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Detection of autoantibodies against platelet glycoproteins in patients with immune thrombocytopenic purpura by flow cytometric immunobead array

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ABSTRACT

Background: The goal of this study is to develop a flow cytometric immunobead array (FCIA) assay to detect platelet autoantibodies commonly present in bleeding patients with immune thrombocytopenic purpura (ITP).
Methods: Polystyrene microbeads coated with antibodies against human platelet glycoproteins (GPs) IX (SZ1), Ib (SZ2), IIIa (SZ21), IIb (SZ22), and P-selectin (SZ51) were incubated with platelet lysate from 50 ITP patients and 86 controls. The platelet antigen–autoantibody complexes were detected by flow cytometry using an FITC-labeled antibody. The results were compared with that of a monoclonal antibody immobilization of platelet antigen (MAIPA) assay.
Results: By FCIA, platelet autoantibodies against GPIb, GPIIb, GPIIIa, GPIX and P-selectin were detected in ITP patients. Mean fluorescent intensity values with antibodies SZ1, SZ2, SZ21, SZ22 and SZ51 were all higher in ITP patients than controls (p values<0.01). In ROC analysis, values of the area under the curve were 0.89, 0.82, 0.93, 0.94 and 0.95, respectively. In ITP diagnosis, the FCIA assay with these five antibodies had better sensitivity and accuracy than the MAIPA assay (96% vs. 44% in sensitivity; 80.9% vs. 64.7% in accuracy, $p<0.01$).
Conclusion: FCIA assays with multiple antibodies against platelet GPs may be used to improve the diagnosis of ITP in hospitals.

1. Introduction

Immune thrombocytopenic purpura (ITP), also called idiopathic thrombocytopenic purpura or immune thrombocytopenia, is a common bleeding disorder characterized by abnormally low platelet counts of unknown cause [1–6]. Studies indicate that autoimmune is a major contributing factor in the pathogenesis of ITP. Autoantibodies against platelet antigens are frequently detected in patients with ITP [7,8]. These autoantibodies often recognize platelet glycoproteins (GP) such as GPIb, GPIIb–IIIa and GP IX that are abundant on the platelet surface [2,9]. The formation of the antigen and autoantibody complex on the platelet surface may promote platelet destruction by macrophage-mediated phagocytosis in the spleen and other organs, which reduces platelet counts in the blood [1–3,5,6]. Autoantibodies or cytotoxic T cells also target megakaryocytes, which may impair megakaryocyte differentiation and maturation in the bone marrow, thereby preventing platelet production [1–3,5,6].

The detection of platelet autoantibodies is an important step in the diagnosis of ITP [5,10,11]. Methods that are commonly used to detect platelet autoantibodies in ITP patients include ELISA-based monoclonal antibody immobilization of platelet antigen (MAIPA) assay and immunobead-based radioimmune assay (RIA) [7,8,12,13]. In general, however, these assays are cumbersome and time-consuming, which may limit their wide use in hospital settings [14].

Flow cytometric immunobead array (FCIA) is a recently developed technique, in which flow cytometry detects antibody-coated polystyrene microbeads that bind to specific antigens [15]. In this technique, each type of microbeads that are coated with a specific antibody has distinguishable fluorescent intensity. As a result, different types of microbeads can be mixed in a single tube to detect multiple antigens simultaneously. As such, the technique reduces sample volumes and assay times compared to more traditional methods such as ELISA and RIA. To date, FCIA-based assays are being developed to measure a variety of proteins in biological samples [15–17].

In this study, we developed an FCIA assay using 5 monoclonal antibodies against human platelet GPs. We showed that the FCIA assay can be used to detect autoantibodies in ITP patients that target platelet GPIb, GPIIb, GPIIIa, GPIX and P-selectin. More importantly, our results showed that the FCIA assay with these monoclonal antibodies improved the sensitivity and accuracy for the diagnosis of ITP.

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2. Materials and methods

2.1. Patient samples

Blood samples were from 50 ITP patients who visited the First Affiliated Hospital of Soochow University in Suzhou, China between October 2011 and March 2012. Among them, 15 were males and 35 females, aged from 4 to 78 y (average 39 y). Their platelet counts were $<100\times10^9$/L. The diagnosis of ITP was based on the guideline by the American Society of Hematology [10]. Control blood samples were from 37 non-ITP patients, whose diagnosis included leukemia (31 cases), anemia (5 cases), and myelodysplastic syndrome (1 case). Additional blood samples were from 49 normal individuals (24 males and 25 females, 22–51 y (average 37 y) who underwent routine health check-ups at the hospital. Blood platelet counts in non-ITP patients and normal controls were all $>100\times10^9$/L. This study was approved by the ethic committee of the hospital. All participants gave written informed consent.

2.2. Isolation of platelets

Venous blood (2 mL) was collected in tubes containing spray-coated EDTA as an anticoagulant (BD Diagnostics, Franklin Lakes, NJ). Blood samples were centrifuged at 200 *g* for 5 min at room temperature. Platelet-rich plasma (PRP) was transferred to new tubes that were centrifuged at $3000\times g$ for 2 min. Platelet pellets were washed twice with a phosphate-buffed saline (PBS) containing 0.05% EDTA. Washed platelets were lysed in a PBS buffer (110 μL) containing 1% Triton X-100. Samples were centrifuged at $3000\times g$ for 20 min and soluble platelet lysate was collected.

