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Original Article

Western blotting was used to detect ZAP-70 molecule from γδT cells in peripheral blood

Keqiang Wang[1*], Yanqiang Hou[5*], Chenxing Gu[6], Dapeng Zhao[1], Yanchao Duan[2], Yiren Wang[7], Zhangshen Ran[3], Xiangqi Li[4]

Departments of [1]Clinical Laboratory, [2]Hematology, [3]Medical Examination Center, [4]Breast Surgery, The Affiliated Hospital of Taishan Medical University, Tai'an, Shandong Province, China; [5]Department of Clinical Laboratory, The First People's Hospital Affiliated to Shanghai Jiaotong University, Shanghai, China; [6]Department of Emergency, Peking University International Hospital, Beijing, China; [7]Medical Laboratory Class, Grade 2016, Medical College, Qingdao University, China. [*]Equal contributors.

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Abstract: Objective: To detect the expression of signal transduction molecule ζ-chain associated protein 70 (ZAP-70) in activated γδT cells from human peripheral blood. Methods: The peripheral blood mononuclear cells (PBMC) obtained from healthy volunteers were stimulated by low molecular peptide antigen of mycobacterium tuberculosis (Mtb-Ag), PBMCs were activated by Mtb-Ag for 10 days and then highly purified γδT cells were obtained by immunomagnetic beads selection. The expression of ZAP-70 in γδT cells was detected by Western blotting. Results: The proportion of γδT cells were 4.7 ± 0.6% in freshly obtained PBMC, and 69.8 ± 5.1% after 10 days of Mtb-Ag activation, and 99.1 ± 5.9% after immuno-magnetic beads selection, as same as the expression of protein ZAP-70 detected by western blotting. Conclusions: ZAP-70 is highly expressed in proliferated and activated γδT cells.

Keywords: γδT cells, ZAP-70 protein-tyrosine kinase, Mtb-Ag, Western blotting

Introduction

ZAP-70 is an important protein tyrosine kinase (protein tyrosine kinase, PTK) for T cells activation and belongs to Syk family. The expression of ZAP-70 is one of the most powerful parameters for the clinical stages and prognosis of chronic B lymphocytic leukemia, which is helpful to the clinical treatment and judgement of the prognosis [1-3]. At present, the detection methods of ZAP-70 mainly focused on two aspects: analytical methods and experimental techniques. Analytical methods included percentage method, fluorescence quantitative method and reference method [4-7]. One important step is to set controls. Experimental techniques included specimen acquisition, storage, detection time, fixation method, antibody and fluorescein selection. There were some differences between laboratory test results. ZAP-70 standardized detection methods have not yet been confirmed. In our study, γδT cells were activated by specific stimulation of Mtb-Ag, and high purity γδT cells were obtained by immunomagnetic positive sorting method. The expression level of ZAP-70 in γδT cells were detected by western blot.

Materials and methods

Reagents and instruments

Equipments used in this study: Flow Cytometry (Coulter EPICSR XL-MCL) from Beckman-counter, USA; Reverse Microscope (XDS-1) from Chongqing Electro-optical Instrument Factory; CO2 Incubator (MCO0175) from Sanyoo Company, Japan; 24-well cell culture plate from Falcon Company, USA; Automatic Enzyme-Label Reader (SLT-II) from Austria; Magnetic Cell Sorter (Miltenyi Biotec, Midi-MACS) from Germany; Electrophoresis apparatus DYY-III Beijing 61 Instrument Factory; Electrophoresis tank, Mini Protean II cell, Bio-Rad Company; Transfer equipment, Mini Trans-blot, Bio-Rad Company.

Low molecular weight peptide antigens from Bovine Mycobacterium tuberculosis (Mtb-Ag) was made according to method from Boom WH [8]; lymphocyte isolation liquid (Cat No 2000408) was manufactured by the Hematology Institute of Chinese Academy of Science; RPMI 1640 cell culture medium: RPMI powder (GIBCO BRI) was dissolved in 500 ml of triple distilled water first, then L-glutamine 2 mmol/L, dithioethanol 5×10^5 mmol/L, sodium acetate 1 mmol/L, penicillin 100 u/ml and streptomycin 100 μg/ml were added in turn, and triple distilled water was added to make the final volume of 1,000 ml. The pH of the medium was adjusted to 7.2 by adding sodium bicarbonate. After filtering through the 0.2 μM filter membrane, the medium was stored at 4°C. Before use, the medium was supplemented with 10% inactivated neonatal calf serum; Recombinant interleukin 2 (rIL-2, PTK company); 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) (FLUKA); fluorescent mouse-anti-human monoclonal antibody TCRγδ-PE (Becton Dickinson); TCR γδ immunomagnetic bead kit (Miltenyi Biotec); ZAP-70 Monoclonal antibody (Transduction Laboratories, USA); Sheep anti mouse IgG-HRP; Two amino amine amine (DAB) concentrated color solution and PMSF (HUA MEI company); Aprotinin, Leupeptin and Pepstin (Roche, Germany).

