Article

Effect of Protein Kinase C on Proliferation and Apoptosis of T Lymphocytes in Idiopathic Thrombocytopenic Purpura Children

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It is well-documented that T lymphocyte proliferation and apoptosis are abnormal in idiopathic thrombocytopenic purpura (ITP) children. However, the underlying regulation mechanisms especially in terms of signal transduction remain unknown. In this paper, we reported the changes of protein kinase C (PKC) activity in peripheral blood T lymphocytes and the effect of PKC on T lymphocyte proliferation and apoptosis. We demonstrated that in ITP children, the activator (PMA) and inhibitor (H-7) of PKC affected on T lymphocyte proliferation and apoptosis dramatically, but they altered little in healthy children. PKC activity was significantly enhanced in ITP children together with an increased expression of FasL on $CD3^+$ T, $CD4^+$ T and $CD8^+$ T cells, resulting in a positive correlation between PKC activity and the expression of FasL on T cells. While the PKC activity and the platelet count were negatively correlated. Taken together, our findings suggest that the PKC activation may enhance T lymphocytes activity, suppress T cell apoptosis and be involve in thrombocytes damage as a mechanism related to immune pathogenesis of ITP. *Cellular & Molecular Immunology*. 2005; 2(3):197-203.

Key Words: ITP, PKC, proliferation, apoptosis, T lymphocyte

Introduction

Idiopathic thrombocytopenic purpura (ITP) is a typical autoimmune disease, its pathogenesis involves in abnormal activation in T lymphocytes and production of T cell-dependent autoantibodies. Previous studies showed that platelet associated immunoglobulins (PAIgs) were harmful to thrombocytes, as major factors (1). However, recent researches noted that lymphocyte apoptosis deficiency and abnormal polarization between Th1 and Th2 cells in peripheral blood might play an important role in ITP pathogenesis. We have analyzed the expressions of Fas and FasL on T cell subsets (2, 3). But we did not know the factors affecting T cell proliferation and apoptosis in ITP patients in signal transduction.

Protein kinase C (PKC) is an important signal molecule in T cell activation and apoptosis (4). Mitogenic stimulants such as phytohemagglutinin (PHA) or anti-CD3 antibodies induce phosphatidylinositol hydrolysis in T cells. Of the hydrolytic products, inositol 1,4,5-trisphosphate (IP3) mobilizes free calcium from intracellular stores, and diacylglycerol (DG) activates PKC. Phorbol myristate acetate (PMA) binds to PKC intracellularly and directly activates PKC resulting in proliferation of T cells. H-7 is a kind of inhibitor for PKC activation and T cell proliferation. Meanwhile, PKC can regulate T cell apoptosis by Fas-mediated apoptosis signaling (5). In this study, we analyzed the effect of PKC-related signal transduction on T cell proliferation and apoptosis to explore the possible role of PKC signal transduction in ITP immunopathogenesis, so that we could look for some new approaches for ITP clinical prevention and cure.

Materials and Methods

Patients and normal subjects

Forty-five ITP children (20 boys and 25 girls) came from the No.1, 2 and 3 affiliated hospitals of Guangdong Medical College fulfilled the diagnostic criteria for ITP (6). The patients had an average age of 4.5 years (ranging from 8.0 months to 12.5 years). Anti-coagulated venous blood by heparin was collected from the patients before treated, and blood was also collected from 40 healthy children (14 boys and 26 girls, ranging from 2 years to 13 years, having an average age of 5.7 years).

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Monoclonal antibodies
Cy5-mouse anti-human CD4 mAb, Cy5-mouse anti-human CD8 and Cy5-mouse IgG1 isotype control immunoglobulin were purchased from EB Company. FITC-mouse anti-human Fas mAb, FITC-mouse anti-human FasL mAb and FITC-mouse IgG1 isotype control immunoglobulin were purchased from AC Company. Cell apoptosis reagent kit (AnnixinV-FITC and PI) was purchased from BioVision Company.

