Eosinophil Granulocyte and NK Cell-Mediated Platelet Destruction in Immune Thrombocytopenia

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Abstract To investigate the contribution and mechanism of eosinophil granulocytes and NK cells-mediated cytotoxicity to the pathogenesis of ITP. Mononuclear cells and platelets were prepared from the bone marrow of 16 ITP patients and 10 healthy controls. Separately, eosinophil granulocytes and NK cells were selected with magnetic microbeads. As the target cells, the autologous platelets were cultured with eosinophil granulocytes and NK cells respectively for 6 h and then stained with annexin V. Ratio of platelets expressing annexin V was determined by flow cytometry. The fraction of NK cells expressing perforin, granzyme B, FasL and TNF were determined by flow cytometry. Human eosinophil cationic protein (ECP) *level in the bone marrow was determined by ECP ELISA kit*. The annexin V positive platelet ratio of the ITP group was significantly higher than that of the control group; the expression rates of granzyme B, perforin, FasL on NK cells of the ITP group were significantly higher than those of the control group. Expression of human ECP in bone marrow of immune thrombocytopenia patients was higher than that of healthy controls. NK cells and eosinophil granulocytes are activated in ITP and might be involved in the pathogenesis of ITP.

Keywords Immune thrombocytopenia · Eosinophil granulocytes · NK cell

Introduction

Immune thrombocytopenia (ITP) is an acquired disorder characterized by mild to severe thrombocytopenia. Historically ITP-associated thrombocytopenia was attributed solely to sequestration of autoantibody-sensitized platelet in the reticuloendothelial system. However, humoral mechanisms cannot account for all observations made in this disorder, and it is increasingly evident that cellular mechanisms contribute to platelet destruction [1, 2].

We have developed a novel system by using eosinophil granulocytes and NK cells to study the cytotoxicity of specific cell towards patient's autologous platelets.

Materials and Methods

Patients

Bone marrow was collected from 16 patients (5 males and 11 females) with ITP and 10 healthy donors. The patients were aged between 14 and 66 (mean 33) years. They were all newly diagnosed and met the diagnostic criteria for ITP as evidenced isolated thrombocytopenia, normal or increased number of megakaryocytes in bone marrow biopsies, absence of splenomegaly and exclusion of other known causes of thrombocytopenia (such as connective tissue disease, malignancy, human immunodeficiency virus (HIV) or drug-induced disorders). The platelet counts of all patients were lower than $100 \times 10^9/L$, ranging between 5 and $60 \times 10^9/L$ at the time of bone marrow sampling, and none of the patients received corticosteroids or other immunosuppressive agent therapy before bone marrow sampling. Control bone marrow was obtained from 10 healthy donors with no history of blood transfusion,

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pregnancies and did not take any medications within three months.

Reagents

The CD16 and CD56 magnetic microbeads were obtained from Miltenyi Biotec GmbH (Bergisch Gladbach, Germany). Annexin V-fluorescein isothiocyanate (FITC), Granzyme B-FITC, Perforin-PE, as well as mAbs Fas ligand (FasL)-phycoerythrin (PE), tumor necrosis factor (TNF)- α -FITC were purchased from Beckman Coulter, Inc. Human eosinophil cationic protein (ECP) ELISA kit was purchased from Biotechnology Co., Ltd. Shanghai enzyme research (Shanghai, China). RPMI Medium 1640 and percoll were obtained from Beijing Solarbio Science & Technology Co., Ltd. RPMI Medium 1640 were supplemented with 10% plasma of normal control or ITP patient before using.

Isolation of NK Cells, Eosinophil Granulocytes and Platelets

NK cells, eosinophil granulocytes and platelets were prepared from ethylenediamine tetraacetic acid (EDTA) anticoagulated bone marrow by density centrifugation on Percoll. Eosinophil granulocytes were negatively selected using CD16⁺ magnetic microbeads, and NK cells were selected using CD56⁺ magnetic microbeads according to the manufacturer's recommendations (Miltenyi Biotec GmbH). Flow cytometric analysis revealed that the NK cells fraction contained >91% CD16⁺CD56⁺ cells. The eosinophil granulocytes fraction contained >90% CD16⁺ cells.

