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Apoptotic Events in Type-I Glanzmann Thrombasthenia Platelets

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ABSTRACT

Background: Activated normal platelets undergo numerous biochemical and morphological changes, some of which are apoptotic. Phosphatidylserine (PS) expression, Δψm depolarization, microparticle (MP) formation, platelet shrinkage, release of cytochrome c, and caspase activation are hallmarks of both platelet activation and apoptosis. In this study, we report the apoptotic responses of type-I Glanzmann thrombasthenic platelets.

Materials and Methods: Platelets from twelve unrelated patients with type I Glanzmann thrombasthenia were examined as washed platelets. Calcium ionophore A23187 was used as an agonist to activate the platelets. Flow cytometry was employed to detect phosphatidylserine expression (Annexin A5 Alexa Fluor), Δψm depolarization (JC-10), platelet-derived MP formation (forward scatter; events <1.0 µm in size), and platelet shrinkage (mean-FSC). Anti-CD42b was used as a platelet-specific marker to distinguish platelets from other particles.

Results: We determined that increased cytosolic calcium significantly increased PS exposure, depolarized mitochondrial inner membrane potential (Δψm), increased microparticle formation, and induced platelet shrinkage in type-I Glanzmann thrombasthenic platelets. Our research showed that type I Glanzmann thrombasthenic platelets exhibit characteristics of platelet apoptosis. GPIIbIIIa deficiency does not limit platelet activation or apoptosis.

Conclusion: We conclude that GPIIb-IIIa-independent mechanisms may be involved in the normal apoptosis of thrombasthenic platelets. Our data deepen the understanding of the role of the platelet fibrinogen receptor in revealing aspects of normal apoptosis. However, this may help explain the normal platelet count among thrombasthenic patients.

Keywords: Apoptosis; Glanzmann thrombasthenia; Glycoprotein IIbIIIa; Flow cytometry; Microparticle

INTRODUCTION

Apoptosis is programmed cell death. Two apoptotic pathways exist in nucleated cells: extrinsic and intrinsic[1]. The signalling networks and cellular events that occur during their activation are very complex and differ between cell types [1]. In platelets, the intrinsic mitochondria-dependent apoptotic pathway has been well-documented, but the contributions of the extrinsic apoptosis pathway (via death receptors) have not been extensively explored[2]. Several external stimuli, including soluble platelet agonists[3], calcium ionophore[4], anti-platelet antibodies[5], as well as high shear stresses[6] and long-term storage of platelets (under blood banking conditions)[7-9], induce platelet activation and reveal manifestations of apoptosis. The markers of apoptosis include ΔΨm depolarization, expression of

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pro-apoptotic Bcl-2 family proteins, caspase-3 activation, PS exposure, platelet-derived microparticle (MP) formation, and platelet shrinkage[1].

Glycoprotein IIb/IIIa (also known as integrin αIIbβ3) is an integrin complex found exclusively on platelets. It is a receptor for fibrinogen and plays a crucial role in platelet activation and aggregation[10,11]. Platelet activation leads to a conformational change in the platelet GPIIb-IIIa receptor, which induces binding of fibrinogen (fibrin)[11].

The exact mechanism of inside-out and outside-in signalling through integrin αIIbβ3 is not fully understood, and the significance of this receptor in platelet apoptosis remains unclear[10,12, 13]. Glanzmann thrombasthenia (GT) is an inherited bleeding disorder characterized by the presence of abnormal platelets deficient in the glycoprotein IIb-IIIa complex[13]. To gain a better understanding of the factors contributing to platelet apoptosis and their survival, we investigated certain markers of apoptosis in type-I GT platelets upon activation by calcium ionophore A23187.