Segregation of peripheral blood T cell
Anti-coagulated venous blood by heparin was collected from ITP children and healthy children. Lymphocytes were segregated by lymphocytic separating medium (AXIS-SHIELD), then incubated in a glass petri dish at 37^oC, so that monocytes were removed out. Erythrocytes were lysised when the samples were incubated at 37^oC for 10 min with hemolysin (0.85% NH_4Cl). Finally, T cells were collected when the samples passed through Human $CD3^+$ T Cell Enrichment Column (BD). The purity of T cells was analyzed by immunofluorescent staining and flow cytometry by anti-CD3 mAb (EB), and the activity of T cells was detected by trypan blue staining. Lymphocytes were adjusted to 2×10^6/ml with RPMI-1640 culture media (containing 20% fetal bovine serum).

T lymphocytes culture
Peripheral blood T lymphocytes from ITP children and healthy children were divided into four groups (A, B, C and D), Group A was blank, PMA (20 nM) was put into group B and PMA + H-7 (5 μM) was put into group C, H-7 (10 μM) was put into group D, each group has two portions and was cultured for 24 h or 72 h under the condition of 37^oC, 5% CO_2, respectively.

Preparation for T cell PKC
PKC was prepared from unstimulated or stimulated cells, PMA and PMA + H-7 stimulation was as follows: T cells were suspended at 2×10^6/ml in complete media and were stimulated with the indicated concentration of PMA or PMA + H-7 at 37^oC. Cells removed at indicated time intervals were washed twice with phosphate-buffered saline (PBS). Unstimulated or PMA, PMA + H-7 stimultaed cells were resuspended in 50 μl buffer A (20 mM Tris, 2 mM EDTA, 0.5 mM EGTA, 1 mM PMSF, pH 7.4,) containing 50 mM 2-mercaptoethanol and sonicated with a Branson Model Sonifier for 45 s at 20 W. The homogenates were centrifuged for 60 min at 100,000 g, the supernatant was purified by applying each sample to a 1.5 ml DEAE-Sepharose column equilibrated with buffer A. The column was washed with 2 ml buffer A and 0.5 ml 0.15 M NaCl, and the eluate by 1 ml 0.15 M NaCl was collected. All of PKC activity was present in this fraction.

Determination of T cell PKC activity
According to determination way of Schondorfand so on (7), PKC activity was detected by PepTag Assay for non-radio-active detection of PKC. The reactive mixture, in a final volume of 25 μl, consisted of 5 μl reaction buffer, 5 μl PepTag C1 (0.4 μg/μl), 5 μl PKC activator solution (DG), 1 μl peptide protection solution and 9 μl sample. Keeping on phosphorylation reaction for 30 min referring to reagent explaination, then 25 μl reactive mixture was used to have electrophoresis on a 0.8% agarose gel at 100 V for about 20 min. After electrophoresis, the PepTagC1 peptides of phosphorylation and non-phosphorylation were detached, phosphorylated PepTagC1 peptide with negative electricity migrated toward the anode (+), but non-phosphorylated PepTagC1 peptide with positive electricity migrated toward cathode (-), the gel was photographed. Electrophoresis strip on anode represented PKC activity and was analysed by quantitation.

Determination of T cell FasL
Purified T cells (100 μl) were added to different tubes. CD4, CD8 and FasL mAb marked with immunoflurescence were added to reasonable tube, then the sample and mAb were shaked to mix, incubated in the dark at 37^oC for 30 min. The samples were washed two times by PBS, then 0.5 ml buffer was added to each tube, which were shaked before they were detected by flow cytometry. The expression of FasL on T cell was detected by FasL/CD4, FasL/CD8 gating.

Determination of T cell proliferation
T cell proliferation was detected by Cell Counting Kit-8 (CCK-8) assay. T cells culture was finished before 3 hours, CCK-8 reagent (10 μl) was added to each pore and continued to culture for 3-5 h, until the media turned yellow. The absorb value (450 nm) of each pore was analysed by enzyme symbolized meter.

Determination of T cell apoptotic rate
When T cells were cultured for 24 h, samples of each group were washed twice by PBS. The concentration of T lymphocytes were adjusted to 2×10^6/ml by 0.9% saline, in each tube, the volume was 100 μl, then 15 μl Annexin V-FITC and 15 μl PI were added to each tube respectively, then the sample and mAb were shaken to mix in the tube, incubated in dark at 37^oC for 30 min. At last T cells apoptotic rate was detected by bicolor flow cytometry.