Flow Cytometric Analysis

Assay for eosinophil granulocytes and NK cell-mediated cytotoxicity toward platelets was performed with a Beckman Coulter Cytomics FC 500 flow cytometer (Beckman Coulter, USA). The eosinophil granulocytes and NK cells were used as effector cells respectively and autologous platelets were used as target cells. Effector cells and target cells were incubated for 6 h in RPMI 1640 culture medium supplemented with plasma of ITP patient at a ratio of 100 platelets to one effector cell at 37 °C in CO₂ incubator.

Thereafter aliquots of the cell suspension were incubated with FITC-conjugated Annexin V. Logarithmic amplifiers were used for fluorescence signals, and 10,000 events were collected. A platelets gate was set in the forward light scatter and side-scatter dotplot to distinguish platelets from effector cells. The fraction of platelets expressing Annexin V was analyzed using the CXP Analysis software (Beckman Coulter, USA).

Immunofluorescence flow cytometry was also used to measure the molecules involved in NK-mediated cytotoxicity. The cell concentration was adjusted to 10⁹/L. Aliquots of the cell suspension were added with 20 μ L PE or FITC-conjugated mAbs, FasL-PE and TNF- α -FITC, respectively. Appropriate isotype matched control antibodies were used. After incubation for 30 min on ice in the dark, the surface expression in CD16⁺ CD56⁺ cells was evaluated by flow cytometry. Logarithmic amplifiers were used for fluorescence signals and 10,000 events were collected. The fraction of NK cells expressing FasL and TNF- α were determined by setting a quadrant gate in the fluorescence dotplot for NK cells.

The Determination of ECP Concentrations

The ECP level in the bone marrow was analyzed with the ECP ELISA kit strictly following the instructions of the kit.

Statistics

Data were reported as mean $\pm$ SD. Differences between groups were evaluated using the Student's *t* test for analysis of completely randomized two-group designs. *P* values <0.05 were considered statistically significant. Sample size was determined by PASS 11.0 for two-sample *t* test power analysis.

Results

1. Cell-mediated lysis of autologous platelets in ITP

Table 1 depicted the data obtained from the 16 ITP patients and 10 healthy donors and the results were from flow cytometry. When platelets were placed in culture with media supplemented with plasma of ITP alone, the differences of Annexin V positive platelets ratios between the ITP patients and the controls were not statistically significant (3.59 ± 1.08 vs 3.50 ± 0.96). Annexin V-FITC positive platelets in ITP group (11.10 ± 1.75 , 14.69 ± 2.21) in which the NK cells or eosinophil granulocytes were used as effector cells were significantly higher than those of normal control (3.62 ± 0.88 , 3.74 ± 0.78).

2. Expression of the molecules involved in NK-mediated cytotoxicity

The cells of ITP patients (*n* = 16) and normal controls (*n* = 10) were labeled using PE or FITC conjugated anti-FasL, anti-human granzyme B, anti-human perforin and anti-TNF- α mAbs, respectively. Flow cytometry was used to measure these molecules levels in NK cells. As shown in Table 2 expression of FasL, perforin and granzyme B in

Table 1 Comparison of annexin V-fluorescein isothiocyanate labeled platelets between the immune thrombocytopenia (ITP) patients and healthy controls

Group	A (%)	B (%)	C (%)
ITP patients (n = 16)	3.59 ± 1.08	11.10 ± 1.75 [▲]	14.69 ± 2.21 [▲]
Controls (n = 10)	3.50 ± 0.96	3.62 ± 0.88	3.74 ± 0.78

A: platelets were placed in culture with media supplemented with plasma of ITP patient alone. B: NK cells were used as effector cells and autologous platelets as target cells. C: eosinophil granulocytes were used as effector cells and autologous platelets as target cells

[▲] $P < 0.05$ compared with healthy controls

Table 2 Expression of Fas ligand (FasL), tumor necrosis factor (TNF), granzyme B and perforin in NK cells of ITP patients and healthy controls

Group	FasL (%)	TNF- α (%)	Granzyme B (%)	Perforin (%)
ITP patients (n = 16)	50.91 ± 7.89 [▲]	0.20 ± 0.10	25.40 ± 3.19 [▲]	99.00 ± 6.90 [▲]
Controls (n = 10)	10.03 ± 3.15	0.19 ± 0.09	5.90 ± 1.80	10.40 ± 4.67

