Investigation of platelet apoptosis in adult patients with chronic immune thrombocytopenia

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ABSTRACT

Objectives: Immune thrombocytopenia (ITP) is an acquired and heterogeneous autoimmune-mediated hematological disease typically characterized by a low platelet count. Emerging evidence over the past several years suggests that platelet biogenesis and ageing are regulated, at least in part, by apoptotic mechanisms. However, the association between decreased platelets and apoptosis in ITP patients is poorly understood. To better understand the role of platelet apoptosis in ITP pathophysiology, we investigated apoptotic markers in platelets acquired from 40 chronic ITP patients. Furthermore, the results of ITP patients were compared to those from 40 healthy individuals.

Methods: Markers of apoptosis, including phosphatidylserine (PS) exposure and mitochondrial inner membrane potentials (ΔΨm), were examined using flow cytometry. The expression of pro-apoptotic molecules such as Bak and Bax and anti-apoptotic molecules such as Bcl-xL were determined using quantitative real-time PCR (qRT-PCR) and Western blotting.

Results: Our study demonstrated that the platelet mitochondrial membrane depolarization in chronic ITP patients tended to be higher than in healthy controls. Additionally, the proportion of platelets with surface-exposed PS in chronic ITP was significantly higher than that of controls. The results showed that the expression levels of Bak and Bax were significantly higher in chronic ITP patients than in healthy controls; Bcl-xL expression levels were significantly decreased in the platelets of chronic ITP patients compared to healthy controls.

Discussion and conclusion: Study indicates that the enhancement of platelet apoptosis observed in patients with chronic ITP may be one of the pathogenic mechanisms of chronic ITP.

KEYWORDS

Platelet; immune thrombocytopenia; apoptosis; thrombocytopenia

Introduction

Immune thrombocytopenia (ITP) is an acquired and heterogeneous autoimmune-mediated haematological disease typically characterized by a low platelet count [1]. The natural history of ITP in children differs significantly from that in adults. Children typically will have the acute form of disease that is self-limited and resolves completely within weeks or months with or without therapy. In contrast, ITP in adults typically has a chronic course (persistent thrombocytopenia with disease durations longer than 12 months), and many will require some form of ongoing therapy. Currently, the precise mechanism of thrombocytopenia remains unclear; however, it has been associated with apoptosis [2].

Apoptosis, a programed process of cell death, is an important process whereby cells are intentionally marked for clearance from the body and is the main mechanism that regulates the life span of the cell and the elimination of damaged or infected nucleated cells [3]. Although platelets are anucleate, previous studies have shown that they contain the necessary components and the intrinsic program for apoptosis, which controls their survival and dictates their lifespan [4,5].

In recent years, it has become increasingly apparent that apoptotic-like events occur in platelets and are linked to the production of platelets and their subsequent life span. Moreover, studies also suggest that antiplatelet antibodies that induce thrombocytopenia in murine models are related to increases in apoptotic markers in platelets [6]. Winkler et al. demonstrated an enhancement of apoptotic processes in the platelets of pediatric patients with acute ITP, which was ameliorated by intravenous administration of immunoglobulin. These findings suggest that apoptosis might also contribute to the pathogenesis of ITP [7]. However, it remains unclear whether platelet apoptosis is involved in the pathogenesis of chronic ITP. In the current...
study, we extensively examined whether apoptosis contributed to the decreased platelet count observed in patients with ITP. Specifically, we sought to determine the expression of apoptosis markers, including PS exposure, ΔΨm and the protein expression of the Bcl-2 family, in ITP platelets to better define the role of apoptotic pathways in thrombocytopenia. Research on the role of platelet apoptosis will provide important insight into the pathogenesis of ITP, the etiopathogenesis of which has not yet been clarified.

Materials and methods

Patients and controls

A total of 40 patients with chronic ITP (22 females and 18 males; median age, 35 years, ranging from 18 to 60 years) and 40 age- and sex-matched healthy donors were recruited from the First Affiliated Hospital of Soochow University after obtaining informed parental consent from subjects. The patients were diagnosed with chronic ITP, which was defined as isolated thrombocytopenia (PLT count below 100 × 10^9/l) lasting more than 12 months in the absence of any underlying cause of thrombocytopenia. The patients had not received glucocorticoids or immunosuppressive treatment in the past 2 weeks.