DNA ladder electrophoresis
Purified T cells of ITP group (Blank group, PMA group and H-7 group, 100 μl) were added to three tubes, and the volume was 100 μl in each tube. T cell DNA of each group was extracted by DNA extract reagent. Then 20 μl TE was added to DNA tube of each group, after DNA was completely lysed, 10 μl DNA solutions were used for electrophoresis on 2% agarose gel.

Statistical analysis
SAS 8.22 and SPSS 11.0 softwares were used for statistical analysis of the results. Data were expressed as the mean ± SD. Statistical differences between the two groups were evaluated by analysis with Student's *t*-test; analysis of linear correlation was used to evaluate the correlation between two variances, *q*-test of analysis of variance (ANOVA) was used to analyse

Table 1. Determination of PKC activity in peripheral blood T lymphocytes and platelet counting in ITP children

Groups	Numbers	PKC (nmol/min·ml)	PLT (× 10^9)
Normal	40	0.55 ± 0.13	203 ± 59
ITP	45	0.97 ± 0.21*	28 ± 17*

Data were presented as mean ± SD. Compared with normal, * $p < 0.05$.

multiple comparison. Values of $p < 0.05$ were accepted as indicating significance.

Results

Determination of T cell PKC activity

Compared with healthy children, T cell PKC activity increased significantly in ITP children ($p < 0.05$), platelet count decreased dramatically, there was negative correlation between PKC activity and platelet count (r = 0.75, $p < 0$ .05) (Table 1 and Figure 1). Meanwhile, PMA and H-7 affected PKC activity in ITP children, PMA can enhance PKC activity, but H-7 can suppress it (Figure 2).

Determination of T cell FasL expression

Compared with healthy children, in ITP children, the expression of FasL on $CD3^+$ T, $CD4^+$ T and $CD8^+$ T cells all increased significantly ($p < 0.05$). There was positive correlation between PKC activity and the expression of FasL on T cells ($r_1 = 0.65$, $r_2 = 0.54$, $r_3 = 0.60$, $p < 0.05$) (Table 2 and Figure 3).

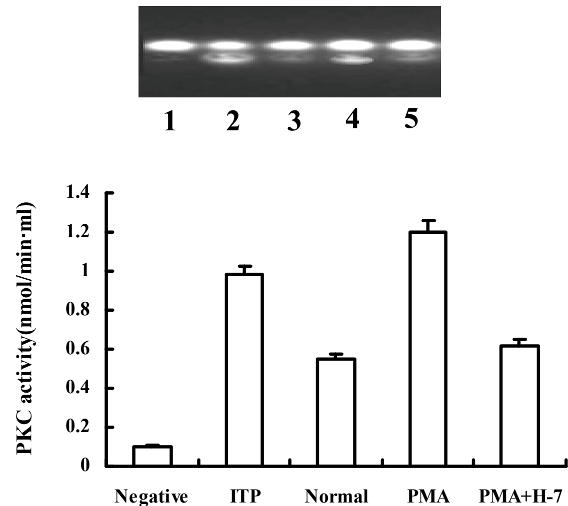

Figure 2. Effect of PMA and H-7 on T cell PKC activity. Anti-coagulated venous blood by heparin was collected from healthy children and ITP children. T cells were segregated by Human $CD3^+$ T Cell Enrichment Column. The concentration of T cells was adjusted to 2 × 10^6/ml with RPMI1640 culture media (containing 20% fetal bovine serum). T cell PKC activity was measured by PepTag assay as described under "Materials and Methods" (the upper: cathode; the down: anode). The effect of PMA and H-7 on T cell PKC activity in ITP children were shown (Lane 1, negative; Lane 2, ITP; Lane 3, normal; Lane 4, PMA; Lane 5, PMA + H-7).