[▲] $P < 0.05$ compared with healthy controls

Table 3 Expression of ECP in bone marrow of immune thrombocytopenia (ITP) patients and healthy controls

Group	ECP (ng/L)
ITP patients (n = 16)	6985.66 ± 2332.25 [▲]
Controls (n = 10)	1068.36 ± 356.55

[▲] $P < 0.05$ compared with healthy controls

Table 4 The percentage of eosinophils in the bone marrow

Group	The percentage of eosinophils (%)
ITP patients (n = 16)	3.50 ± 0.50 [▲]
Controls (n = 10)	0.50 ± 0.30

[▲] $P < 0.05$ compared with healthy controls

NK cells was remarkably higher in ITP, compared with control group (50.91 ± 7.89, 25.40 ± 3.19, 99.00 ± 6.90 vs 10.03 ± 3.15, 5.90 ± 1.80, 10.40 ± 4.67).

3. The determination of ECP concentrations in the bone marrow

According to the manufacturer's instructions, human eosinophil cationic protein (ECP) kit was performed to assay human ECP level in the bone marrow for the 16 ITP patients and 10 controls. As shown in Table 3, ITP patients had higher levels of ECP expression compared with healthy controls (6985.66 ± 2332.25 vs 1068.36 ± 356.55, $P < 0.05$).

4. The percentage of eosinophils in the bone marrow

As shown in Table 4 the percentage of eosinophils of ITP patients in the bone marrow was higher, compared with control group (3.50 ± 0.50 vs 0.50 ± 0.30).

Discussion

Historically ITP-associated thrombocytopenia was attributed solely to sequestration of autoantibody-sensitized platelet in the reticuloendothelial system. Recently, Olsson et al. [2] reported several cytotoxic genes, such as Apo-I/

Fas, granzyme B and perforin, together with genes involve in the Th1 cell response, such as interferon- γ and interleukin-2 receptor- R, showing increased expression in the ITP patients. With a modified ³In release assay, Heyns Adu et al. [3] noted the destruction of autologous platelet by CD14⁺ and CD19⁺ mononuclear cells. The results provided the first evidence that the cell-mediated toxicity might mediate or participate in the pathogenesis of ITP.

Moreover, many authors have reported associations between the increased numbers of eosinophils with platelet dysfunctions, such as increased bleeding time, reduction in platelet aggregation induced by various agonists, among others disorders [4–6]. Recently, Maziero et al. [7] found that the eosinophil cationic protein (ECP) inhibited the platelet aggregation on platelet rich plasma and washed platelets.

These findings indicate that NK cells and eosinophil granulocytes may contribute to platelet destruction. To test this hypothesis we isolated the NK cells and eosinophil granulocytes from ITP patients and co-cultured them with autologous platelets to investigate whether the NK cells or eosinophil granulocytes contribute to cell-mediated lysis of autologous platelets in vivo. We found that the Annexin V positive platelets were highly increased in ITP patients when NK cells or eosinophil granulocytes were used as effector cells, suggesting that eosinophil granulocytes and

NK cell-mediated cytotoxicity contribute to the destruction of platelets in ITP.

FasL and TNF- α belong to TNF superfamily and may induce apoptosis after binding with their respective receptors, leading to immune attack in many autoimmune diseases [8]. In our study, FasL showed increased expression on the surface of NK cells in ITP Patients. We thought that interaction of upregulated FasL with their respective receptors on the surface of target cells may result in apoptosis of autologous platelets.

Furthermore, it is well known that granzyme and perforin are important functional proteins in cell-mediated toxicity [9, 10]. To test the specific mechanism of platelets destructions in ITP, we examined the granzyme B and perforin protein expressed in NK cells and found that granzyme B and perforin expression rate were high, demonstrating that the NK cells were abnormally activated in ITP patients which in turn directly destroyed the platelets by cytotoxicity, leading to thrombocytopenia. Apoptosis and perforin/granzyme-mediated cytotoxicity are two important pathways through which NK cells destruct autplatelets.

Zhang et al. [1] found that platelets from patients with active ITP displayed in vitro lysis when incubated with CD3⁺CD8⁺T effector cells but not with CD3⁺CD16⁺CD56⁺ natural killer (NK) cells. But our study indicated NK cells were abnormally activated, possibly due to the patient's plasma that we have added to the trial. The plasma contains antibodies directed against platelets, so NK cells can act on PLT by antibody-dependent cell-mediated cytotoxicity (ADCC).