Induction and propagation of lymphocytes

Peripheral blood mononuclear cells (PBMCs) were obtained from 5-10 ml of heparinized venous blood from 11 healthy volunteers, which were adjusted to 1×10^6/ml using RPMI 1640 culture medium. one mililitre of the cell suspension and Mtb-Ag at the concentration of 5 μg/ml were added to each well of a 24-well culture plate, and then being cultured at 37°C for 30 min. IL-2 50 U/ml was added to each well every three days to optimize the propagation of the PBMCs and the cell numbers were counted as necessary. After about 10 days, the cells were collected and selected by immunomagnetic beads according to the procedures provided by the manufacturer.

Measurement of the percentage of γδT cells in PBMC

5×10^5 PBMCs were washed three times in ice-cold PBS and fixed in PBS containing 1% formaldehyde, and the cells were immunocytochemically stained and analyzed by flow cytometry assay to determine the percentage of γδT cells.

The preparation of electrophoretic samples

The isolated positive cells were put into frozen cracking liquid. Vortex and vibration immediately, and then cracking for 20 min on ice [9, 10]. Acidified acetone and methanol were used for protein delipidation and precipitation in lysis cells. Equal volume of Acidified acetone and methanol were mixed to form precipitation agent. Five volume of precipitation agent were added into the cell lysis solution, mixed and incubated overnight in the refrigerator of -20°C. The precipitations were obtained after high speed centrifuge for 30 min in 4°C. Samples and buffers mixed at 1:1 and boiled for 5 min for preparation.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

Electrophoresis apparatus were installed, and 10% separation gel was freshly prepared, 0.75 mm comb insert into the newly prepared 5% gum, waiting for solid. Protein molecular weight marker and samples were added, 10 μl per hole. Meanwhile two were added with samples with same method, electrophoresis for 45 min in 100 V. After electrophoresis, one gum was stained by Kaumas Coomassie brilliant blue overnight. 5 times of decolorization solution was used to decolorize for 4~8 hours, and replaced the decolorization solution for 2~3 times. Another gum was used for western blot.

Protein transfer

All the steps were performed according to the instrument specification of BIO-RAD Company as follows, Whatman 3 MM Filter paper and cellulose nitrate (NC) membrane were immersed in transfer buffer. The filter paper and NC film were cut into the same size as the gel before transfer. The sponge layer and filter paper were placed on the two sides of the gel. The rough surface of the NC film was pasted on the glue. The film was marked into the transfer. The NC film side was put in the positive electrode, and the gel side was put in the negative electrode. Transfer was performed overnight on 14 V.

Western blot

After transfer, the NC membrane was removed and the protein molecular weight marker part

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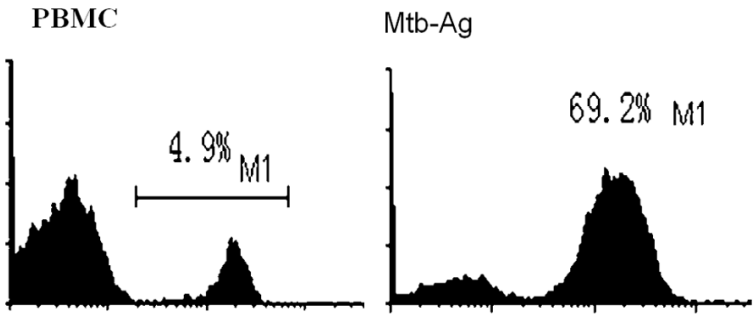

Figure 1. The percentage of γδT cells in PBMCs before and after stimulation in a healthy volunteer.