MATERIALS AND METHODS

Patients

Twelve young to middle-aged patients with known cases of type I Glanzmann thrombasthenia (GT) were included. Data about their diagnosis and therapeutic management were available in their medical records, and GPIIbIIIa deficiency was confirmed by flow cytometry. Control samples were collected under similar conditions from 12 healthy donors with no history of abnormal bleeding either in themselves or in their family. Informed consent was obtained from all patients and healthy donors. The study was approved by the ethical review committee affiliated with the Iranian Blood Transfusion Organization (IBTO).

Plateletpreparation and activation

Blood was obtained from GT patients and healthy volunteers (controls). None of the GT patients or control subjects took 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). Platelet-rich plasma (PRP) was obtained by centrifugation of whole blood at 80 g for 15 minutes at room temperature (RT). Then, two-thirds of the PRP was transferred to a 10 ml silicon tube and centrifuged at 2500 g for 15 minutes at RT. 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 NaH2PO4, 1 mM MgCl2, 5.5 mM glucose, 2 g/L BSA, and 5 mM HEPES, pH 7.4) (1). To prevent fibrin polymerization and platelet aggregation, CaCl2 was omitted from the Tyrode-HEPES buffer during the washing steps. After 30 minutes, platelets were centrifuged at 1200 g for 10 minutes at RT. The 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 suspended in Tyrode-HEPES containing 2 mM CaCl2, and the platelet count was determined by a Sysmex K-1000 automated hematology analyzer (Sysmex Corporation, Kobe, Japan) and adjusted to approximately 150-200×10^9 L^(-1). Platelets were treated with 3 mM calcium ionophore A23187 (Abcam, Cambridge Science Park, Cambridge, England), with mixing done by gentle inversion of the tubes every minute for up to 10 minutes.

Flow Cytometric Assessment

The following fluorophores were added to a suspension of activated platelets: Annexin A5-Alexa Fluor® 488 (A13201, Invitrogen, Life Technologies Corporation, California, USA) for the measurement of phosphatidylserine (PS) exposure[16]; anti-CD42b-PE (clone P2; Beckman Coulter, Fullerton, CA) as a platelet-specific marker. The mitochondrial inner membrane potential-sensitive dye JC-10, enhanced 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolcarbocyanine iodide (AB112133, Abcam, Cambridge Science Park, Cambridge, England), was used to assess depolarization of the mitochondrial inner membrane potential (ΔΨm). JC-10 is a potentiometric cationic and cell-penetrating fluorescent dye sensitive to mitochondrial potential (ΔΨm), which accumulates in the matrix of mitochondria. JC-10 disperses as monomeric molecules in the cytosol (FL1-green) and

aggregated molecules in polarized mitochondria (FL2-red), also known as J-aggregates. Depolarization is characterized by a decrease in the content of JC-10 aggregates in mitochondria, reflected by a decrease in red (FL2) fluorescence. We used the FL2/FL1 ratio (575 nm/530 nm) to determine the depolarization rate of mitochondria in platelets. Platelets were resuspended in JC-10 solution (10 mg/ml) and incubated in the dark for 15 minutes at room temperature before acquisition. Platelet vesiculation and MP shedding are calcium-dependent activation events. Shrinked platelets are the remnants of platelets after MP shedding, with a smaller volume, as indicated by lower mean-FSC compared with unstimulated platelets. Single platelets were identified by their characteristic forward light scatter and high CD42b fluorescence, while platelet-derived microparticles (MPs) and shrinked platelets were identified by their characteristic forward light scatter, CD42b fluorescence, and Annexin A5 as an apoptosis indicator[14,17]. MPs were defined as events less than 1 µm [14]. Beads of 0.5-1.0 µm (Megamix 7801, Biocytex, Linscott's Directory, Linscott's USA) were used as a calibrator. After adding fluorophores, samples were incubated for 40 minutes in the dark at room temperature, then diluted 5-fold with Tyrode-HEPES buffer before acquisition. Samples were treated under the same conditions with minimal time delay from blood sampling to analysis[1,14]. To ensure comparable results between samples and across runs, activation and incubation times were fixed. Flow cytometry was performed on a CyFlow® ML flow cytometer (Partec GmbH, Germany) equipped with a 488-nm argon-ion laser. For analysis, fluorescence parameters and light scattering were adjusted at logarithmic gain, and a threshold was set to eliminate events negative for platelet markers. Quality control procedures were followed, and the flow cytometer settings were identical for all analyses. Negative and positive controls were used in each run. A minimum of 25,000 events was acquired for each sample, with real-time data acquisition and analysis performed using FloMax® software (Partec GmbH, Germany). Resting platelets were used to set baseline gates (microparticles were not excluded). Phosphatidylserine exposure and mitochondrial depolarization were examined in both normal and type-I GT platelets within the total platelet population.