Based on our observation, the percentage of eosinophils of ITP patients in the bone marrow was higher than that of the control group. Besides, our study found *ITP patients had higher levels of ECP* expression in the bone marrow than the healthy controls. ECP is a toxic protein released by activated eosinophils and it is also a specific marker of eosinophil activation. The ECP concentration is related to the number of activated eosinophils, which can directly reflect the number of eosinophils activation [11]. Because eosinophil surface has Fc receptor, we believe that eosinophil can also destroy platelets through ADCC.

Our study only examined the molecular expression related to cytotoxicity. Further studies are needed to identify the expression of their respective receptors so as to confirm and improve our hypothesis. In conclusion, eosinophil and NK-mediated cytotoxicity is an alternative mechanism for platelet destruction in ITP. This finding further enriched the theoretical content of pathogenesis of ITP, and provided a new way for targeted therapy of ITP. Blocking the activation of eosinophil and NK and their cytotoxicity might reduce platelet destruction and thereby

have therapeutic effects. Besides, ECP assay is simple and might be used to monitor the development of ITP and judge prognosis.

Compliance with Ethical Standards

Conflict of interest Wang Shuguo declares that he has no conflict of interest. Liu Fenghai declares that he has no conflict of interest. Zhao Hongguo declares that he has no conflict of interest.

Ethical Standards All procedures performed in studies involving human participants were in accordance with the ethical standards of The Affiliated Qingdao Municipal Hospital of Qingdao University and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Informed Consent Informed consent was obtained from all individual participants included in the study.

References

1. Zhang F, Chu X, Wang L, Zhu Y, Li L, Ma D, Peng J, Hou M (2006) cell-mediated lysis of autologous platelets in chronic idiopathic thrombocytopenic purpura. *Eur J Haematol* 76(5):427–431
2. Olsson B, Andersson PO, Jacobsson S, Carlsson L, Wadenvik H (2005) Disturbed apoptosis of T-cells in patients with active idiopathic thrombocytopenic purpura. *Thromb Haemost* 93(1):139–144
3. Heyns Adu P, Badenhorst PN, Lotter MG, Pieters H, Wessels P, Kotze HF (1986) Platelet turnover and kinetics in immune thrombocytopenic purpura: results with autologous ³In-labeled platelets and homologous 51Cr-labeled platelets differ. *Blood* 67(1):86–92
4. Chin NS, Koong PL (1990) A study of 31 patients with easy bruising from University Hospital, Kuala Lumpur. *Med J Malays* 45(4):325–328
5. Laosombat V, Wongchanchailert M, Sattayasevana B, Kietthubthaw S, Wiriyasateinkul A (2001) Acquired platelet dysfunction with eosinophilia in children in the south of Thailand. *Platelets* 12(1):5–14
6. Lucas GN, Seneviratne TR (1996) A case of acquired platelet dysfunction with eosinophilia. *Ceylon Med J* 41(3):122
7. Maziero AM, Lorenzetti R, Donato JL, Lilla S, De Nucci G (2013) Inhibition of human platelet aggregation by eosinophils. *Life Sci* 93(9–11):416–422
8. Cao JY, Wang H (2004) Role of Fas-FasL in insulinitis in nonobese diabetic mouse. *Chin Med J (Engl)* 117(4):615–617
9. Podack ER, Lowrey DM, Lichtenheld M, Olsen KJ, Aebischer T, Binder D, Rupp F, Hengartner H (1988) Structure, function and expression of murine and human perforin 1 (P1). *Immunol Rev* 103:203–211
10. Shi L, Mai S, Israels S, Browne K, Trapani JA, Greenberg AH (1997) Granzyme B (GraB) autonomously crosses the cell membrane and perforin initiates apoptosis and GraB nuclear localization. *J Exp Med* 185(5):855–866
11. Rasp G, Thomas PA, Bujia J (1994) Eosinophil inflammation of the nasal mucosa in allergic and allergic and non-allergic rhinitis measured by eosinophil cationic protein levels in native nasal fluid and serum. *Clin Exp Allergy* 24(12):1151–1156