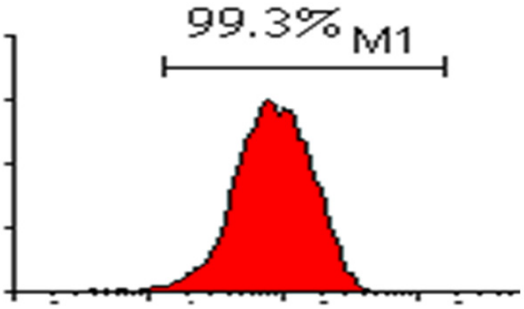

Figure 2. The percentage of γδT cells after sorting by immunomeganetic beads in a healthy volunteer.

was cut down. Staining with amino black for 5-10 min and then observed. The rest of the NC membrane was rinsed with double distilled water, then was sealed with TBS-3% BSA membrane at room temperature for 1-3 h. TBS (Tris 4.84 g, NaCl 58.48 g, added distilled water to 2000 ml, PH7.5) was used to wash membrane for 3 times, 5 min every times. Anti ZAP-70 antibody to mouse was added, (sealing solution dilution 1:2000), 4 degrees overnight. After being washed again, Goat anti mouse IgG-HRP (closed liquid dilution 1:50), room temperature for 2 hours. After being washed, diaminobenzidine (DAB) substrate was added for staining at room temperature for 5 min. Reaction ended by water washing and results were observed.

Statistical analysis

Data are expressed as the mean ± SEM or raw numbers. The normality of the variables was assessed using the Shapiro-Wilk test. For data that were normally distributed, one-way analysis of variance (ANOVA) and the LSD post hoc multiple comparisons test were applied. The Kruskal-Wallis test and the Mann-Whitney U test were performed to compare data that were not normally distributed. The chi-square test was employed to compare expression rate of the cells. All data were processed by SPSS software package for Windows version 13.0 (SPSS, Inc, Chicago, USA), All statistical tests were two-sided, and statistical significance was defined as $P<0.05$.

Results

Lymphocytes propagation induced by Mtb-Ag

The propagation status of lymphocytes was observed using activity determination method. In the first few days after co-stimulation by Mtb-Ag and rIL-2, the lymphocytes grew slowly and showed a substantial amplification from the fourth day on, reaching peak cell growth on the 12th day. At this time, the cell number increased by about 40 times.

The percentage of γδT cells determined by flow cytometry

Flow cytometric analysis on freshly obtained PBMCs and those stimulated by Mtb-Ag for 10 days showed a dramatic increase in the percentage of γδT cells from 4.7 ± 0.6% before stimulation to 69.8 ± 5.1% after stimulation (**Figure 1**). It can be seen that Mtb-Ag can powerfully and specifically stimulate the growth of γδT cells. Moreover, after sorting by immunomagnetic beads, the percentage of γδT cells can be as high as 99.1 ± 5.9% (**Figure 2**).

ZAP-70 molecule detection in γδT cells

ZAP-70 molecule in γδT cells was detected after SDS-PAGE gel electrophoresis, protein transfer and Western blot. Its molecular weight is 70 kDa (**Figure 3**).

Discussion

T cells were divided into αβT cells and γδT cells according to the TCR types. γδT cells is different from αβT cells that the identification, processing and presenting of antigens by γδT cells [11]. γδT is a small subpopulation of T lymphocytes identified in 1986, accounting about 0.5-5% of all PBMCs in adults. These cells are mainly dis-

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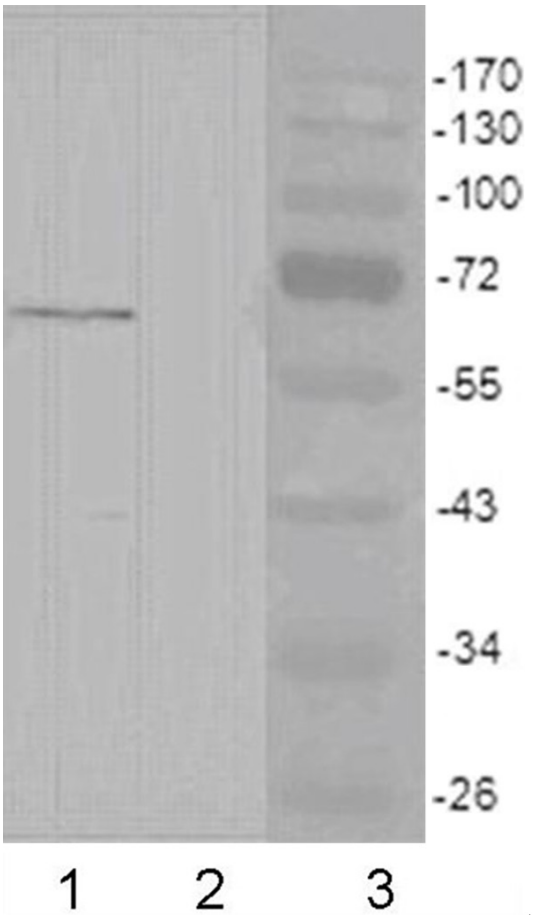