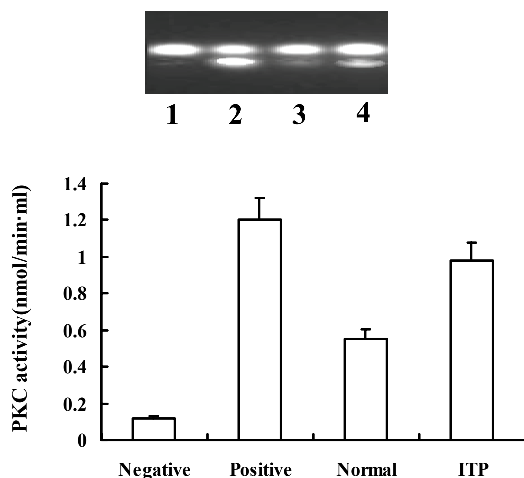

Figure 1. Detection of T cell4s PKC activity by PepTag assay for non-radioactive detection of PKC. Anti-coagulated venous blood by heparin was collected from healthy children and ITP children. T cells were segregated by Human $CD3^+$ T Cell Enrichment Column. The concentration of T cells was adjusted to 2 × 10^6/ml with RPMI1640 culture media (containing 20% fetal bovine serum). T cell PKC activity was measured by PepTag assay as described under "Materials and Methods" (the upper: cathode; the lower: anode). The activity for T cell PKC in healthy children or ITP children was shown (Lane 1, negative; Lane 2, positive; Lane 3, normal; Lane 4, ITP).

Determination of T cell proliferation

In healthy children, T cell proliferation among the four groups (blank group, PMA group, PMA + H-7 group and H-7 group) did not vary significantly ($p > 0.05$). In ITP children, T cell proliferation between blank control group and PMA + H-7 group was unchanged obviously ($p > 0.05$), while there was significant difference between PMA group and the other three groups ($p < 0.05$). Compared with the four groups in healthy children, T cell proliferation increased dramatically only in PMA group in ITP children, the H-7 group decreased ($p < 0.05$), while the other two groups altered little ($p > 0.05$) (Figure 4).

Determination of T cell apoptosis

In healthy children, T cell apoptotic rate among the four groups (blank group, PMA group, PMA + H-7 group and H-7 group) did not vary significantly ($p > 0.05$). In ITP group, T cell apoptotic rate between blank control group and PMA + H-7 group was unchanged obviously ($p > 0.05$), while there was significant difference between PMA group and the other three groups ($p < 0.05$). Compared with the four groups in healthy children, T cell apoptotic rate increased in H-7 group

Table 2. The percentage of FasL on T cell subpopulation in ITP patients

Groups	Numbers	PKC (nmol/min·ml)	$CD3^+$ T FasL (%)	$CD4^+$ T FasL (%)	$CD8^+$ T FasL (%)
Normal	40	0.55 ± 0.13	15.4 ± 5.7	4.7 ± 4.5	10.3 ± 4.2
ITP	45	0.97 ± 0.21*	54.6 ± 12.3*	32.7 ± 3.4*	25.0 ± 4.8*

Data were presented as mean ± SD. Compared with normal, * $p < 0.05$.

in ITP children, but the other three groups all decreased, while the PMA group did more dramatically ($p < 0.05$) (Figure 5).

DNA ladder electrophoresis for T cells in ITP children

In ITP children, T cell apoptotic rate between blank control group and PMA + H-7 group was unchanged obviously ($p > 0.05$), while there was significant difference between PMA group and the former two groups ($p < 0.05$). T cell apoptotic DNA ladder in PMA group was not seen in PMA group, but in H-7 group, there were more apoptotic T cells, the DNA ladder was very obvious, which was more distinct than those of blank group and PMA group (Figure 6).

Discussion

Recent researches show that protein kinase C (PKC) signal transduction may participate in the process of T cell proliferation and apoptosis. PKC is the key enzyme during the cell inner biology signal transduction, which can regulate the expression of IL-2, IL-6, GM-CSF and IL-1β by activating backward position of NF-κB, AP-1 and so forth in the way of phosphorylation. PKC signal transduction may play an important role in the immune inflammation reaction, cell proliferation and apoptosis, self-immunity disease. For example, Xiong reported that PKC played an important role in the mechanism of asthma (8). Tada indicated that PKC activity decreased in SLE patients, which related to the state of illness (9). Tori also found that PKC activity of fibroblasts boost up in the diabetes of type I (10). But the action of PKC signal transduction was unclear in the ITP immunopathogenisis.

Autoimmune diseases are kinds of clinical common diseases, but the causes and mechanisms of them are very complex and still remain unclear. Maybe there are some kinds of self-immunogenicity and/or self-tolerance destruction, which brings about continual and over-degree autoimmune responses.

