The Mechanism of Mitochondria-Mediated Pathway in the Apoptosis of Platelets in Immune-Induced Bone Marrow Failure

Qin Zheng, Yiling Jiang, Aiping Zhang, Lele Cui, Lemin Xia, and Meihong Luo

Department of Hematology, Shanghai Baoshan Hospital of Integrated Traditional Chinese and Western Medicine (Baoshan Branch of Shuguang Hospital Affiliated to Shanghai University of Traditional Chinese Medicine), Shanghai 201999, People’s Republic of China

Abstract

Excessive platelet apoptosis is one of the pathogenic causes of immune-induced bone marrow failure (BMF). The aim of the present study was to explore the role of mitochondria-mediated pathway in the apoptosis of platelets in immune-induced BMF. An immune-induced BMF model was established in mice, which were randomly divided into three groups: normal control (CTL) group, BMF group and cyclosporine (CSA) group (n = 10 in each group). Mice were given 0.027 g/kg CSA daily in the CSA group. Platelet count (PLT), mitochondrial transmembrane potential (ΔΨm), cytochrome C (CytC), phosphatidylserine (PS), calcium ion (Ca2+) and expression of proteins of the mitochondrial apoptotic pathway, including Bak, Bax, caspase-3, caspase-8 and caspase-9, was examined and compared. Compared with the CTL group, the BMF group had significantly a lower level of PLC and ΔΨm, but higher levels of CytC, PS, Ca2+ and higher expression levels of Bak, Bax, cleaved caspase-9 and cleaved caspase-3 (P < 0.05). CSA restored the above changes in the BMF model (P < 0.05). Further studies showed that intravenous injection of the caspase-9 inhibitor Z-LE(OMe)HD(OMe)-fluoromethylketone (FMK) into the mice could significantly inhibit apoptosis of the platelets and the effect of CSA treatment when compared to the BMF group, and exerted a better protective effect from apoptosis if the caspase-9 inhibitor was combined with the CSA treatment. These results revealed that platelet apoptosis may play an important role in the reduction of platelet of immune-induced BMF probably through the mitochondrial pathway.

Key Words: apoptosis, cyclosporine, mitochondrial pathway

Introduction

Bone marrow failure (BMF) is a disease characterized by reduced capability in blood cell regeneration (15), especially regeneration of the platelets, which is life threatening. Over the past decade, it has been recognized that apoptosis also occurs in anucleated cytoplasts and platelets (1, 4, 6, 9-12, 14, 16, 17, 19, 20, 25). Excessive platelet apoptosis is one of the pathogenic causes for immune-induced BMF. Mitochondrial apoptotic pathway is one of the important pathways modulating platelet apoptosis. The mechanisms include early manifestation of reduction in mitochondrial transmembrane potential (ΔΨm) and the formation of mitochondrial perme-
Mitochondria-mediated Pathway in Platelet Apoptosis in BMF

339

ability transition pore (MPTP), followed by multiple transportation of mitochondrial proteins to the cytoplasm to exert pro-apoptotic effects. Pro-apoptotic proteins are translocated to the mitochondria and cytochrome C (CytC) is released from the mitochondrial intermembranous space to the cytoplasm. In the platelet, cysteinyl aspartate specific proteinase (caspase)-8, caspase-9 and caspase-3 are activated subsequently and the cytoskeleton is split. The membrane phosphatidylserine (PS) is then exposed extracellularly and the platelet then undergoes shrinking, dropping off microparticles and apoptosis (7). The commonly used medication for thrombocytopenia is cyclosporine (CSA), which exerts its effect through inhibiting apoptosis via the mitochondrial pathway (8). Our previous pilot study found that the mitochondrial pathway-induced platelet apoptosis could lead to abnormal blood coagulation and hemorrhage (22).

It remained unknown whether thrombocytopenia in BMF is related to the mitochondrial apoptotic pathway. Therefore, we aimed in this study to explore the role of the mitochondria-mediated pathway in the apoptosis of platelets in immune-induced BMF.

Material and Methods

Animal

C57BL/6 mice (male and female in half) aged between 8 and 12 weeks were purchased from Shanghai Slac Laboratory Animal Corporation (Shanghai, China). Mice were housed in the animal center of the company with free access to water and food. The animal experiment approval number was SCXK 2012-0002 (Baoshan Branch of Shuguang Hospital Affiliated to Shanghai University of Traditional Chinese Medicine).