Statistical analysis

The values reported are mean ± standard error of the mean (SEM). The reported mean values are derived from samples (n=12), and statistical analysis was performed using Student's paired t-test to compare differences before and after activation in the control or GT platelets. One-way ANOVA was used to compare differences among groups in control and GT platelets. A p-value of <0.05 was considered significant.

RESULTS

Patient demographics

A total of 12 patients with known cases of type I Glanzmann thrombasthenia (GT) were included in the study, comprising 5 males (41.7%) and 7 females (58.3%). The mean age was 27 ± 15 years for males and 28 ± 12 years for females. CD41/CD61 deficiency was confirmed by flow cytometry (less than 5% of control, data not shown). The average CD41/CD61 value across the cohort was 2.5 ± 2. All patients adhered to a medication avoidance period of more than two weeks prior to data collection.

PS exposure

The expression of phosphatidylserine (PS) on platelets stimulated with calcium ionophore (A23187) was assessed by measuring the mean fluorescent intensity (MFI) of Annexin A5-Alexa Fluor binding to surface-exposed PS. Resting platelets from type I GT patients exhibited a lower proportion of PS exposure compared with controls (GT: 90.8 ± 65.7 mean ± SEM, vs. controls: 100.6 ± 81.9, n = 12, p = 0.746) (Fig. 1A; top set of dot plots). Stimulation with calcium ionophore A23187 resulted in increased expression of PS in GT platelets, with levels comparable to controls (GT: 3070 ± 357 mean ± SEM, vs. controls: 3040 ± 402.5, n = 12, p = 0.820). Overall, PS exposure was observed on about 88.71% of activated GT platelets and 86.1% of activated control platelets (Fig. 1A; bottom set of dot plots). However, the extent of PS exposure on resting and activated type I GT platelets increased similarly to respective

controls (Fig. 1B). Some type I GT patients showed slightly higher PS expression than others. Thus, there was heterogeneity in PS expression among GT patients, as well as among normal control platelets (data not shown).

Elevated cytosolic Ca2+ levels induced by calcium ionophore A23187 are potent inducers of PS expression and other apoptotic events in both control and GT platelets (18). The reactions were not terminated, and by 1 hour, the MFI of Annexin A5 binding significantly increased in platelets stimulated with 3.0 µM A23187 ($P < 0.05$) (Fig. 2A, 2B).

ΔΨm decline

Using JC-10, we demonstrated depolarization of mitochondrial inner membrane potential (ΔΨm) in GT platelets. Resting platelets from GT patients exhibited a lower proportion of depolarized ΔΨm compared with controls (3.9±1.9%, mean±SEM, vs. controls: 2.4±2.5%, n=12, p=0.149) (Fig. 3A: top set of dot plots). We observed that in both control and type I GT platelets, a significant proportion of platelets showed ΔΨm loss after activation with A23187 (53.2±18.7%, mean±SEM, vs. controls: 55.8±19.2%, n=12, p=0.575) (Fig. 3A: bottom set of dot plots; Fig. 3B).

