Effect and mechanism of glycosylphosphatidilinoditol-specific phospholipase D on the adhesion function of bone marrow mononuclear cells separated from myeloid leukemia patients☆

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Abstract

BACKGROUND: There still is rarely report about the effect of glycosylphosphatidilinoditol-specific phospholipase D (GPI-PLD) on the adhesion function of leukemic cells through screening Medline and CNKI databases.

OBJECTIVE: To observe the effect of GPI-PLD on the adhesion function of bone marrow mononuclear cells separated from myeloid leukemia patients, and to investigate the related mechanism.

DESIGN, TIME AND SETTING: This study addressing cytology *in vitro* was conducted at the Hematological Laboratory of Xiangya Hospital from January to June 2004.

MATERIALS: Bone marrow was collected from myeloid leukemia patients at the Department of Hematology, Xiangya Hospital, China.

METHODS: The GPI-PLD activity of bone marrow mononuclear cells separated from myeloid leukemia patients was measured by using GPI-anchored placental alkaline phosphatase as substrate and Triton-X114 partition. By use of 1,10-phenanthroline, the activity of GPI-PLD was inhibited, the experiment was divided into 2 groups: treatment group adding phenanthroline to achieve a final concentration of 1 mmol/L, while control group adding the same amount of phosphate buffered saline. The adhesion rate to the fibronectin and CD24 expression of these cells were measured by MTT and immunohistochemical method, respectively.

MAIN OUTCOME MEASURES: GPI-PLD activity of myeloid leukemic cells, cell adhesion rate, CD24 expression were all measured.

RESULTS: The GPI-PLD activity of bone marrow mononuclear cells separated from myeloid leukemia patients was inhibited significantly after these cells were treated by 1 mmol/L 1,10-phenanthroline for 5 hours compared with control groups [(5.40±2.96)%, (42.08±7.21)%, $P < 0.01$]. At the same time, the adhesion rate of these cells were increased after the GPI-PLD activity was inhibited [(61.19±29.14)%, (49.78±26.73)%, $P < 0.01$], and the CD24 expression was also up-regulated [(18.5±11.14)%, (16.02±9.68), $P < 0.01$].

CONCLUSION: The adhesion rate of bone marrow mononuclear cells separated from myeloid leukemia patients can be promoted by inhibiting GPI-PLD activity. At the same time, the CD24 expression of GPI-anchored proteins on bone marrow mononuclear cells is improved.

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INTRODUCTION

Glycosylphosphatidylinositol-specific phospholipase D (GPI-PLD) is abundant in human plasma. There are many cells and constitutions in human that can synthesis and excrete GPI-PLD. In these loci, bone marrow, hepar and pancreatic gland are the most important organs[1-3]. Under some specific circumstances, only GPI-PLD can hydrolyze and release proteins from their GPI anchors on the plasma membrane of cells[4]. The GPI-anchored proteins are functionally diverse and include cell-adhesion molecules, ectoenzymes, cell surface receptors, lymphocyte differentiation antigens, *etc.* These proteins have many functions involving adhesion, migration, regulation growth of cells. For instance, CD24 expressed on human white blood cells can regulate the adhesion and migration of these cells[5-6]. So we speculated that GPI-PLD might have some effect on adhesion ability of leukemic cells by changing some GPI-anchored protein such as CD24. However, litter is known about the effect of GPI-PLD on the adhesion ability of myeloid leukemic cells through screening Medline and CNKI databases. This report was aimed to investigate the adhesion rate of bone marrow mononuclear cells (BMMNCs) separated from myeloid leukemia patients to fibronectin and CD24 expression after GPI-PLD activity being inhibited by 1,10-phenanthroline, a specific inhibitor for GPI-PLD *in vitro.*

MATERIALS AND METHODS

Design

The *in vitro* cytology study.

Time and setting

The study was conducted at the Hematological Laboratory of Xiangya Hospital from January to June 2004.

Subjects

Fresh bone marrow cells were obtained from 26 patients suffering from myeloid leukemia and admitted in the Out-patient Department and Inpatient Department of Xiangya Hospital, including 18 males and 8 females, aged 11–76 years with a mean of 37.5 years. Myeloid patients were distributed as follows: M_{2a}: 9; M_3: 8; M_4: 2; CML:7. Diagnosis of leukemia was based mainly on the *Standard of Hemopathy Diagnosis and Effect* (The Second Edition) described by Zhang[7]. Inclusive criteria: myeloid leukemia patients with

normal liver function; exclusive criteria: abnormal liver function patients. All patients and their relatives signed the informed consents to the experiment.