CSA Preparation

CSA (S0408, 25 mg × 50 tablets) (Novartis Pharma, Freiburg Area, Germany) was made into 4 mg/ml solution with sterile saline. Lavage solution was then diluted to 0.1 ml per 10 grams of mouse bodyweight.

BMF Animal Model

The BMF animal model was established following the method of Liu et al. (13). Briefly, thymus glands were dissected from DBA/2 mice sacrificed by cervical dislocation, filtered through nylon filters and passed through a size 4 syringe needle to form single-cell suspension. Trypan blue staining was used to assess cell viability and the cell number was determined. A total of 1 × 107/0.2 ml thymic lymphocytes were then administered through the tail vein of a C57BL/6 mouse 4 h after exposure to 5.5 Gy 60Co-γ radiation at a rate of 1.1 Gy/min for 5 min. Three days after the modeling, peripheral blood was drawn from the tail vein of the mouse and analyzed by an automatic blood cell analyzer. When the mouse showed pancytopenia, the model was considered successful.

Study Design

Thirty C57BL/6 mice were randomly assigned to three groups with 10 mice in each group: normal control (CTL) group, BMF control group and CSA group. The CTL mice were healthy mice without the BMF modeling. Mice in the BMF control group were exposed to radiation and cell transfusion, and had no treatment with CSA. The CSA group mice received daily lavage with 0.027 g/kg (0.1 ml/10 g) of CSA. The normal and BMF control mice received daily lavage with the same volume of saline. All lavage intervention continued for 3 consecutive days.

Caspase-9 Inhibitor Rescue Assay

In order to rule out the possibility of the assaulting effects of CSA on the transplanted lymphocytes, and to elucidate the intrinsic mitochondrial pathway-dependent apoptosis inhibitory effects of CSA on the platelets, a caspase-9 inhibitor rescue assay was conducted. The BMF mice were divided into four groups with 8 mice in each group: BMF group, BMF+CSA group, BMF+caspase-9 inhibitor group and BMF+CSA+caspase-9 inhibitor group. Caspase-9 inhibitor rescue was conducted by intravenous injection of the caspase-9 inhibitor (Z-LE(OMe)HD(OMe)-fluoromethylketone (FMK) at a dose of 10 μg/g bodyweight immediately after thymic lymphocytes transplantation. The platelets were analyzed after 3 days.

Platelet Count (PLT) and Preparation for Washed Platelets

After 3 days of lavage, 10% chloral hydrate at 0.005 ml/g bodyweight was intra-abdominally administered for anesthesia. Blood was then drawn from the tail vein for a complete blood count using an autoanalyzer (Sysmex XE-2100, Sysmex Co., Ltd., Kobe, Japan). Platelet-rich plasma (PRP) was obtained by combining 0.7 ml whole blood with 7 ml acid citrate dextrose (ACD) (2.5% sodium citrate, 2.0% glucose, and 1.5% citric acid) and centrifuged at 1,300 rpm for 20 min. Modified Tyrode’s buffer (pH 7.4; 2.5 mM Heps, 150 mM sodium chloride, 2.5 mM potassium chloride, 1 mM calcium chloride, 1 mM magnesium chloride, 12 mM sodium bicarbonate...
and 5.5 mM glucose) was used for re-suspension of the PRP. The washed-platelet suspension was then obtained and a cell counter chamber was used for platelet counting. The washed-platelet suspension was then diluted to a concentration of $3 \times 10^8$ cells/ml and rested at room temperature for 60 min. Blood tests were examined in an automated blood cell analyzer (Sysmex Co., Ltd.).

Flowcytometry Assay

Washed platelets were respectively incubated with tetramethylrhodamine ethyl ester (TMRE), CytC (Becton, Dickinson Co., Franklin Lakes, NJ, USA) and fluorescein isothiocyanate (FITC)-conjugated annexin V (Bender Medsystem Co., Vienna, Austria) at 37°C for 20 min or the indicated time according to a previously described method (19). The cells were then examined by flowcytometry (CYTOMICS FC 500, Beckman Coulter Co., Ltd.).