Their median platelet count at diagnosis was 14 × 10^9/l (range, 3–48 × 10^9/l). None of the subjects (neither patients nor controls) had diabetes, hypertension, cardiovascular disease, pregnancy, active infection or any other autoimmune diseases aside from ITP, as these factors may affect the rate of apoptosis. This study was approved by the hospital ethics committee.

Samples

Peripheral blood was collected from ITP patients and healthy controls with EDTA-anticoagulated vacuum tubes. Platelet-rich-plasma (PRP) was separated by centrifugation of the blood for 10 minutes at 200 g, and platelets were pelleted by centrifugation for 15 minutes at 600 g, followed by resuspension in HEPES-buffer saline (HBS: 10 mM HEPES, 150 mM NaCl, 5 mM KCl, 1 mM MgSO4, pH 7.4). The platelet count was measured using an automated blood counter (Sysmex KX2100, Sysmex Corporation, Hyogo, Japan). Anti-CD45 coupled to magnetic microbeads was used to remove leukocytes according to the manufacturer’s recommendations. Generally, leukocyte contamination in the final purified platelets was approximately 0.01%, which was negligible.

RNA isolation

The total platelet RNA was extracted using a total RNA isolation kit (Beijing CoWinBioscience Co., Ltd, Beijing, China) according to the manufacturer’s protocol. The RNA concentration was determined using a Nanodrop spectrophotometer (ND1000; Saveen & Werner, Limhamn, Sweden). The sample was kept at −70°C for further analysis.

Quantitative real-time polymerase chain reaction

qRT-PCR assay was performed to measure the mRNA expression of Bak, Bax and Bcl-xL using the UltraSYBR One Step RT-qPCR Kit (Beijing CoWin Bioscience Co., Ltd). 5s rRNA was quantified to be used as a control for the normalization of differences in total RNA levels. The preamplification protocol was as follows: 95°C for 10 minutes, 95°C for 15 seconds, 60°C for 1 minute, 40 cycles. The relative quantity of gene expression was obtained by comparing with the relative expression of 5s rRNA using the 2-ΔΔCt method. The melting curve analysis was utilized to test the specificity and quality of the qRT-PCR amplification. The data were processed using StepOne™ software v2.2.2 (Applied Biosystems). The primers were synthesized using the sequences listed in Table 1.

Flow cytometry

For the analysis of ΔΨm depolarization, flow cytometry was carried out using the Mitochondrial Membrane Potential Detection JC-1 kit (BD Biosciences, San Diego, CA, USA) according to the manufacturer’s instructions. ΔΨm depolarization was quantified as the percentage of depolarized cells. PS externalization was assessed with flow cytometry using the FITC Annexin V Apoptosis Detection kit (BD Biosciences, San Diego, CA, USA) according to the manufacturer’s recommendations.

Western blotting

Platelet proteins were separated on 12% BisTrisNuPage gels (Invitrogen, Basel, Switzerland) under reducing conditions, transferred to nitrocellulose membranes (Bio-Rad Laboratories AG, Reinach, Switzerland) and immunoblotted with the following Abs: Bcl-xL (Rabbit monoclonal [E18] to Bcl-XL,ab32370), Bak (Rabbit monoclonal [Y164] to Bak, ab32371), Bax (Rabbit monoclonal [E63] to Bax, ab32503) and β-actin (Rabbit polyclonal to beta Actin, ab8227) (Abcam, Cambridge, MA, USA), followed by incubation with secondary HRP-conjugated Abs. Enhanced chemiluminescence was used for detection.

Statistical methods

Data were presented as the mean ± standard deviation (SD). GraphPadPrism 6 statistical software (GraphPad Software, La Jolla, CA, USA) was used for all statistical
analyses. Comparisons between patients with ITP and controls were assessed using Student’s t-test. Given the non-Gaussian distribution of the results, the non-parametric Kruskal–Wallis ANOVA was used to compare the data from these groups. For all results <0.05 indicated a significant difference.