The main reagents and instruments used are as follows:

Reagent and instrument	Source
Placental alkaline phosphatase	Professor Tang Jian-hua, Biochemical Department, Xiangya Medical School, Central South University, China
Fibronectin, 1,10-phenanthroline	Sigma Corporation, USA
CD24 monoclonal antibody	Xiehe Stem Cell Corporation, Tianjin, China
Ficoll lymphocyte isolation solution	Beijing Dingguo Biotechnology Department Center, China
Immunohistochemistry kit	SABC corporation

Methods

BMMNCs isolation and cell culture

Heparinized bone marrow samples were obtained by aspiration from myeloid leukemia patients and BMMNCs were prepared by density gradient centrifugation. For every specimen, the percentage of leukemic blast cell in BMMNCs was more than 80%.

All cells freshly separated from bone marrow were suspended in RPMI 1640 without serum. These cells were seeded at 1×10^9/L. The experiment was divided into 2 groups, treatment group added with 1,10-phenanthroline to achieve a final concentration of 1 mmol/L, while control group with the same amount of phosphate buffered saline. After cells were cultured at 37 ℃ in humidified air containing 5% CO_2 for 5 hours, the viability of cells was evaluated using Trypan blue dye-exclusion test. Then each group cells were separately used for GPI-PLD activity test, cell adhesion assay and immunohistochemistry test.

Cell adhesion assays

The 96-well tissue-culture plates (Nunc, Roskilde, Denmark) were incubated overnight at 4℃ with 50 μL/well of phosphate buffered saline containing 20 mg/L fibronectin. The coating solution was removed by aspiration. Plates were washed once using phosphate buffered saline, replaced with 100 μL of RPMI 1640 containing 1 g/L bovine serum albumin and incubated for a further 2 hours at 37 ℃. After this blocking step, plates were washed three times using RPMI 1640 containing 5 g/L bovine serum albumin, as cell adhesion medium. Freshly isolated BMMNCs were suspended at 1×10^8 cells/L in cell adhesion medium and 100 μL BMMNCs suspension was added to each well. The experiment was assigned into 5 groups: blank group (without cells), treated total cells group, treated adhesive cells group, control total cells group, and control adhesive cells group. At least 3 wells were used for each data point. Following an incubation with 1 mmol/L 1,10-phenanthroline or with equal volume phosphate-buffered saline for 1 hour at 37 ℃. Unattached cells were removed by 3 washes of 300 μL exchanges of medium. Subsequently 20 μL MTT (5 mg/mL) and 100 μL adhesion media was added to attached cells. Attached cells were incubated at 37 ℃ for 4 hours. 100 μL/well Dimethyl Sulfoxide was added to 96-well tissue-culture plates after discarding superior liquor. Subsequently the absorbance value was measured at 570 nm wavelength by microplate reader.

The adhesion rate of 1,10-phenanthroline treated group was calculated using the following formula:

$$\text{Cell adhesion rate (\%)} = \frac{(A_{570} \text{ in C} - A_{570} \text{ in A})}{(A_{570} \text{ in B} - A_{570} \text{ in A})} \times 100\%$$

A, B, C stand for blank control group, the group that cells were treated by 1,10-phenanthroline and unattached cells were not washed, and the group that cells were treated by 1,10-phenanthroline and unattached cells were washed, respectively.

The adhesion rate of control was calculated using the following formula:

$$\text{Cell adhesion rate (\%)} = \frac{(A_{570} \text{ in E} - A_{570} \text{ in A})}{(A_{570} \text{ in D} - A_{570} \text{ in A})} \times 100\%$$

A, D, E stand for blank control group, the group that cells were not treated by 1,10-phenanthroline and unattached cells were not discarded, the group that cells were not treated by 1,10-phenanthroline and unattached cells were discarded, respectively.

GPI-PLD activity assay

GPI-PLD activity assay was carried out as previously described[8]. 0.16% NP_{40} was used as a detergent according to preliminary test. The reaction mixture of substrate, cells and buffer was incubated at 37 ℃ for 90 minutes.

CD24 expression detected by immunohistochemistry

According to ABC kit, CD24 mAb was used as primary antibody, those stained as brown red were taken as positive cells and the number of positive cells was calculated. For negative controls, the antibody was substituted with phosphate buffered saline.

Main outcome measures

GPI-PLD activity of myeloid leukemic cells, cell adhesion rate, CD24 expression were all measured.

Design, practice and evaluation

This study was designed by the first and second authors, performed by the first author, evaluated by the fourth and fifth authors. All authors received training, and no blind method was adopted.

Statistical analysis

Data processing was performed by the third author using SPSS 11.5 software for windows. All the results were represented the Mean±SD. Significant differences of the results were determined by using paired-samples *t*-test. A level of *P* value less than 0.05 was considered statistically significant.