2.3. Antibodies and polystyrene beads

Monoclonal antibodies against human platelet GPIX (SZ1), GPIb (SZ2), GPIIb (SZ22), GPIIIa (SZ21) and P-selectin (SZ51) were prepared in our laboratory, as described previously [18–23]. Fluorescein isothiocyanate (FITC)-labeled goat anti-human IgG (FITC-GAH) and anti-mouse IgG (FITC-GAM) polyclonal antibodies were from Beckman-Coulter (Suzhou, China). Polystyrene microbeads (4 μm in diameter with 8 fluorescent intensities) were from Spherotech (Lake Forest, IL).

2.4. Antibody coating

Monoclonal antibodies SZ1, SZ2, SZ21, SZ22 or SZ51 (160 μg each) in a carbonate coating buffer (pH 9.5) were incubated with 1×10^6 microbeads of distinguishable fluorescent intensities on a shaker at 4 °C overnight. Antibody-coated microbeads were washed three times with PBS containing 0.05% Tween-20 and then stored in PBS containing 0.02% sodium azide as a preservative. The stored microbeads were tested with FITC-GAM antibodies on days 1, 11, 21, 31, 41 and 181, respectively, for their stability. In this test, FITC-GAH antibodies were used as a negative control.

2.5. FCIA

Monoclonal antibody-coated microbeads (1×10^4) were added to platelet lysate (150 μL) from ITP patients or control individuals and incubated on a shaker at room temperature for 1 h. Samples were centrifuged at $500\times g$ for 20 min, washed once with PBS, and incubated with an FITC-GAH antibody at room temperature for 30 min. The microbeads were washed once with PBS, suspended in 0.5 mL of PBS, and analyzed by flow cytometry using an FITC-GAH antibody (Cytomics FC-500, Beckman-Coulter). Data of fluorescent intensity from 1500–2000 microbeads were analyzed by the CXP software (Beckman–Coulter) to calculate mean fluorescent intensity (MFI) values for individual samples.

2.6. MAIPA

ELISA-based MAIPA assay was performed according to published methods [24]. Five monoclonal antibodies against human platelet GPs (SZ1, SZ2, SZ21, SZ22 and SZ51) were tested individually with samples from ITP patients and non-ITP and normal controls.

2.7. Statistical analysis

Analysis was done using the SPSS 16.0 software (SPSS, Chicago, IL). All data are presented as the mean ± SD. Student's *t*-test was used to compare MFI values between two groups. Chi-square test was used to compare the diagnostic sensitivity, specificity and accuracy between FCIA and MAIPA assays or to compare the results of five monoclonal antibodies combined with that of individual antibodies. A $p<0.05$ is considered to be statistically significant. Receiver operating characteristic (ROC) curve analysis was done to determine cut-off values of detected platelet autoantibodies that yielded the highest combined sensitivity and specificity for predicting ITP.

3. Result

3.1. Antibody coating and MFI

Polystyrene microbeads were coated with monoclonal antibodies SZ1, SZ2, SZ21, SZ22 and SZ51, separately. The coated microbeads were detected by flow cytometry using an FITC-GAM antibody. The MFI values for microbeads coated with these monoclonal antibodies were 15.7 ± 1.2 (SZ1), 23.7 ± 1.5 (SZ2), 26.1 ± 1.2 (SZ21), 12.1 ± 1.2 (SZ22), and 17.4 ± 1.5 (SZ51), respectively.

3.2. Stability

The antibody-coated microbeads were stored at 4 °C and tested over time (up to 181 days) with an FITC-GAM antibody for the stability. The results showed that the MFI values for these antibody-coated microbeads varied ~10% (SZ1: 11.1%; SZ2: 11.1%; SZ21: 11.0%; SZ22: 9.4% and SZ51: 11.2%) within six months, indicating that the antibody-coated microbeads were stable when stored at 4 °C.

3.3. Assay variations

We tested intra- and inter-assay CVs for the MFI value for microbeads coated with each antibody. Intra-assay CVs and MFI values for these antibodies were 9.3% and 3.21 ± 0.30 (SZ1), 5.3% and 4.18 ± 0.22 (SZ2), 3.3% and 2.51 ± 0.08 (SZ21), 2.3% and 2.17 ± 0.05 (SZ22), and 2.5% and 2.21 ± 0.06 (SZ51), respectively, and inter-assay CVs and MFI values for these antibodies were 10.9% and 2.78 ± 0.30 (SZ1), 6.6% and 3.84 ± 0.25 (SZ2), 8.7% and 2.41 ± 0.21 (SZ21), 7.1% and 2.01 ± 0.14 (SZ22), and 8.0% and 1.87 ± 0.15 (SZ51), respectively. These CV values indicated that the results from the antibody-coated microbeads were reproducible.

3.4. Platelet autoantibodies in ITP patients

We used the FCIA assay to examine platelet autoantibodies in ITP patients. All microbeads coated by 5 antibodies had higher fluorescent intensities in samples from ITP patients than that of normal controls, indicating the presence of platelet autoantibodies in ITP patients (Fig. 1). The MFI values for these five antibody-coated microbeads were significantly higher in ITP patients than those in non-ITP patients or normal controls (Table 1). No statistically significant difference in MFI values was found between non-ITP patient and normal control groups (Table 1).