Results
To investigate whether the apoptotic process occurs in the platelets of patients with chronic ITP, we examined apoptotic markers, such as PS exposure and \( \Delta \Psi_m \) in platelets, by flow cytometry. The platelet mitochondrial membrane depolarization of the chronic ITP group (5.62 ± 0.78%), as measured by flow cytometry, tended to be higher than that of healthy controls (3.23 ± 0.32%) (\( p < 0.0001 \)) (Figure 1(b)). Additionally, the proportion of platelets with surface-exposed PS in the chronic ITP group (9.75 ± 1.63%) was significantly higher (\( p < 0.0001 \)) than that of healthy controls (4.32 ± 0.15%). Platelet proportions were assessed using flow cytometry annexin V-staining (Figure 1(a)).

In the present study, the mRNA expression levels of Bcl-xL in platelets from chronic ITP patients (0.68 ± 0.03) were significantly lower when compared to the healthy controls (0.93 ± 0.13, \( p < 0.0001 \)) (Figure 2(b)). The protein expression levels of Bcl-xL were also lower in platelets from chronic ITP patients than in controls; however, this difference was not significant (Figure 3(d)). In addition, the results showed that the mRNA expression levels of Bak and Bax were found to be significantly higher (\( p < 0.0001 \)) in ITP patients (1.42 ± 0.12 and 1.83 ± 0.05, respectively) when compared to those in healthy controls (0.95 ± 0.07 and 1.02 ± 0.06, respectively) (Figure 2(a) and (c)). As seen in Figure 3(b) and (c), the protein expression levels of Bak and Bax were not significantly different between the ITP and control groups. Moreover, the ratio of Bcl-xL to Bak and Bcl-xL to Bax appeared to be decreased in ITP patient platelets when compared to control platelets (Figure 3(e) and (f)).

Discussion
To study whether decreased platelets from chronic ITP patients are associated with platelet apoptosis, we assessed apoptotic markers in the platelets of chronic ITP patients. Furthermore, we compared these results to those from healthy controls. We demonstrated that the expression of apoptotic markers, including PS exposure and mitochondrial depolarization, were increased in the platelets of primary ITP patients. Furthermore, research regarding the expression levels of the Bcl-2 family demonstrated that platelets isolated from adult patients with chronic ITP had significantly decreased levels of Bcl-xL and significantly higher levels of Bak and Bax than those in healthy controls.

Platelets are small anucleate cells derived from bone marrow megakaryocyte precursors that play an essential role in proper hemostasis and thrombosis [8]. Megakaryocytes shed platelets into the blood stream where, in humans, they circulate for approximately 7–10 days. At the end of their life span, circulating platelets are degraded by macrophages, primarily in the liver and spleen. The regulation of platelet survival, which is relatively short once in circulation, remains poorly understood. The process of platelet activation and senescence in different models have been associated with processes that resemble programmed cell death, such as depolarization of mitochondrial membrane potentials, microparticle formation, caspase activation, PS exposure on the platelet surface and Bcl-2 family protein expression [9–11]. The process of apoptosis can be viewed as a sequential and orderly series of cellular events initiated by the accumulation of a death signal, the release of apoptotic factors from the mitochondria, and the activation of initiator/effector caspases, ultimately leading to cell death [12].

The main purpose of this study was to evaluate whether platelet apoptosis is involved in the pathogenesis of ITP. Based on previous findings, it is conceivable that the observed apoptotic events in the platelets of ITP patients might contribute to the thrombocytopenia.

Apoptosis is characterized by a variety of morphological features. One of the most important phenomena is the translocation of the membrane PS from the inner to the outer leaflet of the platelet membrane. This is recognized as a signature event that indicates that cells have entered into the early-to-middle stages of apoptosis [13]. Externalization of PS onto the surface of cells undergoing apoptosis represents the most universal and best characterized ‘eat me’ signal, leads to subsequent clearance of the apoptotic cells by phagocytes and causes or aggravates prevailing thrombocytopenia [13,14]. Annexin V, a calcium and phospholipid binding protein, has a high binding affinity for PS and is therefore used as a measure of the translocation of PS to the outer leaflet [15]. Our data demonstrated unequivocally that the surface-exposed PS levels in