RESULTS

Effect of 1,10-phenanthroline on the GPI-PLD activity, cell adhesion rate and CD24 expression of BMMNCs separated from myeloid leukemia patients (Table 1)

Table 1 The effect of 1,10-phenanthroline on the GPI-PLD activity, cell adhesion rate and CD24 expression of bone marrow mononuclear cell separated from myeloid leukemia patients ($\bar{x}\pm s$, n=26, %)

Group	GPI-PLD activity	Adhesion rate	CD24 expression
Treated	5.40±2.96[a]	61.19±29.14[a]	18.50±11.14[a]
Control	42.08±7.21	49.78±26.73	16.02±9.68

GPI-PLD: glycosylphosphatidylinositol-specific phospholipase D; [a]P < 0.01, *vs*. control group

As 1,10-phenanthroline being a specific inhibitor for GPI-PLD *in vitro*, 1,10-phenanthroline was selected to inhibit GPI-PLD activity in BMMNCs. After BMMNCs were incubated with 1 mmol/L 1,10-phenanthroline for 5 hours at 37 ℃, 5% CO_2, more than 95% cultured cells had viability detected by Trypan Blue dye-exclusion test. The GPI-PLD activity in the treated group was remarkably reduced compared with control group (P < 0.01). Compared with control group, the adhesion rate of BMMNCs to fibronectin was increased significantly after the BMMNCs were incubated with 1 mmol/L 1,10-phenanthroline for 5 hours at 37 ℃, 5% CO_2. Under microscope, the number of cells adhered to 96-well plates was increased and the adhesion rate in the control group and treated group was respectively (49.78±26.73)% and (61.19±29.14)%. The percentages of CD24 positive cells were also enhanced (Figures 1, 2).

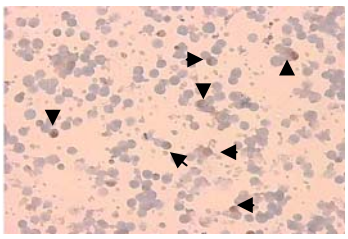

Figure 1 The immunohistochemical result of the cells that were treated by 1 mmol/L 1,10-phenanthroline for 5 hours. The positive cells were marked by arrows (AEC, ×200)

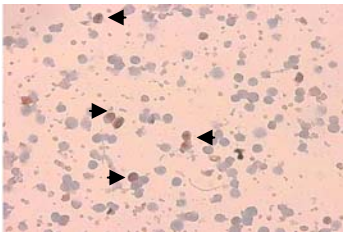

Figure 2 The immunohistochemical result of control group. The positive cells were marked by arrows (AEC, ×200)

DISCUSSION

An increasing number of cell surface proteins have been found to be anchored to the plasma membrane by GPI. These proteins are called GPI-anchored proteins. GPI-anchored proteins are functionally diverse and include cell-adhesion molecules, ectoenzymes, cell surface receptors, differentiation antigens. On the plasma membranes of human hematopoietic cells, approximately 10% of the antigens with defined CD expression pattern are GPI-anchored proteins. GPI-PLD is a secreted mammalian enzyme that specifically cleaves GPI-anchored proteins. There are many evidences for the release of GPI-anchored proteins by intracellular GPI-PLD[9]. GPI-PLD activity or expression may have effect on hematopoietic tumor cells adhesion and migration because some GPI-anchored proteins such as CD24 expressed on hematopoietic cells are also cell-adhesion molecules that facilitate tumor cells extramedullary infiltration[10-11]. Therefore, it is significant to investigate GPI-PLD for cell adhesion, growth, apoptosis and differentiation.

It is well known that many cells and constitutions in human such as liver, brain, pancreas, keratose cells, bone marrow stroma cells and hematopoietic cell lines express GPI-PLD mRNA, but most of it comes from liver, pancreas and bone marrow. Many studies indicate that the mount of GPI-PLD mRNA is correlated to the malignant phenotype of cells[5]. The level of GPI-PLD expression in human ovarian cancer cells and epithelioma cells is higher than that in homologous normal cells. In the same time, a lot of tumor marks in cancer cells such as urokinaseis plasminogen activation receptor is generated. Therefore, GPI-PLD may take part in cancer infiltration and metastasis. Xie *et al* [3] discovered that human bone marrow cell lines K562, HL60, PLB-985, U937, and THP-1 express GPI-PLD. Our former experiment indicated that GPI-PLD activity and mRNA expression in BMMNCs separated from myeloid leukemia patients are higher than that from normal persons. So BMMNCs separated from myeloid leukemia patients were selected as research objects. The GPI-PLD activity could be decreased from (42.08±7.21)% to (5.4±2.96)% after BMMNCs were incubated with 1 mmol/L 1,10-phenanthroline for 5 hours *in vitro* and the adhesion rate increased from (49.78±26.73)% to (61.19±29.14)%. The difference was statistically significant (P < 0.01). These results illustrate that GPI-PLD activity is correlated to the adhesion ability of BMMNCs separated from myeloid leukemia patients. Decreasing GPI-PLD activity can increase the adhesion ability of BMMNCs separated from myeloid leukemia patients. The mechanism may be GPI-anchored adhesion molecules such as CD24 and CD87 increasing because of GPI-PLD activity decreasing.