Acute idiopathic thrombocytopenic purpura (AITP) is a kind of common autoimmune disease in children and young

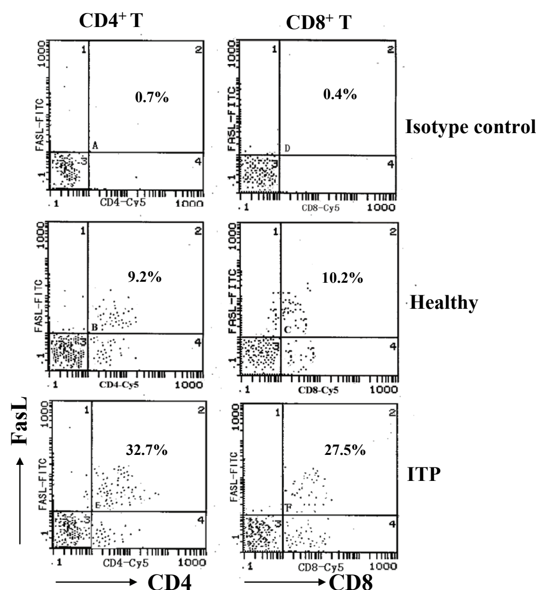

Figure 3. Detection of T cells FasL by FCM. Anti-coagulated venous blood by heparin was collected from healthy children and ITP children. T cells were segregated by Human $CD3^+$ T Cell Enrichment Column. The concentration of T cells was adjusted to 2×10^6/ml with RPMI-1640 culture media (containing 20% fetal bovine serum). T cells FasL was measured by PCM as described under "Materials and Methods". The percentages of the expressions of FasL on $CD3^+$ T, $CD4^+$ T and $CD8^+$ T cells were shown.

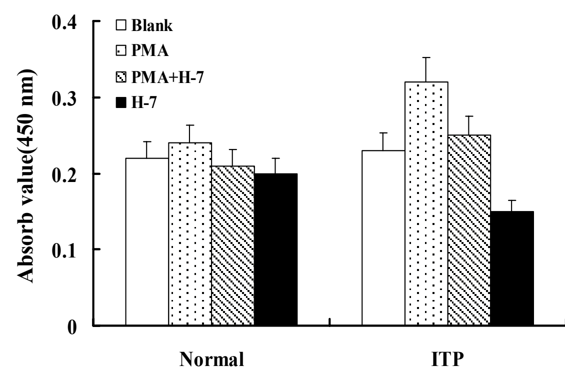

Figure 4. Detection of T cell proliferation by CCK-8 assay. Anti-coagulated venous blood by heparin was collected from healthy children and ITP children. T cells were segregated by Human $CD3^+$ T Cell Enrichment Column. The concentration of T cells was adjusted to 2×10^6/ml with RPMI1640 culture media (containing 20% fetal bovine serum). T cell proliferation was measured by CCK-8 assay as described under "Materials and Methods". The effects of PKC activity on the T cell proliferation in healthy children or ITP children were shown.