Microparticle formation

We observed no significant difference in microparticle (MP) formation between GT platelets and healthy control platelets. Under resting conditions, the percentages of platelet-derived MPs from the patients were low, similar to controls (GT patients: 205±49, vs. controls: 199±41, p=0.734). Calcium ionophore A23187 dramatically increased MP formation in both control and type I GT platelets (GT patients: 995±206; vs. controls: 1002±148, p=0.914) (data not shown).

Platelet shrinkage

Cell shrinkage is a key characteristic of apoptosis in platelets. The PS-exposing type I GT platelets, after activation with A23187, showed no significant difference compared to normal control platelets. In resting platelets, the mean-FSC of platelets from GT patients showed no significant difference compared with controls (GT patients mean-FSC: 4.12±1.3, vs. controls: 4.21±1.0, p=0.861). However, calcium ionophore A23187 significantly increased platelet shrinkage in both control and type I GT platelets, resulting in a decrease in mean-FSC (GT patients: 1.67±0.2; vs. controls: 1.69±0.3, p=0.850) (data not shown).

(A)

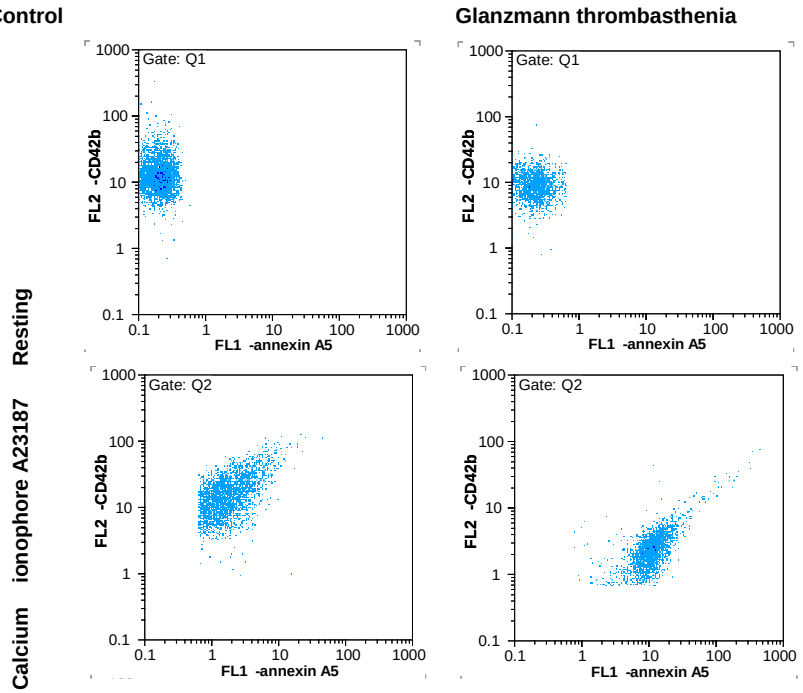


Figure 1. (A) PS exposure on quiescent and activated control and type-I GT platelets. Platelets were activated in washed platelet suspension with calcium ionophore (A23187). Representative dot plots of platelet anti CD42b versus Annexin A5 binding for quiescent platelets and platelets stimulated with A23187.

(B)