| Gene | Primer (5′→3′) | Gene | Primer (5′→3′) |
|------|---------------|------|---------------|
| SSrRNA-F | TACGCCCACCAACCCTGGA | SSrRNA-R | TAACCCACCCCGACCCCTGCT |
| Bak-F | TGATGTTCCAACGAGATCA | Bak-R | AGTCGACCCATGCTGGTAGAC |
| Bak-R | AGGCCCACGTTATGGA | Bak-R | TGGATGAAACCCTGAAGCAA |
| Bcl-xL-F | TACCTGATGACACCTA | Bcl-xL-R | ATTCGCGACTGAAGAGTGA |
| 5SRNA-F | TACGGCCATACCACCCTGGA | 5SRNA-R | TAACCACCCCGACCCCTGCT |
| Bax-F | GACCACCGTTGATGGA | Bax-R | TGGATGAAACCCTGAAGCAA |
| Bax-R | AGTCGACCCATGCTGGTAGAC | Bax-R | TGGATGAAACCCTGAAGCAA |
platelets from chronic ITP patients were significantly higher when compared to the levels measured in healthy donor platelets. Similarly, enhanced PS exposure has been reported in the platelets of adult patients with chronic ITP and pediatric patients with acute ITP [7,16].

Differences in the distribution of ions on the outer and inner mitochondrial membranes give rise to membrane potentials and are a hallmark of mitochondrial inner membrane permeabilization. In addition, ΔΨm plays a crucial role in oxidative ATP generation and reflects the functional status of mitochondria. The reduction in ΔΨm has been linked to the permeability of the transitional pore, inordinate mitochondrial Ca2+ uptake and cytochrome c release from the mitochondria, which activates the key effector caspases and is recognized as an early sign of mitochondrial-mediated apoptosis [10,17]. Our experiments revealed a significantly higher number of depolarized platelets and significantly lower mitochondrial membrane potentials in chronic ITP patients when compared to healthy controls. These results suggest that the mitochondrial dysfunction of platelets gives rise to the chronic ITP condition.

Apoptosis, the physiologically most common form of programmed cell death, plays a fundamental role in development, tissue homeostasis and the pathology of a variety of diseases. Experimental evidence has shown that although platelets are anucleate, they contain at least some of the machinery necessary for apoptosis, for example, mitochondrial DNA and associated mRNAs. There are two distinct and convergent

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**Figure 1.** Platelet apoptosis in ITP (n = 40) vs. Control groups (n = 40). (a) Analysis of platelet phosphatidylserine (PS) exposure using the FITC Annexin V Apoptosis Detection kit; (b) analysis of mitochondrial depolarization using the JC-1 assay kit.

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**Figure 2.** The expression levels of Bcl-2 family mRNA in ITP (n = 40) vs. Control group platelets (n = 40). Relative mRNA expression levels of Bcl-xL, Bak, and Bax were measured using quantitative real-time PCR.
pathways for apoptosis: the extrinsic and the intrinsic (or mitochondrial) pathways. The intrinsic pathway is governed by the BCL-2 family of proteins, which consists of both anti-apoptotic (Bcl-2, Bcl-xL, and Mcl-1) and pro-apoptotic (Bax and Bak) members. Moreover, studies also suggest that platelets absolutely depend on the pro-survival protein Bcl-xL, which functions to restrain the activity of pro-apoptotic Bak and Bax [18,19]. Thus, the platelet life span is governed by the interplay between Bcl-2 family proteins. Furthermore, research on the mutations of Bcl-xL in mice that cause dose-dependent reductions in platelet life span further support that Bcl-xL is the critical regulator of platelet survival [5,19,20]. In conclusion, the results of our studies demonstrated that the expression levels of BCL-xL in platelets from chronic ITP patients were significantly lower than those in controls, while the expression of Bax and Bak were found to be significantly higher. These results further support the claim that the clearance of platelets from circulation via apoptosis may be one of the primary mechanisms of thrombocytopenia. By incubating healthy platelets with plasma from active ITP patients to mimic the in vivo environment, Qiao et al. demonstrated that the unbalanced expression of Bcl-xL and Bax might be the main factor that initiates platelet apoptosis during the development of ITP [21].

