets to produce MPs (<1.0µm) and platelet shrinkage. In apoptotic platelets plasma membrane vesiculate and shed microparticles 12. Shrinked platelets are the remnants of platelets after MP shedding. In normal platelets, this phenomenon occurs concurrently with PS-exposure and other apoptotic features. Shrinked platelets have smaller volume; shown as decreased FSC compared with unstimulated platelets by flow cytometry 10. In the present study, we investigated microparticle formation and platelet shrinkage, two major calcium-dependent apoptotic reactions, in platelets of twelve unrelated previously described type-I GT patients. Flow cytometry was used in washed platelets stimulated with calcium ionophore (A23187) that is capable of optimal platelet activation 13. We assessed PS expression as hallmark of platelet activation and apoptosis.

### Patients and Methods Patients

Twelve young middle-aged known cases of type I GT were included in the present study. Control samples were used in each run-work to ensure the proper testing procedures and were obtained from healthy volunteers who had no history of abnormal bleeding disorders among themselves and their families. Informed consent was obtained from all patients and healthy donors. The study was approved by the ethics committee of the Iranian Blood Transfusion Organization (IBTO).

#### Methods

#### Platelet preparation and activation

Blood was obtained from GT patients and healthy control volunteers. None of the subjects used medication affecting platelet function for two weeks prior to the study. Blood was anticoagulated with CPD-A (citrate phosphate dextrose-adenine) containing 0.32% sodium citrate 14, 15, 16. Platelet rich plasma (PRP) was obtained by centrifugation of the whole blood in 80g for 15 minutes, then two-thirds of PRP was transferred to a 10ml silicon tube and centrifuged at 2500g for 15 minutes at room temperature. Platelet poor plasma (PPP) was discarded and the platelet pellet was suspended in Tyrode-HEPES buffer (137 mM NaCl, 2.7 mM KCl, 11.9 mM NaHCO3, 0.42 mM NaH<sub>2</sub>PO<sub>4</sub>, 1 mM MgCl<sub>2</sub>, 5.5 mM glucose, 2 g/L BSA and 5 mM HEPES), pH 7.4<sup>15, 16</sup>. To prevent fibrin polymerization and platelet aggregation, we omitted the CaCl, from Tyrode-HEPES buffer during washing steps. After 30 minutes, platelets were centrifuged at 1200g for 10 minutes at room temperature. Supernatant was discarded and the platelet pellet was washed again with Tyrode-HEPES buffer to remove residual plasma factors affecting platelet activation. The pellet was then suspended in Tyrode-HEPES containing 2mM CaCl, and then platelet count was determined by a Sysmex K-1000 automated hematology analyzer (Sysmex corporation, KOBE, JAPAN), and adjusted at about 150-200×109 L<sup>-1</sup>. Platelets were treated with 3mM calcium ionophore A23187 (Abcam, Cambridge Science Park, Cambridge, England); Mixing was done by gentle inverting tubes several times and every one minute up to 10 minutes.

# Flow cytometric assessment of platelets in washed platelets

The following fluorophores were added to washed activated platelets suspended in Tyrode-HEPES buffer containing BSA, and CaCl<sub>2</sub> pH 7.4: Annexin V-Alexa Fluor<sup>®</sup> 488 (A13201, Invitrogen, Life Technologies Corporation, California, USA) for the measurement of PS exposure and anti CD42b-PE (clone P2; Beckman Coulter, Fullerton, CA), as the platelet marker. Samples were incubated for 40 min in a dark room and then diluted 5-folds with Tyrode-HEPES buffer before flow cytometry. Negative and positive controls and GT platelet samples were treated at the same condition with the least time delay from blood sampling to analysis <sup>10, 16</sup>.

Analyses were performed on a CyFlow<sup>®</sup> ML flow cytometer (Partec GmbH Otto-Hahn-Straße 32, D-48161 Munster, Germany) equipped with a 488-nm argon-ion laser and 635-nm red-diode laser. For analysis of platelets in washed platelet suspensions, fluorescence parameters and light scattering was adjusted at logarithmic gain, and the threshold was set to eliminate events negative for platelet markers. Quality control procedures were followed; the flow cytometer settings were identical for all analyses and negative and positive controls were done routinely. Single platelets were identified by their characteristic forward light scatter and high CD42b fluorescence.