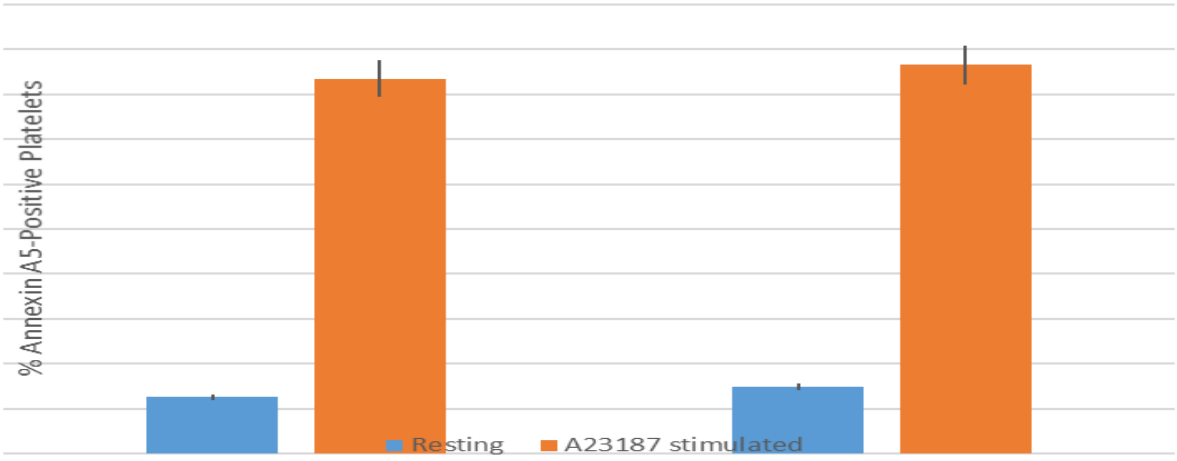


Figure 1. (B) Percent of PS-exposing platelets. Values are mean ± SEM, n =12; for GT Patients and control subjects.PS: phosphatydilserine, GT: Glanzmann thrombasthenia

(A)

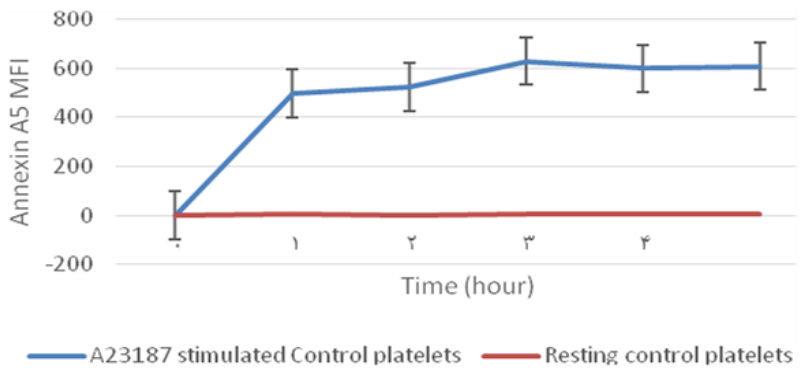
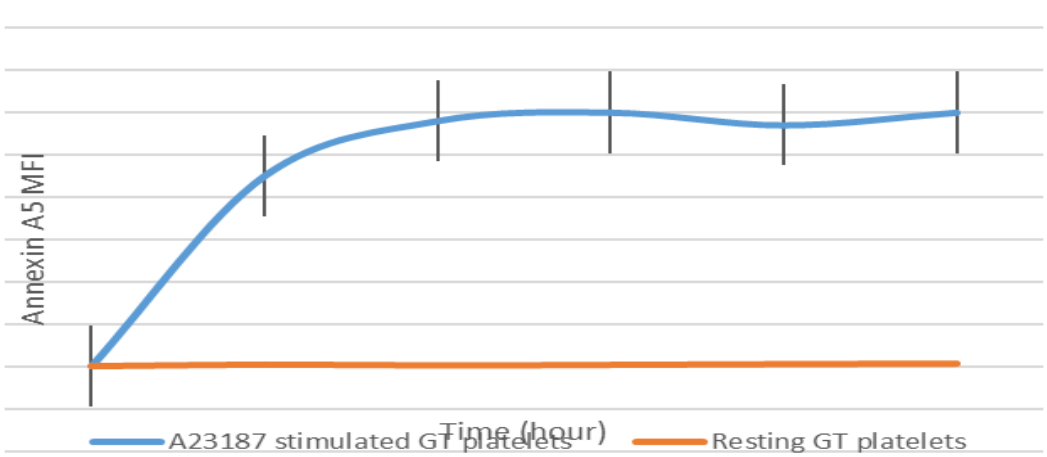


Figure 2. PS Expression up to 4 h after activation with A23187
(A): Surface expression of PS on activated platelets determined by flow cytometry for control, unstimulated platelets, and platelets stimulated with 3 µm A23187