Figure 3. The detection of ZAP-70 of proportion of γδT cells. 1 ZAP-70; 2 negative control; 3 protein molecular weight marker.

tributed in the mucous and subcutaneous region in the body. In humans, 10-18% of small intestine intra-epithelial lymphocytes (IEL) are γδT cells, and in large intestine 25-37% of IEL are γδT cells. In mice large intestine, as many as 50% of IELs are γδT cells. Since the mucous and the epithelia are the first barrier against pathogenic organism invasion and are the most frequent site for malignancy, the high distribution of γδT cells in these regions suggests the important role these cells play in defense against microorganisms, parasites, tumor cells and in comprehensive immunomodulation [12-16]. Recent studies confirmed these observations and drew extensive attention to these cells. As γδT cells neither need MHC restriction in antigen recognition nor require antigen presenting cells for activation, these cells can have faster and more extensive effect than γδT cells, an important property that attracts increasing attention [12-16].

Previous studies have already shown that Mtb-Ag can effectively stimulate the growth of $Vγ9^+/Vδ2^+$ γδT cells, which make up over 80% of the total γδT population. In our study, we used Mtb-Ag to stimulate PBMC obtained from 5-10 ml of peripheral blood. This method is characterized by quick γδT cell proliferation and higher cytotoxicity effect, accompanied with the percentage of γδT cells reaching from 4.7 ± 0.6% before stimulation to 69.8 ± 5.1% after stimulation, suggesting that Mtb-Ab can preferentially stimulateds the amplification of γδT cells and enhance the cytotoxicity effect of them. Cultured cells obtained by immunomagnetic positive sorting, to obtain high purity γδT cells, we found that after the separation of high purity γδT cell ratio as high as 99.1 ± 5.9%, so the expression changes of ZAP-70 protein can be detected from high purified γδT cells. In summary, as a practical method with minimal blood volume required, high specificity, efficiency and no requirement of sophisticated equipment, this method can be utilized as a good technique to provide sufficient γδT cells for the study of γδT cell biology and adopted immunotherapy strategy.

ZAP-70 is an important protein tyrosine kinase (protein tyrosine kinase, PTK) for T cells activation and belongs to Syk family. Currently research of αβT cells showed that ZAP-70 was associated with phosphorylated ζ-Chain and CD3 molecules after stimulation of TCR. At the same time, tyrosine phosphorylation occurred on its own molecules. As so far, the signal transduction pathway after ZAP-70 activation are as followed, ZAP-70-VAV-RAC pathway, ZAP-70-Grb2. Shc. Sos-Ras-Raf-MEK-Erk pathway, ZAP-70-PLCγ1-IP3 pathway, ZAP-70-PLCγ1-DAG-PKC pathway [17].

At present, the detection methods of ZAP-70 mainly focused on two aspects: analytical methods and experimental techniques. Analytical methods included percentage method, fluorescence quantitative method and reference method [4-7]. One important step is to set controls. Experimental techniques included specimen acquisition, storage, detection time, fixation method, antibody and fluorescein selection. There were some differences between laboratory test results. ZAP-70 standardized detection methods have not yet been confirmed. We used western blot method to detect ZAP-70 molecules, and some satisfactory re-

sults were acquired. Our studies showed that ZAP-70 molecules existed in γδT cells and played a methodological foundation for the detection and analysis of other molecules in γδT cells. It also played a foundation for further detection of activation and function of ZAP-70 molecules in the processing of γδT cells activation and signal transduction.

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Disclosure of conflict of interest

None.

Address correspondence to: Dr. Xiangqi Li, Department of Breast Surgery, Affiliated Hospital of Taishan Medical University, Tai'an 27100, Shandong Province, China. Tel: +86-538-6230822; Fax: +86-538-8420042; E-mail: drlixqi@hotmail.com; Dr. Keqiang Wang, Department of Clinical Laboratory, The Affiliated Hospital of Taishan Medical University, Tai'an, Shandong Province, China. Tel: +86-538-6236422; Fax: +86-538-8420042; E-mail: wkqsd@163.com

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