Upon activation in a buffer containing calcium,

platelets undergo platelet shrinkage and MP formation <sup>9, 10</sup>. Platelet derived MPs were identified by their characteristic forward light scatter and high CD42b fluorescence as well as Annexin A5 as apoptosis indicator <sup>10, 17</sup>. MPs were defined as events less than 1 $\mu$ m in MP gate <sup>10, 17</sup>. In this study 0.5-1.0 $\mu$ m beads were used as calibrator. Platelet shrinkage was identified by the mean-FSC and high CD42b fluorescence as well as Annexin A5 in whole platelet population gate.

A minimum of 25,000 events was acquired on each sample; real-time data acquisition and analysis, as well as real-time data display, was performed using FloMax<sup>®</sup>, the Windows<sup>™</sup>-based flow cytometry software (Partec GmbH Otto-Hahn-Straβe 32, D-48161 Munster, Germany). Resting platelets were used to set baseline gates (MPs were not excluded). MP formation and shrinkage were examined on platelets of both normal and type-I GT platelets within total platelet population.

#### Statistical analysis

Values reported are mean  $\pm$  Standard Error of the Mean (SEM). Reported mean values are obtained from samples (n=12), and statistical analysis was performed using Student's paired T-test to compare differences before and after activation in GT platelets. p<0.05 was deemed to be statistically significant.

Characteristic (n = complete data)	No. of Patients (%)	Mean ± SD	
Gender			
Male	5(41.7)		
Female	7 (58.3)		
Age (years)			
Male		27±15	
Female		28±12	
CD41/CD61		2.5±2	
Last platelet transfusion (days)		60±22	
Last FVIIa* use (days)		40±12	
Medications** avoidance	>2 weeks		

Table 1: Patients' demographics.

\*Recombinant factor FVIIa (Novoseven)

\*\*Medications affecting platelet functioning.

Apoptotic responses	n	Buffer treatment	A23187 treatment	P-value
MP formation (N)	12	39±24 (14.18±5.4%)	567±344 (34.31±15.2%)	<0.005
Platelet shrinkage (mean FSC)	12	4.12±1.3	1.67±0.2	<0.0024

Table 2: MPs Formation and platelet shrinkage in platelets of type I GT patients.



**Figure1 (A-D):** Microparticle formation and platelet shrinkage in GT and control platelets. Platelets and MPs are stained with anti-CD42b-PE. (A to D) shows the dot plot and gating for platelets and MPs. (A) Resting control platelets, (B) Resting GT platelets, (C) Control platelets stimulated with A23187, (D) GT platelets stimulated with A23187. MPs were counted from microparticle gate as CD42b+ events with low-FSC. PLTs gate using FSC histogram and average values of FSC (mean FSC) was used to assess platelet shrinkage.



**Figure 2:** Platelet shrinkage in GT platelets. FSC histogram and average values of FSC (mean FSC) was used to assess platelet shrinkage. This figure shows the forward scatter of GT platelet before (1) and after (2) activation.

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Twelve young middle-aged known cases of type I GT were included in the present study. Seven patients were young middle aged females (28±12 years) and other GT patients were males (27±15 years). CD41/ CD61 deficiency was confirmed by flow cytometry (less than 5% of control). All 12 patients had normal platelet counts and abnormal aggregometry results corresponding to Glanzmann thrombasthenia <sup>1</sup>. Other data about patients' diagnosis and therapy managements are presented in table1.

#### **PS** exposure

Quiescent platelets from type I GT patients, analyzed in washed platelet suspensions, were observed to have as low proportion of PS-exposing platelets as controls (90.8  $\pm$  65.7 mean $\pm$ SEM, vs. controls: 100.6  $\pm$  81.9, n =12, p=0.746). Stimulation with calcium ionophore A23187 resulted in increase of PS-exposing platelets, MPs, and shrinked platelets (3070  $\pm$  357, mean $\pm$ SEM, vs. controls: 3040  $\pm$  402.5, n =12, p=0.820). By this method we ensured that platelets were adequately activated.