(B)



(B): GT platelets activated with A23187 showed PS expression during first minutes and up to 4 h similar to control platelets

(A)

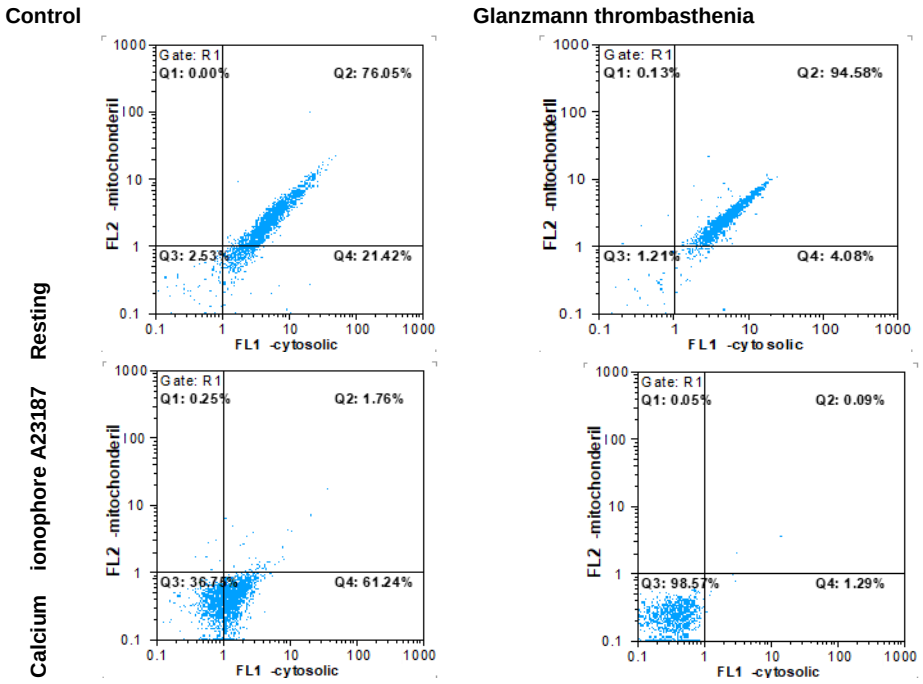
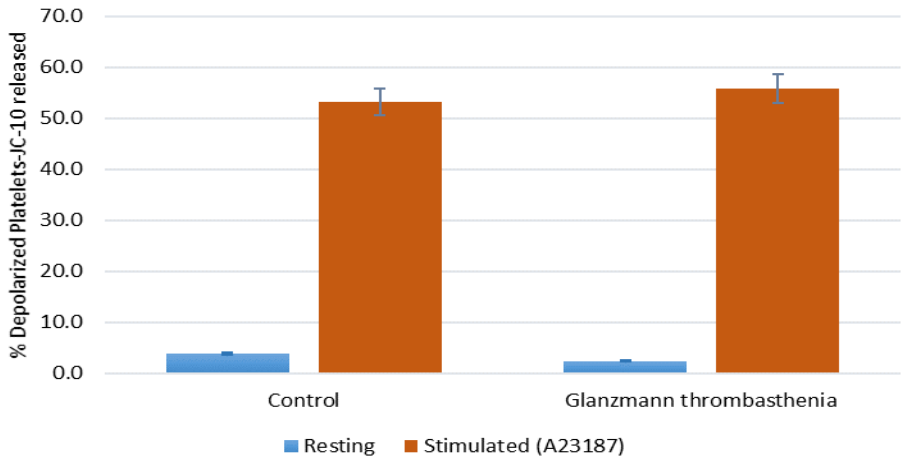


Figure 3. (A): Resting and Calcium ionophore A23187-induced *ΔΨm* depolarisation determined by JC-10 probe. Representative dot plots are shown for platelets treated with Tyrode buffer (quiescent) and 3 µm calcium ionophore A23187. Depolarisation is characterized as the decrease in the content of JC-10 aggregates, as reflected in the decrease of RN2/RN1 ratio.