Western Blotting

Total protein extracts were prepared from the washed platelets with radioimmunoprecipitation assay (RIPA) lysis buffer (Beyotime Institute of Biotechnology, Shanghai, China) in the presence of 1 mM phenylmethanesulfonylfluoride and 0.1 to 2.0 mM sodium orthovanadate (Beyotime Institute of Biotechnology). Total protein extracts were separated in a 10% sodium dodecyl sulfate (SDS)-polyacrylamide gel and blotted onto nitrocellulose membranes (Bio-Rad, Inc, Hercules, CA, USA). The membranes were immunoblotted with antibodies specific to the following proteins: Bak, Bax, caspase-3, caspase-8 and caspase-9, and the β-actin control (Santa Cruz Co, Dallas, TX, USA). Anti-mouse IgG (Santa Cruz Co) was used as the secondary antibodies. β-actin was used as a protein loading control. A BandScan software was used for analyzing the signal intensity of each protein band and for calculating the relative expression level.

Histological Observation

Tissue samples of the left lower limb tibia from the mice were fixed in 2.5% glutaraldehyde followed by 1% osmic acid, dehydrated with epoxy resin, and then made into ultrathin sections for staining with uranyl acetate and lead citrate. Pathological changes were observed under a transmission electron microscope. Hematoxylin and eosin (H&E) staining was also performed to examination the pathological changes of bone marrow biopsies derived from three groups of mice.

Statistical Analysis

All data are presented as mean ± standard deviation (SD). Data analysis was performed by SPSS 19.0 software (SPSS Inc., Chicago, IL, USA). The differences among the four groups were tested using analysis of variance (ANOVA) with Student-Newman-Keuls (SNK)-q test. $P < 0.05$ was considered as statistically significant.

Results

The Establishment of BMF Model

To prove if the model of BMF was established, hemoglobin (Hb), white blood cell count (WBC), platelet count (PLT) and bone marrow mononuclear cells (BMMNCs) were measured. Compared to the CTL group, the Hb, WBC, PLT and BMMNC values decreased significantly in the BMF group (Table 1 and Fig. 1A). In addition, the pyknotic, irregular platelet epicyte and the empty, big bubble cytoplasm were shown under electron microscope in the BMF group (Fig. 1B), which showed that the BMF model

<table>
<thead>
<tr>
<th></th>
<th>CTL</th>
<th>BMF</th>
<th>BMF+CSA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hb (g/L)</td>
<td>156.60 ± 5.21</td>
<td>115.30 ± 11.75</td>
<td>151.50 ± 6.04</td>
</tr>
<tr>
<td>WBC ($10^9$/L)</td>
<td>10.11 ± 1.31</td>
<td>0.19 ± 0.05</td>
<td>3.99 ± 1.55</td>
</tr>
<tr>
<td>PLT ($10^9$/L)</td>
<td>614.20 ± 53.90</td>
<td>352.40 ± 53.56</td>
<td>580.30 ± 88.86</td>
</tr>
<tr>
<td>BMMNCs ($10^6$/femur)</td>
<td>5.43 ± 0.61</td>
<td>0.76 ± 0.15</td>
<td>2.51 ± 0.23</td>
</tr>
</tbody>
</table>

CTL, normal control group; BMF, bone marrow failure group; CSA, treatment with cyclosporine; Hb, hemoglobin; WBC, white blood cell count; PLT, platelet count; BMMNCs, bone marrow mononuclear cells. In each group, n = 10.
Mitochondria-mediated Pathway in Platelet Apoptosis in BMF

341

had been successfully established. CSA could restore the PLT and microstructure in the BMF model (Fig. 1B & 1C), possibly an indication of curing thrombocytopenia. The HE staining of the bone marrow biopsy showed that the integrity of the hematopoietic tissue was maintained, and the hematopoietic cells were distributed uniformly in the bone marrow of the CTL mice (Fig. 2A). The volume of the hematopoietic tissue and the number of hematopoietic cells were significantly reduced in the BMF mice compared to the CTL group (Fig. 2B), but were significantly higher in the BMF+CSA group than in the BMF mice (Fig. 2C). The results, thus, demonstrated that the model of BMF associated with immunity was successfully established, and that CSA could alleviate the severity of BMF.

Apoptosis of Platelets Plays an Important Role in BMF

To show whether apoptosis of platelets plays an important role in BMF, the ΔΨm and biochemical indices were examined by flowcytometry. Compared to the CTL group, ΔΨm was significantly decreased in the BMF group, which showed the initiation of platelet apoptosis in the BMF model (Fig. 3I). Levels of biochemical indices of CytC, PS and Ca²⁺ were significantly higher in the BMF group than in the CTL group (Figs. 3I-III), which showed that apoptosis of platelets might induce thrombocytopenia in the BMF model. CSA which could alleviate thrombocytopenia through inhibiting apoptosis to restore changes of the ΔΨm and biochemical indices in the BMF model.