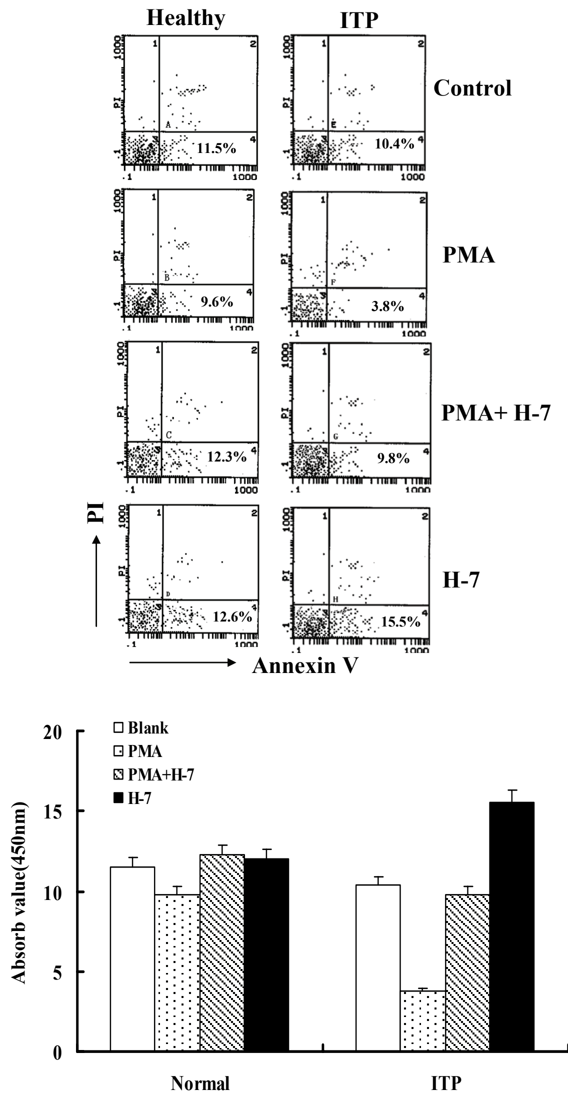

Figure 5. Detection of T cell apoptotic rate by FCM. Anti-coagulated venous blood by heparin was collected from healthy children and idiopathic thrombocytopenic purpura children. T cells were segregated by Human $CD3^+$ T Cell Enrichment Column. The concentration of T cells was adjusted to 2×10^6/ml with RPMI-1640 culture media (containing 20% fetal bovine serum). T cell apoptotic rate was measured by FCM as described under “Materials and Methods”. The T cell apoptotic rates in healthy children or ITP children were shown.

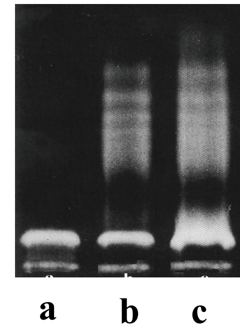

Figure 6. Effect of PKC activity on DNA ladder electrophoresis. Anti-coagulated venous blood by heparin was collected from idiopathic thrombocytopenic purpura children. T cells were segregated by Human $CD3^+$ T Cell Enrichment Column. The concentration of T cells was adjusted to 2×10^6/ml with RPMI-1640 culture media (containing 20% fetal bovine serum). DNA ladder for T cell in ITP children was measured by electrophoresis as described under “Materials and Methods”. The T cell DNA ladders in ITP children were shown. (Lane a, PMA group; Lane b, Blank group; Lane c, H-7 group).

females. Thrombocytes decrease extremely in serious patients, some of whom turn to be chronic or refratory ITP. How to cure the chronic or refratory ITP completely is a difficult problem puzzling many doctors. Recently, some researchers found that many peripheral activated lymphocytes, which apoptosis decrease, relate to pathogenisis of ITP (11), but the regulation mechanism of T cell proliferation and apoptosis is not clear so far. PKC is a kind of important key enzyme in cell signal transduction. According to the reports, PKC signal transductin may take part in the mechanism of autoimmune disease and correlate to the T cell proliferation and apoptosis (12, 13).

PKC is expressed on T lymphocytes selectively and is necessary for T cell antigen receptor (TCR)-triggered activation of mature T cells. Productive engagement of T cells by antigen-presenting cells (APCs) results in recruitment of PKC to T cell-APC contact area, where it interacts with several signal molecules to induce activation signals essential for productive T cell activation and IL-2 production. The transcription factors, NF-κB and AP-1, are the primary physiological targets of PKC, and efficient activation of these transcription factors by PKC requires integration of TCR and CD28 costimulatory signals. Cooperating with the Ser/Thr phosphatase, calcineurin, PKC can lead to activation of JNK, NFAT, and the expression of IL-2 gene. PKC also promotes T cell cycle progression and proliferation (14). In this study, we found that PKC activity was enhanced in ITP children, and there was positive correlation between PKC activity and the expression of FasL on T cell. T cells were more sensitive to PMA stimulation in ITP children than in healthy children, which resulted in more T cells activation and thrombocytes damage. So we think that PKC signal transduction involved in the immunopathogenisis of ITP, PKC can be an attractive drug target for immunosuppression in ITP and other auto-immune diseases in the future.