(B)



(B): The percentage of platelets with collapsed *ΔΨm* in resting and activated control and GT platelets. For controls, values are mean ± SEM, *n=12*. JC-10: enhanced 5, 5', 6, 6'-tetrachloro-1, 1', 3, 3'-tetraethylbenzimidazolcarbocyanine iodide

DISCUSSION

We investigated critical apoptotic events in platelets of 12 type I Glanzmann thrombasthenia (GT) patients. Our study showed that quiescent GT platelets manifest PS exposure, mitochondrial depolarization, microparticle (MP) formation, and platelet shrinkage, similar to quiescent control platelets.

PS exposure

Phosphatidylserine (PS) exposure is considered a representative event in both platelet activation and apoptosis and plays an important role in the clearance of senescent platelets[18,19]. Activated GT platelets showed PS externalization similar to control platelets. We demonstrated that A23187 is able to activate GT platelets to expose PS, and we hypothesize that GPIIbIIIa is not necessary for the phospholipid scrambling that results in PS exposure. In GT platelets, the extent of PS exposure was also similar to control platelets, suggesting that the lack of GPIIbIIIa has no significant impact on the cell cycle of platelets. This result is consistent with findings in GT patients where platelets activated by collagen plus thrombin also showed similar PS expression[17]. Despite the use of different agonists, similar results were obtained in both conditions.

Topalov et al. [20] reported a new function for GPIIbIIIa in PS externalization. They showed two types of procoagulant platelets formed upon physiological activation that were controlled by GPIIbIIIa: type 1 PS+/Ca+ containing high cytoplasmic calcium, and type 2 PS+/Ca– with low intracellular calcium. Their evidence suggested that GPIIbIIIa plays a critical role in the formation of PS+/Ca– platelets, as the formation of PS+/Ca– platelets was prevented by GPIIbIIIa antagonists and in GT patients. They explained that the absence of PS+/Ca– platelets might contribute to the severe bleeding observed in GT patients and the antithrombotic action of αIIbβ3 inhibitors, which were shown to inhibit PS+/Ca– platelets in their study. Thus, it is not surprising that A23187-induced GT platelets exposed PS, which we hypothesize are type 1 PS+/Ca+ platelets, as suggested by Topalov.

Schoenwaelder et al.[21] noted that PS expression on the surface of platelets occurs via two pathways: platelet activation via surface receptors (calcium-dependent pathway and mitochondria) or direct induction of apoptosis (calcium- and mitochondria-independent pathway). Direct apoptosis induction by materials such as ABT-737, without involvement of mitochondria and calcium, and solely through activation of Bax and Bak proteins and activation of caspase-3, leads to PS expression. Platelet activation via surface glycoproteins or other platelet-activating agents, however, increases cytosolic ionic calcium, and the mitochondrial pathway activates to express PS.

Leung et al.[22] showed that stimulation of normal platelets for 4 hours with lower concentrations of A23187 (i.e., 0.5 and 1 µM) was associated with a significant increase in PS-exposing platelets compared with 10 minutes, with increases greater than platelets stimulated with physiologically relevant agonists. Similarly, we found that PS exposure increased dramatically in both GT and control platelets stimulated with 3 µM A23187, with the percentage of PS-exposing platelets remaining unchanged up to 4 hours.

Rand et al.[23] explained that PS exposure is a fundamental component of the coagulatory activity of platelets. Although phosphatidylserine is expressed on platelets lacking GPIIbIIIa, the absence of this glycoprotein prevents platelet aggregation. In GT patients, coagulation reactions occur normally, suggesting that in GT patients, PS expression—an essential platform for coagulation reactions—is normally organized and formed.