It is increasingly clear that, like nucleate cells, platelet apoptosis induced by physiological or chemical compounds or platelet storage occurs in a number of

Figure 3. Expression levels of Bcl-2 family proteins in ITP (n = 40) vs. Control group platelets (n = 40). (a) The pro-apoptotic proteins Bak and Bax, and the anti-apoptotic protein Bcl-xL were measured in platelets using Western blotting. (b) Differences in Bak levels were not significant between the ITP and Control groups. (c) Differences in Bax levels were not significant between the ITP and Control groups. (d) Bcl-xL expression was lower in the platelets of ITP patients when compared to controls; however, this difference was not significant. (e) The ratio of Bcl-xL to Bak appeared to be decreased in ITP patient platelets when compared to controls. (f) The Bcl-xL to Bax ratio appeared to be decreased in ITP patient platelets when compared to controls.
in vitro and in vivo models [22–24]. Until now, most of the platelet apoptotic events have appeared to arise from the mitochondrial pathway. Emerging evidence over the past several years suggests that macrophage-mediated clearance of Ab-coated platelets or cell-mediated cytotoxic lysis of platelets might contribute to decreases in the number of platelets in ITP patients [25,26]. Moreover, other studies suggest that genetic defects or environmental disturbances of the regulated pathways are linked to peripheral platelet destruction [27,28]. Thrombocytopenia occurs when the rate of platelet destruction exceeds the pace of production of newly formed platelets.

Our data revealed that platelet apoptosis was increased in patients with chronic ITP when compared to the control group. Therefore, we assume that the observed apoptotic events might play a role in the pathogenesis of thrombocytopenia in chronic ITP. The results presented in our study were similar to those from previous research. Piguet and Vesin demonstrated that platelet apoptosis may be involved in the development of ITP, which can be induced by pro-apoptotic agonistic antibodies and prevented by apoptosis inhibitors and anti-apoptotic antibodies [2]. Leytin et al. demonstrated that the MWReg30 anti-GPIIb antibody directly triggered platelet apoptosis, suggesting an involvement of GPIIbIIIa integrin in pro-apoptotic signaling in platelets and an association of thrombocytopenia in murine ITP models with platelet apoptosis [6]. Catani et al. reported that platelets isolated from adult patients with chronic ITP had a significantly higher level of PS exposure than platelets from healthy adult volunteers [16]. Stephan et al. suggested that genetic or induced dysregulation of these programmed cell death pathways may lead to thrombocytopenia or ineffective thrombopoiesis in ITP [29].

It should be noted that platelet apoptosis was more heterogeneous than previously understood. Although the proportion of platelets with increased apoptosis markers was higher in ITP, this was not the case in all ITP patients, even when their platelet count was greatly decreased. Some ITP patients exhibited decreased or unchanged expression during platelet apoptosis. The possibility should be considered that the remaining platelets in circulation selected for analysis were apoptotic resistant platelets because the majority of non-resistant platelets were destroyed in chronic ITP. Jiaan-Der et al. reported that the percentage of platelets with positive apoptosis markers was not increased in pediatric patients with chronic ITP when compared to controls. This may be related to platelet apoptosis resistance [30]. Furthermore, in some chronic ITP patients, peripheral tolerance may be induced by preventing platelet apoptosis. Another cause of decreased platelet apoptosis in patients with chronic ITP may be altered levels of anti-apoptotic or apoptotic molecules caused by some hematologic disorders, however the exact mechanism remains unclear. In summary, our data revealed that platelet apoptosis was increased in patients with chronic ITP when compared to the control group. Therefore, we assume that the observed apoptotic events might play a role in the pathogenesis of thrombocytopenia in chronic ITP.

We found that these apoptotic responses were triggered in human platelets, beginning with an early mitochondrial ΔΨm depolarization and ultimately culminating in terminal biochemical and morphological alterations, such as exposure to PS. However, the exact mechanisms associated with thrombocytopenia in chronic ITP patients should be further investigated. More direct evidence is still needed to clarify the link between platelet apoptosis and thrombocytopenia. Better insight into these mechanisms might lead to novel therapeutic approaches and better patient selection for the treatment of ITP.

Disclosure statement
No potential conflict of interest was reported by the authors.

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