Ruiz reported that effect of PKC on T cell apoptosis relate to Fas/FasL signal ways (12). Fas/Apo-1(CD95) is a kind of type I transmembrane glycoprotein with molecular weight about 48 kD. Fas is mainly expressed on peripheral $CD4^+$ T cells, $CD8^+$ T cells, some NK cells and monocytes. Fas ligand (FasL) is a family member of tumor necrosis factor (TNF). FasL is a kind of type II transmembrane glycoprotein with molecular weight about 40 kD, and it is mainly expressed on activated T cells and B cells, and it is

one of the T cell activated markers. When membrane FasL (mFasL) crosslinks with membrane Fas (mFas), celluar apoptosis can be induced by Fas/FasL pathway, TNFR/TNF pathway, p53 pathway and glucocorticoid system, and so forth; Fas/FasL pathway that induces cellular apoptosis disorder is the main factor of the disturbance between cellular immunity reaction and humoral immunity reaction. Fas and FasL participate in the regulation of lymphocytes quantity in immune response, correlating with peripheral activated lymphocyte apoptosis. There are increasing expressions of FasL on activated lymphocytes, with the increase of expression of Fas at the same time. After finishing their immune function, activated lymphocytes start to apoptosis immediately through Fas/FasL apoptotic pathway, so that autoimmune diseases will not happen. If there is a disorder in Fas/FasL apoptotic pathway, autoimmune diseases might take place. Recent researches revealed that many activated lymphocytes existed in ITP patients, and thrombocytes were destructed through direct killing function mediating by activated lymphocytes and cellular apoptosis inducing by lymphocytes (15). Yoshimura (16) noticed that there was a close relation between Fas/FasL apoptotic pathway and ITP. Yang found that the expressions of Fas and FasL on T cell subsets in ITP children increased, but the regulated factors of the expressions of T cell Fas/FasL in ITP patients remained to be reported little recently, especially no one studied the effect of PKC signal transductions on expression of Fas/FasL in ITP patients. In this study, it was found out that PKC signal transduction had positive correlation with the expression of T cell FasL in ITP children significantly, as show that PKC inhibitor (H-7) can suppress T cell proliferation and improve T cell apoptotic rate. Guo also reported PKC activation can suppress apoptosis in Jurkat T cells (17). The possible mechanism is as follows: activation of PKC triggers cellular signals that inhibit Fas/CD95-induced cell death in T cells, which alters Fas/CD95 signal from the plasma membrane to the activation of caspases by exerting a profound action on survial/cell death decision. PKC activation blocked FADD recruitment, caspase-8 activation and DISC formation. However, inhibition of PKC promoted the opposite effect on the Fas/FasL pathway by increasing FADD recruitment, caspase-8 activation and DISC formation rapidly (5). Besides, PKC activation can stimulate superoxide anion production system, which can protect Fas/CD95-induced T cell apoptosis, if PKC activation is inhibited, T cell apoptotic rate is upregulated by Fas/FasL signaling pathways.

In the past, we have proved that abnormal expression of Fas and FasL might play an important role in abnormal polarization of T cell subsets, abnormal activation of T cell subsets, apoptosis of T cell subsets, abnormal polarization of T cell subsets and disorder of Fas/FasL might be involve in ITP immunopathogenesis (18, 19). In this study, we had studied PKC activity of T cells in ITP children and effect of PKC signal transduction on T cell proliferation and apoptosis. Our results displayed that: (1) PKC activity of peripheral blood T cell increased in ITP children and PMA could improve PKC activity but H-7 could suppress PKC activity; (2) There was positive correlation between the changes of PKC activity and expressions of T cell FasL in ITP children, and negative correlation between PKC activity and platelet count; (3) PKC activity affected T cell proliferation and activation in ITP children, proliferation of T cells stimulated with PMA increased significantly, but that of T cells stimulated with H-7 decreased; (4) PKC activity affected the T cell apoptotic rate in ITP children, apoptotic rate of T cells stimulated with PMA decreased more, but that of T cells stimulated with H-7 increased significantly. So we think PKC signal transduction might involve in ITP immunopathogenesis. Furthermore, to study effect of PKC on T cell proliferation and apoptosis in ITP children, we will do well to treat immune diseases including ITP.

PKC signal transductions are anfractuosity, for PKC has many subtypes with different functions. So we still do not know which subtype affects the pathology mechanism of ITP. In the future, we will make use of antisense nucleic acid of PKC subtypes to study PKC signal transduction and backward position molecule NF-κB, AP-1 and so forth (20), and how to adjust the balance of T cell activation, proliferation and apoptosis in ITP patients. From these studies, we want to find some new evidence for ITP cure, meanwhile, we will discuss immunopathogenesis of other autoimmune diseases by the way of signal transduction.

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