Platelet Activation and Coagulation

GPIIbIIIa antagonists as anti-thrombotic therapies have received considerable attention over the past two decades[24]. Shpilberg et al.[25] showed that although GT patients lack GPIIbIIIa, they are not protected from atherosclerosis. Ten Cate et al.[26] declared that patients with Glanzmann thrombasthenia may suffer from deep venous thrombosis. PS expression results in the formation of prothrombinase and tenase complexes to convert fibrinogen to fibrin. We have shown that PS is expressed on the surface of Glanzmann platelets, and it is plausible that GT platelets have implicit potential for thrombus formation similar to normal platelets via coagulation factor activation.

The formation of thrombi is a multifactorial event, with coagulation being one of the important factors in thrombus formation, which occurs subsequent to the emergence of PS on the activated platelet surface. Our study showed that the lack of GPIIbIIIa not only does not prevent PS expression but is one of the multiple pathways through which PS is expressed. There are other pathways that externalize PS to initiate the coagulation cascade, resulting in thrombus formation. It should be noted that GT patients express at least a small amount of GPIIbIIIa on their platelets, which should be considered in these processes.

Mitochondrial depolarization

Dissipation of ΔΨm (mitochondrial inner membrane potential) is an early apoptotic event that occurs before PS exposure in platelets and happens rapidly upon platelet activation[23]. To study the effect of platelet activation via the mitochondrial pathway, we measured mitochondrial inner membrane depolarization. Our study showed that the mitochondria in both control and Glanzmann's platelets were almost completely polarized, with no significant difference observed between the two groups in the resting state. Activated GT and control platelets showed about 50% dissipation of ΔΨm, with no significant difference between the two groups. Notably, Rand et al. [14] reported that the level of mitochondrial depolarization in platelets of Bernard-Soulier syndrome (BSS) is slightly higher than in control platelets. We demonstrated that, unlike BSS, GPIIbIIIa deficiency does not induce spontaneous platelet activation and apoptotic responses. In line with our experiment, Hong Wang et al.[17] observed that activated GT platelets exhibited the apoptotic marker of ΔΨm loss upon stimulation with collagen and thrombin (C+T). It is worth noting that both calcium ionophore A23187 and C+T are potent platelet inducers capable of activating the mitochondrial pathway.

Platelet Vesiculation and Microparticle Formation

Platelet vesiculation and MP shedding are calcium-dependent activation events. Shrinked platelets are the remnants of platelets after MP shedding [27]. In our study, we demonstrated that A23187 induced GT platelets to undergo MP formation and platelet shrinkage (data not shown). Contrary to our result, other studies have shown a central role for GPIIbIIIa in supporting the near-total inability of type I Glanzmann thrombasthenic platelets to vesiculate in response to physiological agonists such as thrombin, ADP, and collagen[27]. They found that MP formation in Glanzmann's platelets was reduced with these agonists for times up to 60 minutes. However, Nomura et al.[28] declared that both normal and thrombasthenic platelets showed a similar time-dependent release of microparticles when activated with A23187. In agreement with our results, Hong Wang et al. [25] showed that microparticle formation by GT platelets was similar to controls when activated with C+T. We conclude that increased cytosolic calcium activates GT platelets to produce MP and shrinked platelets, and the controversy between investigators may be due to different experimental approaches.

CONCLUSION

The data demonstrate that type I Glanzmann thrombasthenic platelets exhibit apoptotic events, including PS exposure, ΔΨm depolarization, microparticle formation, and platelet shrinkage. These findings reveal a novel perspective, suggesting that at least some aspects of normal platelet apoptosis are ongoing in Glanzmann thrombasthenic platelets. This may explain the normal platelet count in these patients. Although the exact contribution of GPIIbIIIa in apoptosis remains to be elucidated, these data support the notion that apoptosis-associated events are vital components of the platelet activation process, even in GT platelets.

CONFLICTS OF INTEREST

There is no conflict of interest, according to the authors, regarding the publication of this work.

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