Pro-apoptotic Protein Expression in Mitochondrial Apoptotic Pathway

To demonstrate that the mitochondrial apoptotic pathway may play a key role in BMF in association with immunity, expression of key pro-apoptotic proteins in the mitochondrial apoptotic pathway was examined by western blotting (Fig. 3IV). Compared to the CTL group, the BMF mice had significantly higher expression levels of Bak, Bax, cleaved caspase-9 and cleaved caspase-3. CSA could significantly reduce the expression of these four proteins compared to the BMF group.

Inhibiting Caspase-9 Activity Could Reduce the Apoptosis of Platelets

Activation of caspase-9 is essential for triggering the mitochondrial pathway-dependent apoptosis. Therefore, we explored whether inhibiting the activation of caspase-9 could reduce the apoptosis of platelets. Flow cytometric results showed that intravenous injection of the caspase-9 inhibitor Z-LE(OMe)HD(OMe)-FMK into the mice could significantly inhibit the apoptosis of platelets as well as the effect of CSA treatment when compared to the BMF group (Fig. 4). Thus, a better protective effect from apoptosis could be achieved if the use of the caspase-9 inhibitor was combined with CSA treatment (Fig. 5).

Discussion

Thrombocytopenia in BMF is associated with an increased risk of hemorrhage, and is a thrombocytopenia include transfusion of platelets and γ-globin and glucocorticoid therapy. However,
blood transfusion, commonly used in patients with severe thrombocytopenia, is limited to blood supply. More importantly, blood transfusion can induce platelet antibodies, which hamper treatment efficacy (3). γ-Globin transfusion is expensive and glucocorticoid therapy has multiple side effects. Therefore, searching for a new therapeutic approach is of high importance. Abnormality in platelet number and function is the leading cause for platelet-associated diseases (21). Recent researches have shown that platelet apoptosis is regulated mainly through endogenous pathways (24). Previous studies also indicated that mice with immune-induced BMF had peripheral cytopenias, including thrombocytopenia, accompanied with reduction in the expression of apoptotic proteins such as caspase-8 and caspase-3, suggesting that the etiology of thrombocytopenia in BMF was related to apoptosis modulation (2, 18). Current researches have drawn much attention targeting at platelet apoptosis pathways in the hope of developing new drug-invention protocols (5).

The present study showed that the BMF mice had significantly lower levels of platelet count and ΔΨm, but higher levels of CytC, PS and Ca^{2+}, and higher expression levels of Bak, Bax, cleaved caspase-9 and cleaved caspase-3 compared to the CTL group. We also demonstrated that the therapeutic effect of CSA in treating immune-induced BMF was through inhibiting platelet apoptosis via the mitochondrial pathway. CSA treatment restored the ΔΨm level, reduced the translocation of pro-apoptotic proteins, hampered the release of CytC from the mitochondrial intramembrane space to the cytoplasm and the lysis of cytoskeleton, and finally, inhibited platelet apoptosis.

Fig. 3. ΔΨm, CytC, PS and Ca^{2+} was examined by flowcytometry. (I-IV) (A) CTL group; (B) BMF group; (C) BMF+CSA group; (D) Quantification of the data. *P < 0.05, compared with the CTL group; #P < 0.05, compared with the BMF group.
Intravenous injection of Z-LE(OMe)HD(OMe)-FMK into the mice could significantly inhibit the platelet apoptosis and the effect of CSA treatment when compared to the BMF group. The observation that a much better protective effect from apoptosis was achieved if the caspase-9 inhibitor was used in combination with the CSA treatment suggested that the therapeutic effect of the CSA treatment was achieved through inhibiting the mitochondrial pathway-dependent apoptosis of the platelet. The roles of Fas/FasL (Fas ligand) and perforin/granzyme B pathways in BMF have previously been defined (2). However, our observation showed that the pathogenesis of thrombocytopenia caused by BMF was more than what has been reported.

In conclusion, data of this work indicate that platelet apoptosis may play an important role in the reduction of platelets of immune-induced BMF through the mitochondrial pathway.

Acknowledgments

This work was supported by Research Project of Shanghai Municipal Health and Family Planning Commission (No. 201640144), and Baoshan Health Systems of Young Medical Talent Training Project (No. bswsyq-2016-A11), and National Nature Science Foundation Project of Shanghai Baoshan Hospital of Integrated Traditional Chinese and Western Medicine (No. GZRPYJJ-201601).
Conflict of Interests

The authors declare that they have no conflict of interests related to this work.

References