CD24 is a small heavily glycosylated glycosylphosphatidylinositol-linked cell surface protein, which is expressed in hematological malignancies as well as in a large variety of solid tumors such as non-small cell lung cancer, breast cancer, liver cancer, ovarian cancer[10-13]. CD24 regulates cell adhesion by interaction with activated platelet and P-selectin on endotheliocyte[14]. Many studies have revealed a highly significant association of increased cytoplasmic CD24 expression with shortened patient survival[15]. The interaction of CD24 on tumor cells and P-selectin on activated platelet and endotheliocyte may facilitate cancer metastasis. Inhibiting

GPI-PLD activity in BMMNCs promotes cells adhesion ability and CD24 expression. This may be due to CD24 promoting adhesion of cells with fibronectin.

In order to explore the effect of GPI-PLD activity on leukemic cell adhesion ability, we inhibited GPI-PLD activity *in vitro* by 1,10-phenanthroline, a special inhibitor for GPI-PLD. The adhesion rate of BMMNCs to fibronectin and GPI-PLD activity were measured after GPI-PLD activity was inhibited by 1,10-phenanthroline and incubated by equal volume phosphate buffered saline. The results revealed that the adhesion rate of BMMNCs to fibronectin was increased significantly by inhibiting GPI-PLD activity. We think the increased adhesion ability of BMMNCs to fibronectin may due to increased cell adhesion molecules that expressed on BMMNCs because GPI-PLD activity was inhibited. So the CD24 expression was measured with Immunohistochemistry method. The result was in coincidence with our speculation. CD24 expression was increased after GPI-PLD activity had been inhibited.

In summary, on the basis of our studies, the adhesion ability of BMMNCs separated from myeloid leukemia patients can be promoted by inhibiting GPI-PLD activity. Therefore, we have reason to speculate that GPI-PLD activity may be related to the prognosis and extramedullary infiltration of leukemia. But we still do not know if we can lower the infiltration ability of tumor cells and regulation the homing of hematopoietic stem cells by alteration GPI-PLD activity.

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糖基化磷脂酰肌醇特异性磷脂酶 D 对髓系白血病骨髓细胞黏附功能的影响及其机制☆

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摘要

背景：据作者查新检索 medline，cnki 等数据库，鲜见国内外有关体外研究糖基化磷脂酰肌醇特异性磷脂酶 D(glycosylphosphatidilinoditol-specific phospholipase D，GPI-PLD)对白血病细胞黏附功能影响方面的报道。

目的：观察 GPI-PLD 对髓系白血病骨髓单个核细胞黏附功能的影响及其机制。

设计、时间及地点：细胞学体外实验，于 2004-01/06 在湘雅医院血液实验室完成。

对象：骨髓标本来自于髓系白血病患者，由湘雅医院血液科提供。

方法：利用具有完整糖基化磷脂酰肌醇(GPI)结构的胎盘碱性磷酸酶做底物，通过 TX-114 分相，定量检测骨髓单个核细胞中 GPI-PLD 活性，利用 1，10-二氮杂菲抑制 GPI-PLD 的活性，实验分 2 组：处理组加二氮杂菲，使其终浓度为 1 mmol/L，对照组加入等量的 PBS。用 MTT 方法检测处理组和对照组骨髓细胞对纤维连接蛋白的黏附率、免疫组织化学方法检测 GPI-锚定蛋白 CD24 的表达。

主要观察指标：髓系白血病细胞 GPI-PLD 活性，细胞黏附率及 CD24 的表达。

结果：1 mmol/L 1，10-二氮杂菲作用髓系白血病骨髓单个核细胞 5 h 细胞的 GPI-PLD 活性较对照组降低[(5.40±2.96)%，(42.08±7.21)%，$P < 0.01$]；GPI-PLD 的活性被抑制后处理组细胞的黏附率较对照组增加[(61.19±29.14)%，(49.78±26.73)%，$P < 0.01$]，同时 CD24 的表达率上调[(18.5±11.14)%，(16.02±9.68)，$P < 0.01$]。

结论：降低 GPI-PLD 活性能增加骨髓单个核细胞对纤维连接蛋白的黏附率，同时骨髓单个核细胞上 GPI-锚定蛋白 CD24 表达增强。

关键词：髓系白血病；GPI-PLD；细胞黏附

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