#### **Microparticle Formation**

Microparticles were defined as <0.1 $\mu$ m polystyrene particles in forward scatter <sup>10</sup>, and were counted from microparticle gate as CD42b+ events with low-FSC. Under resting conditions, the percentage of platelet-derived MPs from GT patients did not show a difference with controls (GT patients: 14.18 ± 5.4%; vs. controls: 15.6 ± 5.7% p=0.734). While calcium ionophore A23187, dramatically increased MP formation by type-I GT platelets up to 14.5 fold increase over baseline (Buffer treated: 14.18 ± 5.4% vs. A23187 treated: 34.31 ± 15.2% p<0.005) (Table 2, Figure 1A-D).

#### **Platelet shrinkage**

We used mean-FSC of platelet gate to measure the FSC decrease in shrinked platelets. Under resting conditions, the mean-FSC of shrinked platelets from the patients was high, similar to controls (GT patients: 4.12±1.3% vs. controls: 4.21±1.0% p=0.861). While calcium ionophore A23187, increased platelet shrinkage by type-I GT platelets and mean-FSC decreased (Buffer treated: 4.12±1.3 vs. A23187 treated: 1.67±0.2 p<0.0024) (Table 2, Figure 1A-D, Figure 2). Microparticle Formation and Platelet Shrinkage ...

## Discussion

We investigated two critical events of apoptosis in platelets of 12 type I Glanzmann thrombasthenia patients. We found that calcium ionophore A23187 that is one of the most potent, non-physiologic platelet activators, increases cytoplasmic ionic calcium and induces platelet activation and reveal some aspects of apoptosis in type I GT platelets, although without aggregation.

Gemmell et al. <sup>8</sup> showed a central role for GPIIbIIIa in supporting the near total inability of type I Glanzmann's thrombasthenic platelets to vesiculate in response to physiologic agonists as thrombin, ADP, and collagen. They showed that MP formation in Glanzmann's platelets was reduced with these agonists for times up to 60 min, the maximal being less than 5% above that spontaneously generated in resting samples <sup>8</sup>. Nomura et al. <sup>18</sup> in 1993 declared that both normal and thrombasthenic platelets showed a similar time-dependent release of microparticles when activated with A23187.

Herein, we demonstrated that A23187 induced GT platelets undergo aspects of apoptosis such as MP production and platelet shrinkage. We conclude that increased cytosolic calcium activates GT platelets, and apoptosis occurs via mitochondrial pathway.

In normal control platelets, PS exposure is a prelude to MP formation that by itself is another aspect of cellular response to platelet activation that occurs during apoptosis <sup>9, 10, 19</sup>.

We suppose that different classes of agonists differ in the way they affect platelet procoagulant activity, the stronger the agonist the harder the response. Our results in using A23187 are in accordance with Hong Wang et al. <sup>20</sup>. They showed that microparticle formation by GT platelets was similar to controls when activated with a combination of Collagen+Thrombin <sup>20</sup>. They explained the synergism of two almost strong agonists to activate platelets to produce MPs <sup>20</sup>.

It has been proposed that patients with GPIIbIIIa deficiency are protected from arteriosclerosis <sup>21, 22,23</sup>. However, some other studies have suggested that this is not the case <sup>3, 22, 23, 24</sup>. Patients with GT also are not protected against venous thrombosis since platelet aggregation is not the only factor, where plasma coagulation and other thrombogenic factors are of primary importance <sup>4, 25</sup>.We observed that stimulated

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GT platelets do produce MPs and shrinked platelets after induction so we think there must be determinants other than GPIIbIIIa that result in thrombus formation and arteriosclerosis.

Overall, we recognized that PS exposure on platelets could occur because of platelet activation or via apoptosis. We also suppose that mechanisms of microparticle formation and platelet shrinkage in these patients are not completely limited to GPIIbIIIa complex but because of their GPIIbIIIa deficiency they are unable to aggregate. Spreading out MPs that do not carry GPIIbIIIa may be a description for bleeding diatheses of GT patients <sup>20, 26, 27</sup>.

## Conclusion

This study shows that, type I Glanzmann thrombasthenia platelets demonstrate platelet apoptosis considering two apoptotic targets, including micro particles formation and platelet shrinkage. We conclude that in thrombasthenic Glanzmann platelets at least some aspects of normal apoptosis is ongoing, and this may explain normal platelet count among these patients.

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